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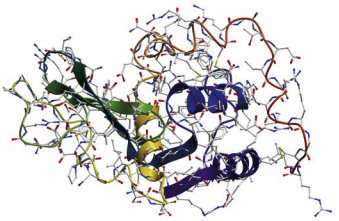

Production, Applications, and Future Prospects

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ENZYMES IN FOOD BIOTECHNOLOGY

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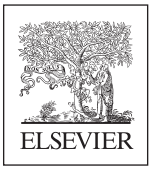

ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
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Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN 978-0-12-813280-7

Publisher: Andre Gerhard Wolff
Acquisition Editor: Patricia Osborn
Editorial Project Manager: Billie Jean Fernandez
Production Project Manager: Prem Kumar Kaliamoorthi
Cover Designer: Mark Rogers

Typeset by SPi Global, India

Dedicated to my beloved family

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Foreword

I am delighted to write the foreword for the book *Enzymes in Food Biotechnology*. Due to the vast application of enzymes in the food industry, there was a need for a comprehensive book on the enzymes used in food production and processing. Dr. Mohammed Kuddus met that need, representing the production and application of various food enzymes from different sources. In addition to exploring enzymes used in the food industry, chapters also include enzyme engineering, immobilization techniques, and extremozymes. Each chapter contains background information and concluding remarks for better understanding of the subject matter. The editor has selected well over 45 prominent chapters related to enzymes involved in food processing. Even though these represent only a small section of the world of enzymes, they sufficiently demonstrate the importance of enzymes in the food industry. This book will provide a basic overview of food enzymes and referenced resources to food biotechnologists, researchers, and post graduate students; indeed, anyone who is fascinated with the world of enzymes. It is also a unique tribute to the many professionals who are involved in enzyme research and in food processing industries.

Pramod W. Ramteke
SHUATS, Allahabad, India
February, 2018

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Preface

Food is the fuel for life. The relationship between population and food supply is not new. Today, the food crisis is a major problem in the world. United Nation's Department of Economic and Social Affairs predicted that due to increasing world population food demand is expected to increase up to 70% by 2050. Food biotechnology could provide an efficient solution for global food security. Scientists have already identified potential uses of enzymes in the food industry, from bioconversion to genetic engineering. The assimilation of enzymes in food processing is well known, and devoted research is still ongoing to solve the food crisis worldwide. Enzymes are proficiently involved in improving food production and food components including flavor, aroma, color, and nutritional value. Recent developments in enzyme biotechnology, along with genetic engineering, will allow the production of more nutritious, safer, tastier, and healthier food. The lack of knowledge of scientific progress and most recent technologies in enzyme engineering contributes significantly to the slow progress of food production. In this perspective, the aim of this book is to offer a broad and updated review regarding the potential impact of enzymes on the food sector. This book brings together the novel resources and technologies regarding enzymes in food production, food processing, food preservation, food engineering, and food biotechnology.

This book contains 49 chapters and includes scientific progress and recent technologies in food production and processing. The chapters highlight potential applications of various enzymes in food industries such as fruit, beverage, dairy, meat, animal feed and functional food, starch processing, and pharmaceuticals. Other chapters include the contribution of enzymes from different sources, such as plants, animals, and microorganisms in the field of bioconversion of agro-industrial waste, sustainable crop production, bioactive peptide production, and antibiotics production as therapeutic enzymes. These chapters explain how and what types of organisms/enzymes, including food enzymes from extreme environments, are involved in the respective process. Some chapters also discuss the use of enzymes in various tools and analytical techniques commonly used for detection of food quality and food-borne pathogens. Other chapters contain literature about the role of enzymes in biosensors, enzyme engineering, enzyme immobilization; food enzymes and nanotechnology, along with future prospective of enzyme technologies in the food industry. These chapters also present future research directions for the development of specific and more active enzymes for food production and processing.

In conclusion, I can say that this book is a comprehensive reference in the most progressive field of food technology and will be useful for professionals, scientists, and academics in the food and biotech industries, along with students studying in food-related courses. And last, but not least, I express my deepest gratitude and regards to my family. Words are not sufficient to express my feelings to them for their love and moral support which helped me in completing this

book. I would also like to thanks my friends and all the authors who have eagerly contributed their chapter in this book. I am confident that these chapters will be beneficial for academics and the scientific community working in the field of food biotechnology. I also want to extend my special gratitude to Ms. Billie Jean Fernandez, Elsevier Inc., Cambridge, for her efforts to get this book published. Finally, I would also like to express my sincere gratitude to the Elsevier Publication House for providing this opportunity.

Mohammed Kuddus
Hail, Saudi Arabia
February 2018

CHAPTER

1

Introduction to Food Enzymes

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ABBREVIATIONS

IUB	International Union of Biochemists
IU	International Units
EC	Enzyme Commission
°C	degree celsius
pH	potential of hydrogen
ES complex	enzyme substrate complex
EIS complex	enzyme inhibitor substrate complex
K_m	Michaelis constant
V_{max}	maximum velocity
NAD	nicotinamide adenine dinucleotide
ATP	adenosine triphosphate

1.1 ENZYMES

The presence of enzymes in nature have been well known for over a century. However, the first enzyme urease was isolated in crystalline form from the jack bean by James B. Sumner in 1926 (Sumner, 1926). Enzymes, also known as biocatalysts, are a biological substance that initiates or accelerates the rate of a biochemical reaction in a living organism, without itself being consumed in the reaction. Even though enzymes are produced inside the living cells, they can work actively in vitro, making them useful in industrial processes. Enzymes are complex protein molecules and are nature's own biocatalysts produced by living organisms to catalyze the biochemical reactions required to sustain life. Mostly enzymes are proteins, but not all. RNA and antibodies can also act as catalysts known as ribozymes and abzymes, respectively. The literature suggested that >5000 biochemical reaction types are catalyzed by the enzymes (Schomburg et al., 2013). Similar to other chemical catalysts, enzymes are also highly effective in increasing the rate of biochemical reactions that otherwise proceed very slowly, or in some

https://doi.org/10.1016/B978-0-12-813280-7.00001-3

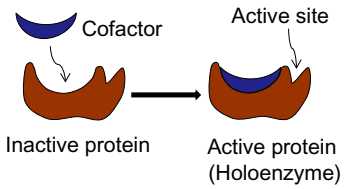

FIG. 1.1 Holoenzyme.

cases, not at all. A common example is the breakdown of foods, which includes mainly proteins, carbohydrates and fats, into their basic constituents. It is normally accomplished within 3–6 h depending on the type and amount of food. However, in the absence of enzymes, this breakdown of foodstuffs would take >30 years. In comparison to chemical catalysts, enzymes are more specific in action and possess high catalytic properties. Also, enzymes can be immobilized on inert support material without loss of activity that facilitates their reuse and recycling.

Most enzymes, but not all, require a small molecule to perform their activity as a catalyst. These molecules are known as cofactors or coenzymes. Cofactors are non-proteinaceous chemical compounds that are bound to an inactive protein part of enzyme (apoenzyme) in order to increase the biological activity of the enzyme required for its function. The active complex of apoenzyme (protein part) along with cofactor (coenzyme or prosthetic group) is referred to as holoenzyme (Fig. 1.1). Cofactor is also considered a "helper molecule" because it assists in biochemical transformations. There are two types of cofactors: coenzymes and prosthetic groups. Coenzymes are a specific type of cofactor and are organic molecules that bind to enzymes and help in their functions. The organic molecules are simply the molecules that contain carbon. Many coenzymes are derived from vitamins. These molecules often attached to the active site of an enzyme and assist in the conveyance of a substrate or product and can also shuttle chemical groups from one enzyme to another. Importantly, coenzymes bind loosely to the enzyme but another group of cofactors do not. Prosthetic groups (organic molecules or metal ions) are also cofactors that often bind tightly to proteins or enzymes by a covalent bond. One of the significant characteristic of enzymes is their specificity for substrates or reactions they catalyze, which make them so important as a research and industrial tool. The specificity of enzymes may be of different types, such as absolute (that catalyze only one reaction), group (that act only on molecules that have specific functional groups), linkage (that act on a particular type of chemical bond) or stereo-chemical (that act on a particular steric or optical isomer).

1.2 NOMENCLATURE AND CLASSIFICATION OF ENZYMES

To date, >6000 different types of enzymes are known (http://www.enzyme-database.org/stats.php). The names of commonly used enzymes are based on the type of reaction they catalyze followed by the suffix *-ase*. For example, the hydrolysis of proteins is catalyzed by proteases. There are also some trivial names for the initially studied enzymes such as trypsin, pepsin, rennin, etc. However, trivial names give no indication of source, function or reaction catalyzed by the enzyme. Thus a variety of different names have been used for the same enzyme and different enzymes were known by the same name. Also, due to lack of consistency

in the nomenclature and rapid growth in enzyme discovery, there was a need for a systematic way to name and classify enzymes. The International Union of Biochemists (IUB) developed an unambiguous system of enzyme nomenclature in which each enzyme had a unique name and four-digit code number, prefixed by EC and separated by points that identify the substrate acted upon and the type of reaction catalyzed. These four-digit code numbers, prefixed by EC, provide the following information: (1) the first number of EC code indicates six main classes of the enzyme, (2) the second number of EC code denotes subclass, (3) the third number indicates sub-subclass, and (4) the fourth number of EC code is the serial number of the enzyme in its sub-subclass. This is now in widespread use and the approved list of classified enzymes can be found at http://www.enzyme-database.org. Enzymes are classified into six main classes based on the type of chemical reaction catalyzed.

CLASS 1: OXIDOREDUCTASES (EC 1)

The enzymes of this class are involved in redox reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules. The enzymes included in this class are dehydrogenases, which are involved in hydride transfer, oxidases responsible for electron transfer to molecular oxygen, oxygenases involved in oxygen transfer from molecular oxygen, and peroxidases that facilitate transfer of electron to peroxide. For example: glucose oxidase (EC 1.1.3.4).

CLASS 2: TRANSFERASES (EC 2)

The enzymes of this class catalyze transfer of specific functional groups (e.g., alkyl-, glycosyl-, etc.) between two molecules, but excluding oxidoreductases and hydrolases, for example, aspartate aminotransferase (EC 2.6.1.1).

CLASS 3: HYDROLASES (EC 3)

The enzymes of this class are also known as hydrolytic enzymes and are involved in hydrolytic reactions and their reversal (use water to cleave chemical bonds). Examples of common hydrolases include proteases, glycosidases, esterases, nucleosidases, and lipases, for example, alkaline phosphatase (EC 3.1. 3.1).

CLASS 4: LYASES (EC 4)

The enzymes of this class are involved in elimination reactions. This is nonhydrolytic removal of a group of atoms from the substrate. The enzymes of this class are decarboxylases, aldolases, dehydratases, and some pectinases but do not include hydrolases, for example, histidine carboxy-lyase (EC 4.1.1.22).

CLASS 5: ISOMERASES (EC 5)

The enzymes of this class catalyze molecular isomerization (transfer of groups within the molecules). Examples of isomerases include epimerases, racemases and intramolecular transferases, for example: xylose isomerase (EC 5.3.1.5).

CLASS 6: LIGASES (EC 6)

The enzymes of this class are also known as synthetases and are involved in condensation reaction. The joining of two molecules involves covalent bond formation, along with hydrolysis of a nucleoside triphosphate, for example, glutathione synthase (EC 6.3.2.3).

1.3 ENZYME UNITS

The amount of an enzyme in a specific biochemical reaction is usually determined by measuring the rate of the reaction. The activity of an enzyme is expressed in enzyme units. The Enzyme Commission of the International Union of Biochemistry recommends expressing it in International Units (IU) which is the most widely used unit of enzyme activity. One IU is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate per minute under standard conditions of temperature, optimal pH, and optimal substrate concentration. Also, in order to adhere to SI units, the Commission on Biochemical Nomenclature recommended that the reaction rates should be expressed in moles per second and proposed another unit of enzyme activity known as *katal* (kat). One *katal* is defined as the amount of enzyme that catalyzes the conversion of one mole of substrate per second. The magnitude of the katal is so big (1 IU = 16.7 *katals*); therefore, in practice, there is a reluctance to use it. The former IU is still more widely used and the magnitude of the IU is appropriate to measure most enzyme preparations (Illanes, 1999). The specific activity of an enzyme is the number of enzyme units per milligram of protein. Therefore, the value of specific activity is mentioned as units/mg and it is an important measure of enzyme purity.

1.4 MECHANISM OF ENZYME ACTION

In order for a reaction to take place, the reactant molecules (substrate) require some amount of energy to cross the transition state of the reaction and become products. This energy is known as activation energy and is defined as the minimum amount of energy required to activate all the atoms or molecules in 1 mol of substance to attain the transition state at the top of the energy barrier at a given temperature. It is assumed that enzymes reduce the activation energy of enzymes catalyzed in chemical reactions and therefore increases the rate of reaction ranging from 10^6 to 10^{24}-fold in comparison to nonenzymatic reactions (Illanes, 1999). In other words, enzymes are thought to reduce the "path" of the reaction that requires less energy for each molecule of substrate to be converted into products. Therefore, the reaction is said to go faster in a given period of time (Fig. 1.2).

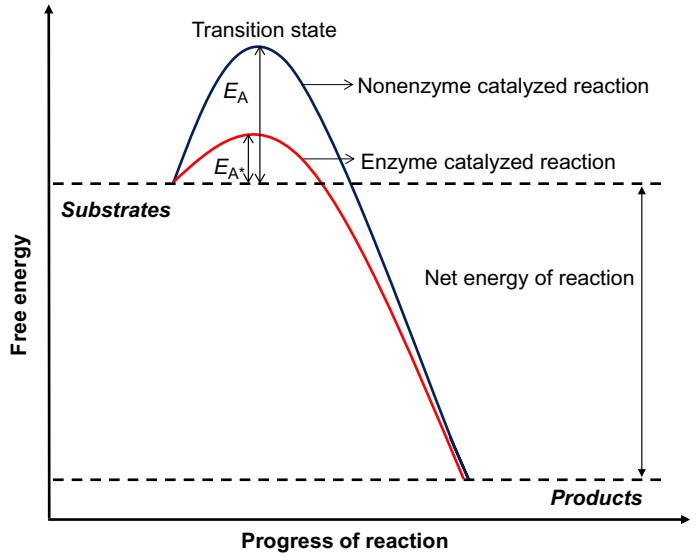

FIG. 1.2 A schematic diagram comparing enzyme catalyzed reaction and nonenzyme catalyzed reaction (E_A is the activation energy for nonenzyme catalyzed reaction and $E_A{}^*$ is the activation energy for enzyme catalyzed reaction).

In 1888, the explanation of the catalytic action of an enzyme was proposed by the Svante Arrhenius, a Swedish chemist. He proposed that the substrate and enzyme combined to form some intermediate compound known as the enzyme-substrate complex. This complex decomposed into a product and active enzyme. The overall enzyme catalyzed reaction can be represented as:

$$\underset{\text{Substrate}}{S} + \underset{\text{Enzyme}}{E} \rightarrow \underset{\text{ES complex}}{ES} \rightarrow \underset{\text{Product}}{P} + \underset{\text{Enzyme}}{E}$$

In general, the enzyme-catalyzed reactions occur in the following steps:

a) The substrate molecule contacts the active site of enzyme by noncovalent bonds. The active site is the region on an enzyme that combines with the substrate.
b) The substrate and enzyme form an enzyme-substrate complex.
c) The substrate molecule is transformed into a product by either rearrangement of atoms or by breakdown of substrate/joining of substrate with other molecule.
d) The breakdown of ES complex leads to product formation which is released from the active site of enzyme.
e) The nature of the enzyme is unchanged and can catalyze a new reaction.

The mechanisms of enzyme action are generally explained by two proposed models:

1) Lock and Key Model

In 1894, Emil Fischer proposed this theory and suggested that both a substrate and an enzyme have specific geometric shapes that fit exactly into each other. It specifies that the active site of an enzyme has a unique conformation. This active site is complementary to

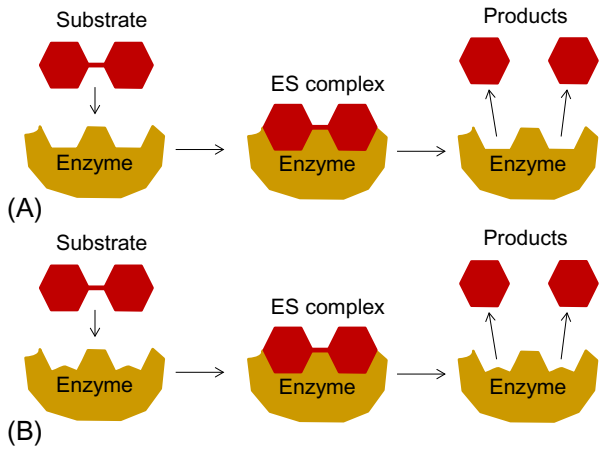

FIG. 1.3 (A) Lock and key model of enzyme action. (B) Induced-fit model of enzyme action.

the substrate structure and consequently allows the two molecules to fit together, called the lock and key hypothesis (Fig. 1.3A). This model recommended rigidity of the enzyme's catalytic site.

2) Induced-Fit Model

In 1958, another scientist named Daniel Koshland proposed some modification in the previously explained lock and key hypothesis. He suggested that the essential functional groups on the active site of the free enzyme are not in their optimal positions for promoting catalysis. Because enzymes are so flexible, when the substrate molecule binds to it the active site of the enzyme adopts a favorable geometrical shape to form the transition state. So, as per Koshland's suggestion, the substrate induces a conformational change in the enzyme which aligns the amino acid residues or other groups for substrate binding and catalysis. This assumption is known as induced-fit model of enzyme action (Fig. 1.3B).

1.5 FACTORS AFFECTING ENZYME ACTIVITY

The activity of an enzyme or rate of an enzyme's catalyzed reaction is affected by several factors. Some of these factors are temperature, pH, substrate concentration, enzyme concentration, and the presence of any inhibitors or activators.

1.5.1 Effect of Temperature

Temperature affects enzyme activity or rate of the enzyme's catalyzed reaction. All the enzymes have a range of temperatures at which they are normally active. The temperature at which enzymes work optimally, or at which the rate of reaction is maximum, is known as optimum temperature (Fig. 1.4A). This temperature is different for different enzymes. As most of the enzymes are proteins, they are denatured by heat and become inactive as the temperature increases beyond a certain point (above 60°C). Some enzymes lose their activity

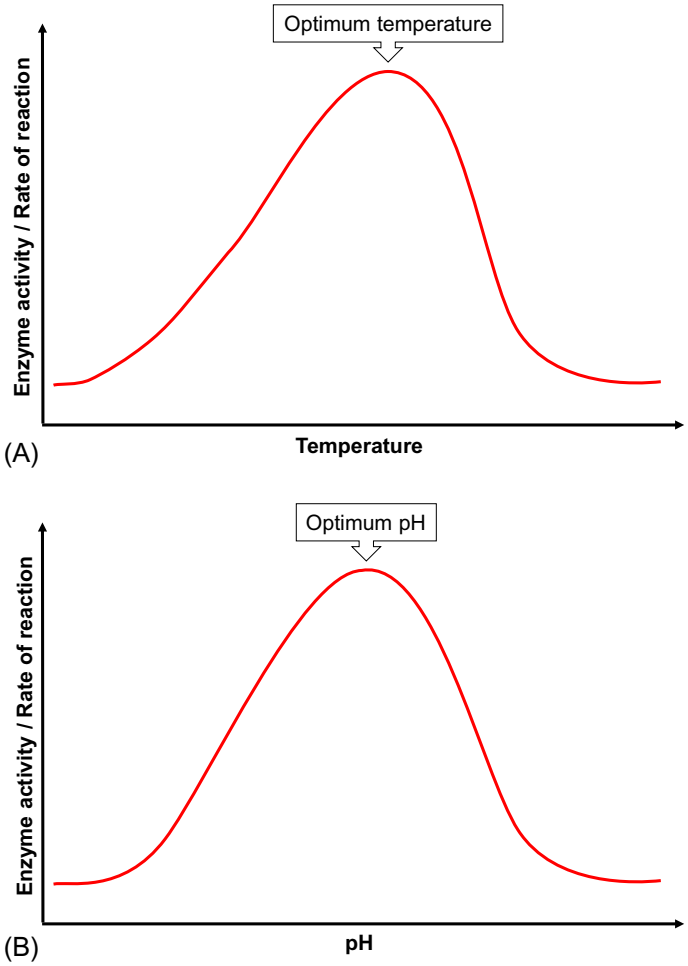

FIG. 1.4 (A) Effect of temperature on enzyme activity or enzyme catalyzed reaction. (B) Effect of pH on enzyme activity or enzyme catalyzed reaction.

when frozen. However, some enzymes work really well at lower temperatures, such as 4°C (known as cold-adapted enzymes), and some work really well at higher temperatures, like 95°C (known as thermo-stable enzymes). At low temperatures, enzymes have low activity. However, the rate of enzyme-catalyzed reactions roughly doubles with a 10°C rise in temperature over a limited range (Fig. 1.4A).

1.5.2 Effect of pH

Along with temperature, pH also affects enzyme activity or rate of enzyme-catalyzed reactions. The acidity or basicity of a solution is determined by its pH value which ranges from pH 1 to pH 14. Acidic solutions have pH values of <7, and basic (alkaline) solutions have pH

values of >7. Deionized water has a pH of 7, which is known as a neutral solution. Most of the enzymes have a characteristic pH value at which their activity is maximum. This pH is known as optimum pH, and above or below it, the activity of enzymes, or rate of enzyme catalyzed reaction, decreases (Fig. 1.4B). Different enzymes have different optimum pH values. Small deviations in pH from their optimum value do not denature an enzyme permanently. Although extreme changes in pH cause enzyme activity loss and will permanently denature the enzyme. Enzymes present at different locations have different optimum pH values due to their different environmental conditions. Also, some enzymes work really well at acidic pH levels (pH <7), termed as acidophilic enzymes, and some work best at an alkaline pH (pH >7), termed as alkaliphilic enzymes.

1.5.3 Effect of Substrate Concentration

Substrate concentrations also affect the rate of an enzyme-catalyzed reaction. When the concentration of substrate is increased, the velocity of the reaction recorded forms a rectangular hyperbola if other factors such as pH, temperature, and enzyme concentration are at optimum levels (Fig 1.5A). At the initial stage, the velocity of the reaction is nearly proportional to the substrate concentration. Further, if substrate concentration is increased, the rate of reaction slows down and finally becomes constant. In the initial phase, increasing the substrate concentration increases the rate of reaction because more substrate molecules are binding with enzyme molecules, so more products formed. At maximum velocity, all the enzyme molecules are saturated with substrate molecules so that further increases in substrate concentration cannot results in an increased reaction rate.

1.5.4 Effect of Enzyme Concentration

In a biochemical reaction, concentration of the enzyme is important as it is needed to react with the substrate molecules. If there are more enzymes, the reaction will be faster until all the substrates are used up (Fig. 1.5B). In comparison to substrate concentration, the enzyme concentration is always very low on a molar basis. Thus, increasing the enzyme concentration will always increase the rate of the enzyme-catalyzed reaction up to a point, after which any increase will not affect the rate of reaction (if no more substrate is available).

1.5.5 Effect of Inhibitors

Enzyme inhibitors are molecules that interact with enzymes (temporary or permanent) in some way and reduce the rate of an enzyme-catalyzed reaction or prevent enzymes to work in a normal manner. The important types of inhibitors are competitive, noncompetitive, and uncompetitive inhibitors. Besides these inhibitor types, a mixed inhibition exists as well. Competitive enzyme inhibitors possess a similar shape to that of the substrate molecule and compete with the substrate for the active site of the enzyme. This prevents the formation of enzyme-substrate complexes. Therefore, fewer substrate molecules can bind to the enzymes so the reaction rate is decreased. The level of inhibition depends on the relative concentration of substrate and inhibitor. This is a reversible process (temporary binding). In the case

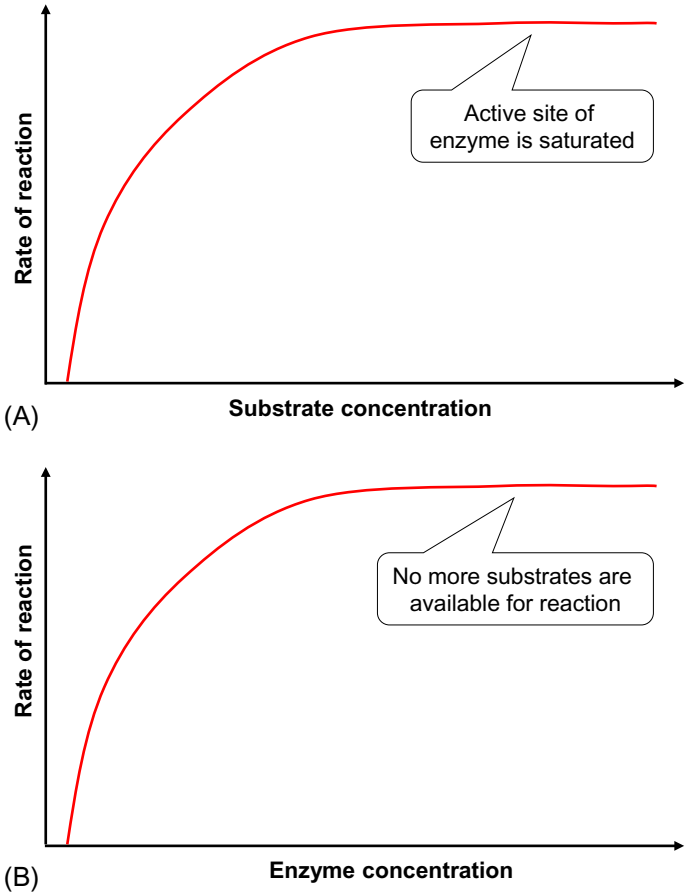

FIG. 1.5 (A) Effect of substrate concentration on enzyme catalyzed reaction. (B) Effect of enzyme concentration on enzyme catalyzed reaction.

of competitive inhibition, K_m is increased but V_{max} is not altered. Noncompetitive enzyme inhibitors bind to a site other than the active site of the enzyme, called an allosteric site. Due to this binding, it deforms the structure of the enzyme so that it does not form the ES complex at its normal rate, and it prevents the formation of enzyme-product complexes, which leads to fewer product formations. Because they do not compete with substrate molecules, noncompetitive inhibitors are not affected by substrate concentration. In the case of noncompetitive inhibition, V_{max} is lowered but K_m is not altered. Uncompetitive inhibitor cannot bind to the free enzyme, but only to the ES complex. The resulting ES complex is enzymatically inactive. This type of inhibition is rare but may occur in multimeric enzymes. Some enzyme inhibitors covalently bind to the active site of the enzyme and inhibit its total activity, thus known as enzyme poison. This type of inhibition is irreversible (permanent). Some enzyme inhibitors can be used as a medicine or as metabolic poison in the treatment of a particular disease.

1.5.6 Cofactors

Most of the enzymes require other compounds, known as cofactors, which are needed for their catalytic activity. This whole unit of protein and cofactor is referred as holoenzyme, as mentioned in Section 1.1. Cofactors usually bind to the close proximity of an enzyme's active site. Cofactors may be either inorganic (e.g., metal ions) or organic compounds (e.g., flavin). Organic cofactors may be either prosthetic groups or coenzymes (e.g., NADH, ATP). These tightly bound cofactors are usually found near the active site of the enzyme and are involved in the catalysis (Oort, 2009).

1.6 SOURCES OF ENZYMES

Enzymes are present in all living organisms. However, commercial enzymes are basically obtained from three primary sources, namely animals, plants and microorganisms. Out of these sources, microorganisms are preferentially used as sources of industrial enzymes due to the following reasons: (1) low production cost, (2) more predictable and controllable enzyme contents of microbes, (3) easy availability of raw materials with constant composition for their cultivation, and (4) plant and animal tissues contain more potentially harmful materials than microbes. Microbes are isolated from natural habitats and used for production of the desired enzyme by optimizing its growth conditions. The process is known as fermentation. Some important food enzymes and their sources are as follows: Enzymes from animals: catalase, lipase, rennet; Enzymes from plants: actinidin, α-amylase, β-amylase, β-glucanase, ficin, lipoxygenase, papain; Enzymes from bacteria: α-amylase, β-amylase, glucose isomerase, protease, pullulanase; Enzymes from fungi: α-amylase, catalase, dextranase, glucose oxidase, lactase, lipase, pectinase, protease, raffinase; Enzymes from yeast: invertase, lactase, lipase, raffinase.

1.7 ENZYMES IN FOOD TECHNOLOGY

Since ancient times, enzymes have played an important role in the production of different foods (Kirk et al., 2002). One of the most common examples is production of beverages such as whiskey, beer, and wine by using an industrial enzyme. Nowadays, the food industry uses a wide-range of screening technologies to discover new food enzymes, but it's become challenging for food scientists and biotechnologists to deliver new food enzymes for commercial applications. However, biotechnology has also emerged as an advance tool for food industries. This technology is providing new products, improving nutritional value, lowering production costs, improving food processing, as well as dealing with waste problems, food safety problems, and packaging issues. This is also going to play an important role in future food production and processing industries. Today, production of almost all commercial foods or their ingredients include enzymes or enzyme-catalyzed reactions. Some common examples of such food/process are alcoholic beverages, syrups, sweeteners, chocolates, infant foods, bakery products, cheese and dairy products, egg products, fruit juice, soft drinks, candy, flavor development, meat tenderization, etc.

Enzymes have many advantages in food production and processing. The most important is that enzymes are used as alternatives to traditional chemical-based technology. This leads to lower energy consumption in processing, creates biodegradable products, and results in less environmental impact. Moreover, enzyme-catalyzed processes produce less waste products (byproducts) due to specific action of enzymes compared to chemical catalysts. Also, enzymes can catalyze reactions under very mild conditions and thus do not destroy valuable elements of food and food components. Although plants, animals, and microbes produce most of the food enzymes, the enzymes produced by microbial sources are more advantageous than their plant and animal counterparts. The reason for preferential use of microorganisms for enzyme production is already mentioned in the previous section. Some of the most common enzymes used in many food applications are presented in Table 1.1 (Avendano et al., 2016; Guomundsdottir and Bjarnason, 2007; Oort, 2009; Singh et al., 2016; Wong, 1995).

TABLE 1.1 Enzymes in Food Applications

Enzymes	Function and Applications
α-Acetolactate	Converts acetolactate to acetoin Reduction of wine maturation time
α-Amylase	Wheat starch hydrolysis Dough making Increased bread volume Production of corn syrup (liquefaction) Aid in the production of sugars for yeast fermentation Brewing (solubilization of barley and other cereal carbohydrates)
β-Amylase	Production of high maltose syrup
β-Glucanase	Hydrolyzes β-glucans in beer mashes Filtration aids, haze prevention in beer production
Acetolactate decarboxylase	In wine making (reducing maturation time by converting acetolactate to acetoin)
Aminopeptidase	Releases free amino acids from N-terminus of proteins and peptides Debittering protein hydrolyzates accelerating cheese maturation
Amyloglucosidase	Hydrolyzes starch dextrins to glucose (saccharification) Production of "lite" beers One stage of high fructose corn syrup production
Catalase	Breaks down hydrogen peroxide to water and oxygen Oxygen removal technology, combined with glucose oxidase
Cellulase	Hydrolyzes cellulose Fruit liquefaction in juice production Ethanol or single cell protein production Conversion of cellulose wastes to fermentable feedstock
Chymosin	Hydrolyzes κ-casein Coagulation of milk for cheese making
Cyclodextrin glucanotransferase	Synthesize cyclodextrins from liquefied starch Cyclodextrins are food grade microencapsulants for colors Flavors and vitamins

Continued

TABLE 1.1 Enzymes in Food Applications—cont'd

Enzymes	Function and Applications
Glucoamylase	Conversion of dextrins to glucose Production of corn syrup (saccharification) Brewing of light beer (conversion of residual dextrins to fermentable sugar)
Glucose isomerase	Converts glucose to fructose Production of high fructose corn syrup (beverage sweetener).
Glucose oxidase	Oxidizes glucose to gluconic acid Oxygen removal from food packaging Removal of glucose from egg white to prevent browning In food packaging (removal of O_2 to potentially protect against oxidative deterioration)
Hemicellulase and xylanase	Hydrolyzes hemicelluloses Bread improvement through improved crumb structure
Hexose oxidase	Oxidative breakdown of glucose Baking industry
Invertase	Sucrose breakdown Beverages industry
Lactase (β-galactosidase)	Hydrolyzes milk lactose to glucose and galactose Sweetening milk and whey Manufacture of lactulose Improving functionality of whey protein concentrates An additive (in dairy products for lactose intolerant individuals) Breakdown of lactose in whey products for manufacturing polylactide
Lipase and esterase	Hydrolyzes triglycerides to fatty acids and glycerol Hydrolyzes alkyl esters to fatty acids and alcohol Synthesis of flavor esters Cheese ripening and flavor enhancement in cheese products Production of specialty fats (improved qualities)
Lipoxygenase	Oxidation of polyunsaturated fatty acids Dough texture improvement
Lysozyme	Antimicrobial preservative
Papain	In meats (as a tenderizer) Brewing (to prevent chill haze formation by digesting proteins)
Pectinase	Hydrolyzes pectin Fruit juice production (treatment of fruit pulp to facilitate juice extraction and for clarification and filtration of juice)
Pectinesterase	Removes methyl groups from galactose units in pectin With pectinase in depectinization technology
Pentosanase	Hydrolyzes pentosans (soluble nonstarch polysaccharides in wheat flours) Part of bread dough improvement technology

TABLE 1.1 Enzymes in Food Applications—cont'd

Enzymes	Function and Applications
Protease	Hydrolysis of κ-casein Hydrolysis of animal and vegetable food proteins Hydrolysis of wheat glutens Milk coagulation for cheese making Bread dough improvement Hydrolyzate production for soups and savory foods
Pullulanase	Hydrolyzes 1–6 bonds that form branches in starch structure Starch saccharification (improves efficiency)
Transglutaminase	Crosslinking of glutamine and lysine residues in proteins Dairy, meat and baking industry
Xylose (glucose)	Isomerization of glucose to isomerase fructose Production of high fructose corn syrup
Xylanase	Xylan breakdown Baking industry

1.8 COLD-ACTIVE ENZYMES IN THE FOOD INDUSTRY

There is a tremendous scope of cold-active enzymes in the food industry and in biotechnology (Gerday et al., 2000). Some of the important applications are in the dairy, juice, meat, and baking industries. In the dairy industry, for example, cold active β-galactosidase is used to decrease the amount of lactose in milk. Lactose, a disaccharide sugar, is accountable for severe intolerances in most of the world's population. Pectinases are used in the fruit juice industry during the juice-extraction process to reduce the viscosity and refine final product. In the meat processing industry, cold-active proteases are used in the meat tenderization process. Some enzymes, including proteases, amylases, and xylanases, are helpful in baking processes to reduce dough fermentation time, along with retention of aromas and moisture levels in baked products. Other cold enzymes may also be used as substitutes for mesophilic and thermophilic enzymes in the brewing and wine industries, cheese manufacturing, animal feed, and so on (Gerday et al., 2000). In food biotechnology, psychrophilic enzymes are mostly used in meat tenderization, food processing, flavoring, baking, brewing, cheese production and animal feed, as summarized in Table 1.2. Analysis of the literature reveals that cold-active enzymes offer several advantages over mesophilic/thermophilic enzymes (Feller and Gerday, 2003; Javed and Qazi, 2016; Kuddus et al., 2011). Most of the cold-active enzymes are characterized by their high-catalytic efficiency at low and moderate temperatures at which homologous mesophilic enzymes are not active (Kuddus, 2015; Kuddus and Ramteke, 2012). The use of psychrophilic enzymes can be advantageous not only for their high specific activity, thereby reducing the amount of enzyme needed, but also for their easy inactivation (Kuddus and Ramteke, 2012). Three important characteristics of cold-active enzymes that increased opportunity of these enzymes in biotechnology are: (1) They are cost effective, as

TABLE 1.2 Some Cold Active Enzymes and Their Source Along With Applications (1995 Onwards)

Cold-Active Enzymes	Source	Possible Application in Food Industry	Reference
α-Amylase, glucoamylase	Various microbes	Cheese ripening Single-cell protein from shellfish waste	Gerday et al. (2000) and Kuddus et al. (2011)
β-Galactosidase	*Paracoccus* sp.	Lactose hydrolysis in milk	Wierzbicka-Wos et al. (2011)
β-Galactosidase	*Arthrobacter* sp. 20B	Dough fermentation Bakery products	Białkowska et al. (2009)
β-Galactosidase	*Carnobacterium piscicola* BA	Dairy industries	Coombos and Brenchley (1999)
β-Galactosidase	*Pseudoalteromonas haloplanktis*	Lactose reduction	Hoyoux et al. (2001)
β-Galactosidase	*Arthrobacter psychrolactophilus*	Conversion of cheese byproduct to glucose and galactose	Nam et al. (2011)
β-Galactosidase	*Guehomyces pullulans*	Hydrolyze lactose	Nakagawa et al. (2006)
β-Lactamase	*Psychrobacter immobilis* A5	Antibiotic degradation	Feller et al. (1997)
Alanine racemase	*Bacillus psychrosaccharolyticus*	Food storage Antibacterial agent	Yokoigawa et al. (2001)
Cellulase	*Fibrobacter succinogenes* S85	Animal feed	Iyo and Forsberg (1999)
Chitinase	Various microbes	Meat tenderizing	Dahiya et al. (2006) and Cavicchioli et al. (2011)
Chitinase A	*Arthrobacter* sp. TAD20	hydrolysis of chitin	Lonhienne et al. (2001)
Chlamysin (lysozyme-like)	*Chlamys islandica*	Antibacterial agent Food preservation	Nilsen et al. (1999)
Laccase	Various microbes	Removal of lactose from milk Conversion of lactose in whey into glucose and galactose in dairy industry	Kunamneni et al. (2008) and Joseph et al. (2008)
Laccase	*Pseudomonas fluorescens* P38	Formation of butyl caprylate as flavor compound	Tan et al. (1996)
Laccase	*Candida antarctica*	Formation of antioxidants to be used in sunflower oil	Buisman et al. (1998)
Lipase	*Aspergillus nidulans*	Food processing	Mayordomo et al. (2000)
Lipase	Various microbes	Protein polymerization and gelling in fish flesh Improvement in food texture Flavor modification Production of fatty acids and inter-esterification of fats	Joseph et al. (2007), Cavicchioli and Siddiqui (2004), and Jaeger and Eggert (2002)

TABLE 1.2 Some Cold Active Enzymes and Their Source Along With Applications (1995 Onwards)—cont'd

Cold-Active Enzymes	Source	Possible Application in Food Industry	Reference
Lipase	*Pseudoalteromonas haloplanktis* TAC125	Animal feed for the improvement of digestibility and assimilation	Tutino et al. (2009)
Metalloprotease	*Sphingomonas paucimobilis*	Bakery Cheese-making Meat tenderization Haze removal from beer Production of fermented foods	Turkiewicz et al. (1999)
Pectate lyase	*Pseudoalteromonas haloplanktis* ANT/505	Cheese ripening Fruit juice and wine industry	Truong et al. (2001)
Pectinase	Psychrophilic yeasts	Starch hydrolysis	Nakagawa et al. (2004)
Pectinase	Various microbes	Degradation of pectin in food processing	Adapa et al. (2014) and Martin and Morata de Ambrosini (2013)
Pectinase (Poly-galacturonase)	*Sclerotinia borealis*	Preparation of cheese Wine and fruit nectar	Takasawa et al. (1997)
Pectinases	*Bacillus* sp. CH15	Red wine making	Martin and Morata de Ambrosini (2013)
Pectin methylesterase	*Penicillium chrysogenum*	Food industry	Pan et al. (2014)
Phytase	*Erwinia carotovora*	Food processing	Huang et al. (2009)
Polygalacturonase	*Cystofilobasidium capitatum* PPY-1	Degradation of pectin compounds	Nakagawa et al. (2005)
Polygalacturonase	*Pseudoalteromonas haloplanktis*	Pectin degradation	Ramya and Pulicherla (2015)
Proteases	Various microbes	In beer, bakeries and cheese industry Tenderization of meat Functional food ingredients in the form of soluble protein hydrolysates	Kuddus and Ramteke (2012)
Xylanase	*Cryptococcus adeliae*	Dough rising Wine and beverages production	Petrescu et al. (2000)
Xylanase	*Pseudoalteromonas haloplanktis* TAH3A, *Flavobacterium* sp. MSY-2	Xylan hydrolysis and improving bread quality	Dornez et al. (2011)
Xylanase	*Pseudoalteromonas haloplanktis*	Baking industry Hydrolyse xylan to xylotriose and xylotetraose	Collins et al. (2005)

fewer enzymes are required to meet the activation energy requirement; (2) They are proficient without additional thermal aid; (3) Due to thermal lability, their selective inactivation can be achieved with less heat input (Javed and Qazi, 2016). Therefore, we can conclude that cold-active enzymes have much more to contribute in the field of food biotechnology. Even though the cold-active enzymes have high-specific activity but short half-life, there is a major disadvantage in the exploitation of these enzymes at the commercial level. To cope with the commercial expectations of cold-active enzymes, various molecular approaches such as protein engineering, r-DNA technology, and the metagenomic approach could be established to achieve qualitative and quantitative improvements and develop radically novel cold-active enzymes. Genetically improved microbial strains, appropriate for specific cold-active enzyme production, would play an important role in food industry.

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CHAPTER

2

Microbial Enzyme in Food Biotechnology

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2.1 INTRODUCTION

The application of enzymes and microorganism in food processing is traditionally a well-known approach. Enzymes and microorganism have been used in the brewing of beer, bread baking, and the cheese and wine making process for ages (Pandey et al., 1999; Pedro, 2010). Food biotechnology is offering various ways to improve the processing of raw materials to convert them into high nutritional value food products (Underkofler et al., 1957). Enzyme technology is improving food quality in various ways, like enhancing functionality, nutritional value, and the flavor and texture of food products. The sources of enzyme production are animal, plant, and microorganism, with microorganisms (bacteria, fungi, yeasts, and actinomycetes) being the best and most suitable sources of commercial enzyme production. Globally, enzymes viz. α-amylase, glucoamylase, lipase, pectinase, chymosin, and protease are use in food-processing industries. The α-amylase enzyme converts starch into dextrins and produces corn syrup for various applications, such as enhancing the sweetness of various food products. The brewing process uses a solubilization of complex carbohydrates into simple form using barley and other cereals grains. In production of good-quality light beer, glucoamylase (hydrolytic enzymes) convert dextrins into glucose in the form of corn syrup, changing the residual dextrins into fermentable sugars. Lipases are fat-hydrolytic enzymes that enhance flavor, shorten the time for cheese ripening, and produce special fat products with better qualities. Pectinase is a hydrolytic enzyme that is used in extraction, clarification, and filtration of fruit juice. Chymosin enzymes help break down kappa-caseins in the milk curding process. Bacterial and fungal proteases enzymes are applied in the production of fish meals, meat extracts, texturized proteins, and meat extenders. Lactase enzymes break down the lactose present in whey and milk products to produce polyactide. Glucose oxidases are used to change glucose into gluconic acid to stop maillard reactions. Acetolactate decarboxylase converts acetolactate to acetoin to minimize the maturation time in wine making. Cellulase is a nonstarch polysaccharide-solubilizing enzyme that helps in the conversion of

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https://doi.org/10.1016/B978-0-12-813280-7.00002-5

cellulose to glucose in cell walls in the breakdown of various grains; they facilitate better extraction of cellular products and release nutrients to increases the fiber content in foods (Christopher and Kumbalwar, 2015). Enzyme technologies are positively affecting the food manufacturing industry by providing new and valuable products, lowering production costs, and improving processes.

2.2 SOURCES OF ENZYMES

Initially, enzymes were extracted from the stomach of calves, lambs, and baby goats, but now are produced by microorganisms like bacteria, fungi, yeast, and actinomycetes. Enzymes obtained from microorganisms are better than those of animal and plant origin. Microorganisms can be genetically manipulated to improve the production of commercial scale (Pandey et al., 1999; Sabu, 2003). Enzymes can hydrolyze complex molecules into simple monomer units, like carbohydrates into simple sugars, which are natural substances involved in all types biochemical processes. Every enzyme is substrate, pH, and temperature-specific for catalyzing the reaction to convert a reactant into a product (Afroz et al., 2015; Shuang et al., 2012). The food-processing industry uses more than 55 different microbial enzymes (Table 2.1).

2.3 PRODUCTION PROCESSES OF ENZYMES

There are two main parts of industrial enzyme production: (i) screening of potential microbial strains; (ii) the fermentation process. Production media composition is another important component of commercial enzyme production; suitable media contains carbon sources, nitrogen sources, and micronutrients which support the growth of microorganisms in the fermentation process. Once fermentation is complete to start the downstream process, like recovery of the enzyme, purification and formulation of products can occur within a suitable carrier (Fish and Lilly, 1984). The fermentation process is categorized into three groups: (i) batch process,

TABLE 2.1 An Overview of Enzymes Used in Food Processing Industry

S.N.	Class	Enzyme	Role
1.	Oxidoreductases	Glucose oxidase	Dough strengthening
		Laccases	Clarification of juices, flavor enhancer (beer)
		Lipoxygenase	Dough strengthening, bread whitening
2.	Transferases	Cyclodextrin	Cyclodextrin production
		Glycosyltransferase	
		Fructosyltransferase	Synthesis of fructose oligomers
		Transglutaminase	Modification of viscoelastic properties, dough processing, meat processing

TABLE 2.1 An Overview of Enzymes Used in Food Processing Industry—cont'd

S.N.	Class	Enzyme	Role
3.	Hydrolases	Amylases	Starch liquefaction and sachcarification, Increasing shelf life and improving quality by retaining moist, elastic and soft nature, Bread softness and volume, flour adjustment, ensuring uniform yeast fermentation, Juice treatment, low calorie beer
		Galactosidase	Viscosity reduction in lupins and grain legumes used in animal feed, enhanced digestibility
		Glucanase	Viscosity reduction in barley and oats used in animal feed, enhanced digestibility
		Glucoamylase	Saccharification
		Invertase	Sucrose hydrolysis, production of invert sugar syrup
		Lactase	Lactose hydrolysis, whey hydrolysis
		Lipase	Cheese flavor, in-situ emulsification for dough conditioning, support for lipid digestion in young animals, synthesis of aromatic molecules
		Proteases	Protein hydrolysis, milk clotting, low-allergenic infant-food formulation, enhanced digestibility and utilization, flavor improvement in milk and cheese, meat tenderizer, prevention of chill haze formation in brewing
		Pectinase	Mash treatment, juice clarification
		Peptidase	Hydrolysis of proteins (namely, soy, gluten) for savoury flavors, cheese ripening
		Phospholipase	In situ emulsification for dough conditioning
		Phytases	Release of phosphate from phytate, enhanced digestibility
		Pullulanase	Saccharification
		Xylanases	Viscosity reduction, enhanced digestibility, dough conditioning
4.	Lyases	Acetolactate decarboxylase	Beer maturation
5.	Isomerases	Xylose (Glucose) Isomerase	Glucose isomerization to fructose

Source: Bloom, J.D., Meyer, M.M., Meinhold, P., Otey, C.R., MacMillan, D., Arnold, F.H., 2005. Evolving strategies for enzyme engineering. Curr. Opin. Struct. Biol. 15, 447–452; Fernandes, P., 2010. Enzymes in food processing: a condensed overview on strategies for better biocatalysts. J. Enzym. Res. 2010, 1–19; Riberiro, D.S., Henrique, S.M.B., Oliveira, L.S., Macedo, G.A., Fleuri, L.F., 2010. Enzymes in juice processing: a review. Int. J. Food Sci. Technol. 45, 224–230.

(ii) fed batch process, and (iii) continuous process. In the batch process, all media components are added at the start of fermentation. Fed batch fermentation is similar to batch fermentation but the production strain is fed with an additional nutrient medium during the fermentation process. Continuous fermentation is a steady state reached by supplying the fresh medium with a simultaneous harvest from the fermenter. The fermentation process can be conducted in

one of two ways—either solid-state fermentation, or submerged fermentation (Rana and Bhat, 2005). Bacterial enzyme production is almost exclusively achieved by submerged fermentation because bacterial cell growth and enzyme secretion are more suitable in a submerged condition than during solid-state fermentation (Aunstrup, 1979). In submerged fermentation, sterilized production media are inoculated by bacterial strains and maintain proper fermentation parameters like aeration, agitation, dissolved oxygen, rotation, temperature, and pH for 48–72 h, depending upon the bacterial strain. However, solid-state fermentation processes are suitable for fungal strains to produce valuable industrial enzymes for various food processing techniques. Fungal filament prefers surface fermentation, or solid-state fermentation, for the production of enzymes, and others use full metabolite. Sterilized solid substrate, like wheat bran, rice bran, and many other grains, support the growth of fungal cell mass at a suitable temperature, humidity, and moisture of the fermentation system. After specific periods of fermentation, the products are harvested and continue downstream processing, like filtration of cell debris, purification, and formulation of enzymatic products. Generally, downstream processing of intracellular enzymes is fairly complex as compared to extracellular enzyme. The purification of enzymes is a very expensive step in downstream processing; techniques like chromatography are mainly used in the purification of enzymatic proteins at the commercial level (Linder et al., 2004). The main issue of the formulation of purified enzymes with a suitable carrier to secure enzymatic activity and stability, to insure enzyme easily release at the site of application. Otherwise the efficiency of the enzymatic product is decreased.

2.4 APPLICATION OF ENZYMES IN FOOD PROCESSING

Enzymes are secreted by nearly all living cells for catalysis of their own specific biochemical reactions in the metabolic process. Enzymes are playing an important role in food processing techniques for improving nutritive value and flavor of processed food. The food-processing industry—the making of cheese, leavened bread, wine and beer, yogurt, and syrup—is successfully using enzymes at the commercial level (Dewdney, 1973).

2.4.1 Glucose Oxidase

The glucose oxidase enzyme is commercially produced from *Aspergillus niger* and *Penicillium glaucum* through a solid-state fermentation method. Muller was first to report the catalyzation of glucose oxidase and the breakdown of glucose into gluconic acid in the presence of dissolved oxygen (Muller, 1928). Fungal strains *Aspergillus niger* are able to produce notable amounts of glucose oxidase. Glucose oxidase enzymes are used to remove small amounts of oxygen from food products or glucose from diabetic drinks. Glucose oxidase is playing an important role in color development, flavor, texture, and increasing the shelf life of food products (Khurshid et al., 2011).

2.4.2 Laccase

Laccase enzymes were first obtained from the cell sap of the Japanese lacquer tree. Laccase enzymes are isolated from plants, bacteria, fungi, and insects (Saqib et al., 2012). Laccase is

responsible for discoloration, haze, wine stabilization, baking, and flavoring in food processing (Rosana et al., 2002). Laccase improves the baking process through an oxidizing effect, and provides an additional development in the strength of dough and baked products, including enhancing crumb structure and increasing softness and volume. Another diverse application of laccase is in environmental sectors, which degrade various ranges of xenobiotic compounds.

2.4.3 Cyclodextrin Glycosyl Transferase

Cyclodextrin glycosyl transferase (CGTase) enzymes catalyze the change of starch into nonreducing cyclic sugars (cyclodextrin) (Coelho et al., 2016). Cyclodextrins (CD) are cyclic homogeneous oligosaccharides of glucose residues, which are composed of 6–8 D-glucose units linked by a -1,4 glycosidic bond. Cyclodextrins are being used in the food-processing industry for preparation of reduced-cholesterol products and rising bioavailability of desired molecules, because cyclodextrins facilitate hydrophobic-hydrophilic interactions within protein-protein and other molecules. Production of Cyclodextrin glycosyl transferase (CGTase) is reported in different bacterial groups; major CGTase producers belong to the genus Bacillus. However, *Klebsiella pneumonia*, *Micrococcus luteus*, *Thermococcus*, *Brevibacterium* sp., and hyperthermophilic archaea are reported as major CGTase-producing strains (Mori, 1999; Szerman et al., 2007; Tachibana, 1999).

2.4.4 Transglutaminase

Transglutaminase enzymes catalyze reactions to alter proteins by merging amine, crosslinking, and deamination. Transglutaminase is responsible for acyl transfer, deamidation, and the inter- and intra-molecular crosslink between amino acid residues of glutamine and lysine (Chanyongvorakul et al., 1995; Christensen et al., 1996; Kuraishi et al., 1997). The commercial application of transglutaminase enzymes in the food-processing industry is improving the protein-emulsifying capacity, gelatation, viscosity, and production of various types of protein ingredients to enhance the quality of food products. Transglutaminase is enhancing the water-holding capacity, softness, foam formation, and stability of food products. Extracellular transglutaminase is isolated from cultural filtrate of *Strepto verticillium* spp., *Strepto verticillium mobarens*, *Strepto verticillium ladakanum*, and *Strepto verticillium lydicus* (Ando et al., 1989; Dickinson, 1997; Jiang et al., 2000; Motoki et al., 1984; Tsai et al., 1996; Zhu, 1995). Intracellular transglutaminase is secreted by common microbial species *Bacillus subtilis* and *spherules* (Tsai et al., 1996).

2.4.5 Lactase

Lactase enzymes catalyze the breakdown of the milk sugar lactose into simple sugar monomer units like glucose and galactose. Lactases are obtained from plants, animal, bacteria, fungus, yeasts, and molds. Commercial production of lactase enzymes are developed from *A. niger*, *A. oryzae*, and *Kluyveromyces lactis* (Mehaia, 1987). Fungal origin lactases have optimum activity at acidic pH ranges, and yeast and bacterial-originated lactases have optimum pH ranges near to neutral (Gekas and Lopez-Levia, 1985). The lactase enzyme is predominantly

rich in infancy and is called a brush border enzyme. Some people do not produce enough of the lactase enzyme so they do not properly digest milk. This is called lactose intolerant, and people who are lactose intolerant need to supplement the lactase enzyme to aid in the digestion of milk sugar. Another useful application of the lactase enzyme is it increases the sweetness of lactase-treated milk, and assists in the manufacturing of ice cream and yogurt preparation.

2.4.6 Catalase

Catalase enzymes break down hydrogen peroxide (H_2O_2) to water and oxygen molecules, which protects cells from oxidative damage by reactive oxygen species. Commercial catalases are produced from *Aspergillus niger* through a solid-state fermentation process (Fiedurek and Gromada, 2000). The major applications of catalase in the food-processing industry include working with other enzymes like glucose oxidase, which is useful in food preservation and egg processing, and sulphydryl oxidase, which under aseptic conditions, can eliminate the effect of volatile sulphydryl groups, that is, they generate from thermal induction and are responsible for the cooked/off-flavor in ultra-pasteurized milk (Maur, 1996).

2.4.7 Lipase

Lipases catalyze the hydrolysis of ester bonds in lipid substrates and play a vital role in digestion and the transport and processing of dietary lipids substrate (Svendsen, 2000). Lipases catalyze the biochemical reaction like esterification, interesterification, and transesterification in nonaqueous media which frequently hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Microorganism like *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcoccus aureus*, and *Bacillus subtilis* are the best sources of lipase enzymes. Lipases are widely used in pharmacological, chemical, and food industries. The commercial applications of lipases in the food industry are the hydrolysis of milk fats, pronounced cheese flavor, low bitterness, and prevention of rancidity. Lipases may combine with many other enzymes like protease or peptidases to create good cheese flavor with low levels of bitterness (Wilkinson, 1995).

2.4.8 Protease

Proteolytic enzymes are also termed as peptidases, proteases, and proteinases, which are able to hydrolyze peptide bonds in protein molecules. Proteases are generally classified as endopeptidases and exopeptidases. Exopeptidases cut the peptide bond proximal to the amino or carboxy termini of the protein substrate, and endopeptidases cut peptide bonds distant from the termini of the protein substrate. Proteases are obtained from diverse groups of organisms such as plants, animals, and microorganisms, but commercially viable proteases are obtained from microorganisms, especially bacterial and fungal species. Microorganisms secrete the extracellular and intracellular proteases in both the submerged and solid-state fermentation process. Bacillus species of bacteria, like *Bacillus licheniformis, Bacillus subtilis,* and *Aspergillus* species of fungus like *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. oryzae*, are the best sources of protease enzyme. Broad working range of temperature (10–80°C) and pH (4–12) of protease enzymes increases their application in the food-processing industry, the

major role in cheese and dairy product manufacturing. Aminopeptidases are significantly improving the flavor in fermented milk products. Other basic applications of proteases in the food-processing industry are to increase the nutritive value of bread, baked goods, and crackers (Law and Haandrikman, 1997).

2.4.9 α-Amylase

Amylase enzymes hydrolyze complex starch molecules into simple monomer units of glucose. Sources of α-amylase are plants, animals, and microorganisms, but commercially viable amylases are produced from microorganisms, especially bacterial and fungal species. Thermostable α amylase is produced by some potential bacterial species like *Bacillus licheniformis* and *Bacillus stearothermophillus*, *Pseudomonas*, and the *Clostridium* family. Starch-converting properties of α-amylases are playing an important role in the food, beverage, and sugar industries. α-Amylase is improving the quality of breads that have reduced size and poor crust color, and compensates for the nutritional deficiencies of the grain. α-Amylase also degrades the starch in wheat flour into small dextrins, thus allowing yeast to work continuously during dough fermentation, proofing, and the early stages of the baking process. α-Amylases are also employed in many other aspects of the food industry like clarification of beer, fruit juices, and pretreatment of animal feed to improve the digestibility of fiber (Ziegler, 1999).

2.4.10 Pectinase

Pectinase breaks down pectin components, which are found in the middle lamella of plant cell walls. Pectin is made up of complex colloidal acid polysaccharides with a back bone of galacturonic acid residue with a α-1-4 linkage. Pectinase therefore helps to break down plant cell walls to extract cell sap. Potential microbial strains like *Moniliella SB9*, *Penicillium* spp. and *Aspergillus* spp. are good sources of commercial pectinase (Priya and Sashi, 2014). Pectinases are now an essential part of the fruit juice industry, as well as having various biotechnological applications in the fermentation of coffee and tea, the oil extraction processes, and the treatment of pectic waste water from the fruit juice industry. Pectinase is lowering down the viscosity of fruit juice during the clarification process through the degradation of pectin substance in fruit juice and getting better pressing ability of pulp, simultaneously jelly structure are breaking down and increases the yields of fruit juice (Dupaign, 1974). Another significant application of pectinase enzymes in industrial processes is the refinement of vegetable fibers during the starch manufacturing process, such as the curing of coffee, cocoa and tobacco, canning of orange segments, and extracting sugar from date fruits.

2.4.11 Acetolactate Decarboxylase

Acetolactate decarboxylase catalyzes the conversion of acetolactate into acitoine and release carbon dioxide, a type of decarboxylation reaction. α-Acetolactate decarboxylase is commercially produced by the submerged fermentation of *Bacillus subtilis*, genetically improved *Bacillus brevis* and *Enterobacter aerogenes* strain 1033. In conventional brewing procedures, α-diacetyl is produced from α-acetolactate and this further reduces to acetoin over

a 2–4 week maturation period, but α-acetolactate decarboxylase causes direct decarboxylation of α-acetolactate to acetoin and avoiding maturation period.

2.4.12 Xylose (Glucose) Isomerase

Xylose isomerase (D-xylose ketol-isomerase) catalyzes the isomerisation reaction of D-xylose into xylulose. This is initial step of xylose metabolism in microbial cell physiologies (Wovcha et al., 1983). Xylose isomerases are also referred to as glucose isomerases because of their capability to exchange D-glucose into D-fructose. Microorganisms are most suitable sources of xylose isomerase; some potential microbial species are *Streptomyces olivochromogenes*, *Bacillus stearothermophilus*, *Actinoplanes missouriensis*, *Thermotoga maritime* and *Thermotoga neapolitana*, known xylose isomerase procurers. Xylose isomerase loses its catalytic activities up to 50% under acidic conditions (Oshima, 1978). The greatest application for glucose isomerase is in the food-processing industry; it mainly catalyzes two significant reactions such as reversible isomerization of D-glucose to D-fructose, and D-xylose to D-xylulose.

2.5 FUTURE ASPECTS OF ENZYMES IN FOOD PROCESSING

Food and feed processing areas are having great success using biological agents like enzymes and microorganisms for the manufacturing of valuable food products (Pedro, 2010). In the future, recombinant strains will be playing a significant role in the production of industrial enzymes, but currently there is some hesitation applying them to genetically modified food products. There is no doubt that the genetically engineered production culture is superior to the wild strain. The sophisticated fermentation techniques and downstream processing will also provide support to the manufacturing of pure and large-scale food processing enzymes. There is a need to develop a novel food processing technology to reduce the cost and time of manufacturing processed food items. Therefore, more advanced research is needed in the area of recombinant DNA technology for improvement of production strains, commercially viable enzyme production, and the development of new food-processing techniques for maximum cost-effective product formulation.

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CHAPTER

3

Enzymes in the Beverage Industry

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3.1 INTRODUCTION

There is a growing interest in food and beverage industries due to increasing consumer demand around the world. One of the largest food processing industries is the beverage industry. The food and beverage processing sector are classified into two main groups: alcoholic and nonalcoholic drinks. Alcoholic drinks comprise beer, wine, whiskey, etc., whereas nonalcoholic drinks consist of fruit juice and soft drinks. Other beverages are coffee and tea. Carbonated soft drinks (48.8 gal per person) are consumed the most, followed by bottled water (29.1 gal per person), coffee (24.6 gal per person), and beer (21.8 gal per person) (Chandrasekaran et al., 2016). Food and beverage processing sectors use raw food and materials via some important steps (extraction and maceration). Food, especially fruit and vegetables, contain polysaccharides such as pectin and starch which cause a fouling problem during filtration process (Rai et al., 2004). The presence of cell debris and small insoluble pectin species are known to be responsible for the immediate turbidity in freshly pressed fruit juices, whereas haze development may result from prior polymerization or condensation leading to the formation of polymeric complexes between polysaccharides, sugars, metal ions, and proteins. Protein-polyphenol interactions are considered as the most frequent cause of haze formation in beer, wine, and clear fruit juices. For commercial use, fruit juices are processed with chemical-fining agents such as bentonite and gelatin (Siebert and Lynn, 1997). However, use of enzymes instead of chemicals is a cost-effective option, which improves yields in terms of reducing carbon footprint, energy consumption, and environmental pollution (Chandrasekaran et al., 2016). Enzymes are used in food as food additives and processing aids. Most food enzymes are used as processing aids, whereas a few are used as additives (such as lysozyme and invertase) (Adrio and Demain, 2014). Among all, amylases, pectinases, and cellulases are extensively used in the beverage industry for improving yield of extraction, clarification, aroma enhancement, and other uses.

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00003-7

In this chapter, various aspects of enzymes used in beverage production (alcoholic and nonalcoholic drinks) are mentioned with a focus on three points. First, the current status of representative enzymes is stated with respect to their role and action of mechanism, source, and advantages in beverage production. Second, enzyme applications in beverage industries are reviewed. Third, the economy and possibilities for further improvement are discussed at the end of the chapter.

3.2 ENZYMES USED IN BEVERAGE INDUSTRIES

3.2.1 Enzymes Used in Fruit and Vegetable Juice Industries

In recent years, there has been an increase in the consumption of natural fruit juices. Based on the National Nutrient Database for Standard Reference (US), carbohydrate content is very high in raw apples (13.8%), pineapples (13.1%) and tomato (4.0%). Fruits and vegetables contain high amounts of polysaccharides such as pectin, cellulose, hemicellulose, and lignin, which may lead to haze and turbidity in the freshly pressed juice (Table 3.1) (Vaillant et al., 2001). As in other juice manufacturing processes, clarification is a fundamental step in the processing of juices to remove turbidity, haze, and sediments in the final product, and it is often achieved through enzymatic treatment, membrane filtration, or the addition of clarifying aids such as chitosan, gelatin, bentonite, polyvinyl pyrolidone, or synergistically combining two clarifying aids or compounds (Gainvors et al., 1994). Of all, enzymatic treatment is

TABLE 3.1 Composition of Some Fruits and Vegetables

	Polysaccharides			
Fruit or Vegetable	**Cellulose (%)**	**Hemicellulose (%)**	**Pectin (%)**	**Reference**
Cabbage	63.0	15.2	6.8	Kahlon et al. (2008)
Cauliflower	35.0	13.6	5.5	Femenia et al. (1997)
Olive	31.9	21.9	16.2	Rodriguez et al. (2008)
Carrot pomace	51.6	12.3	3.88	Nawirska and Kwasniewska (2005)
Tomato pomace	19.0	12.0	7.55	Andres et al. (2017)
Black currant	12.0	25.3	2.73	Wawer et al. (2006) and Nawirska and Kwasniewska (2005)
Grape pomace	17.8	31.0	4.0	Botella et al. (2005)
Mango pulp	11.5	7.0	3.9	Gourgue et al. (1992)
Peach pomace	30.0	20.0	23.8	Augusto et al. (2011)

Modified from Toushik, S.H., Lee, K.-T., Lee, J.-S., Kim, K.-S., 2017. Functional applications of lignocellulolytic enzymes in the fruit and vegetable processing industries. J. Food Sci. 82 (3), 585–593.

TABLE 3.2 Some Enzymes Used in Food Beverage Industry in Terms of Their Mode of Action and Applications

Industry	Product	Enzyme	Action Mechanism	Application
Juice Industry	Juice	Amylases Glucoamylases	Breaks down starch into glucose	Sugar production and increases yield of fruit jucies
		Pectinases	Degrading pectins	Fruit juice clarification
		Cellulases, Hemicellulases	Hemicellulose hydrolysis	Acting on soluble pectin hydrolysis and on cell wall composition with pectinases, lowering viscosity
Brewing	Mashing	α-Amylases β-Amylases	Hydrolyzing glucans into soluble oligomers	– –
	Wort	Pullulanases	Hydrolyzing α-1,6 branch points of starch	–
	Beer	Amyloglucosidases		Increasing glucose content in light beer
Instant tea	–	Tannase	–	Improved solubility
Coffee	–	Pectinase, hemicellulase	–	Coffee bean fermentation
Wine	Wine	Anthocyanase	–	Decolorization of wine

advantageous, providing an increase in extraction yield, reducing sugars, soluble dry matter content, and galacturonic acid content of the products (Joshi et al., 1991).

Table 3.2 lists some of the enzymes used in the food and beverage industries and their modes of action and applications. A considerable challenge in fruit processing is the occurrence of a stable cloud in juice due to high pectin content (Sakhale et al., 2016). Thus clarification of fruit juices is desirable and required for compliance with international standards. Effective enzymatic clarification subsequently leads to a higher juice yield and short clarification process (Sandri et al., 2011; Sandri et al., 2013). The total value of the food and beverage enzyme market is expected to grow to $6.30 billion by 2021. Leading food enzyme manufacturers around the world are Denmark (especially Novozymes), Switzerland, Germany (AB Enzymes), the Netherlands, and the United States (especially DuPont) (Chandrasekaran et al., 2016). Among the food enzymes, pectinases (or pectinolytic enzymes) account for 25%. Pectinolytic enzymes are produced from plants and microorganisms such as bacteria, yeasts, and molds (Khaimar et al., 2009). The major sources of plant pectinases are tomatoes and oranges (Torres et al., 2005). In contrast to plant and animal sources, pectinases derived from microorganisms have advantages such as cheap production, easier gene manipulations, faster product recovery, and they are free of harmful substances over plant- and animal-derived pectinases (Chaudhri and Suneetha, 2012). Almost all commercial pectinolytic enzymes are produced by fungi, namely, *Aspergillus* sp., *Aspergillus japonicus*, *Rhizopus stolonifer*, *Alternaria mali*, *Fusarium oxysporum*, *Neurospora crassa*, *Penicillium italicum* ACIM F-152, and many others (Jayani et al., 2005). Some of the bacterial species producing

pectinases are *Agrobacterium tumefaciens*, *Bacteroides thetaiotamicron*, *Ralstonia solanacearum*, and *Bacillus* sp. (Jayani et al., 2005). Pectinolytic enzymes are produced by two different methods: submerged fermentation (SmF) and solid-state fermentation (SSF). The industrial application of SSF suffers from complicated product purification resulting from a heterogeneous fermentation medium, difficulty of scale up, and losses of enzyme in the solid residues (Pedrolli et al., 2009). However, SmF is easier to control at a large scale and is already used for production of various metabolites since the 1940s. Moreover, enzyme production should be cost-effective. For this purpose, abundant and low-cost raw materials should be utilized. For SmF to be commercially viable, pectinases were produced on low-cost carbon sources such as *Citrus limetta* peel (Joshi et al., 2013), orange peel extract (Rangarajan et al., 2010), mix of apple pulp and corn flour (Mojsov, 2010), wheat bran (Ahlawat et al., 2009), pumpkin oil cake (Pericin et al., 2007), and other agricultural wastes. Therefore, it is of great importance to produce cost-effective commercial enzymes in order to meet the increasing demands toward these novel biocatalysts. Kertesz (1930) reported that the first commercial pectinase had been used in the clarification of apple juice. A list of manufacturers for a few commercial pectinases is given in Table 3.3.

3.2.1.1 Pectinase

Pectin is a complex polysaccharide, which constitutes linear chains of α-(1–4)-linked D-galacturonic acid D-xylose (xylogalacturonan) or D-apiose (apiogalacturonan), branching from the D-galacturonic acid backbone. Pectin is found in cell wall and the middle lamella of fruits and vegetables (Saad et al., 2007). The pectic substances account for about 0.5%–4% of the weight of fresh material (Jayani et al., 2005). It is hard to extract juice by pressing due to highly viscous fruit purees after the fruits have been mechanically crushed. The pectin separates between the liquid and the pulp particles to cause an increase in the viscosity of the juice and makes water retention easy (Lanzarini and Pifferi, 1989). To enhance the juice yield with aromatic and nutritional quality, it is necessary to degrade the pectin. For this reason, pectic enzymes help to degrade the pectin. Pectins bind the water due to presence of

TABLE 3.3 Commercial Pectinases and Their Manufacturers

Product Name	Trade Supplier	Source Microorganism	Recommended pH/Temp.	Action Pattern
Grindamyl3PA	Danisco, Denmark	*A. niger*	4.0/55°C	PL
Pectinase CCM	Biocon, India	*A. niger*	4.0/50°C	PG
			6.0/40°C	PL
Pectinex 3XL	Novozyme, Denmark	*A. niger*	4.7/50°C	PG
			5.0–6.5/35°C	PL
Rapidase C80	Gist Brocades, Holland	*A. niger*	4.0/55°C	PG
			6.0/40–45°C	PL

Modified from Pedrolli, D.B., Monteiro, A.C., Gomes, E., Carmona, E.C., 2009. Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. Open Biotechnol. J. 3, 9–18.

hydrophilic (–OH) groups. Pectic enzymes disrupt these bonds to release the water during extraction process (Scott and Eagleson, 1988). Moreover, this enzyme degrades the plant cell wall to release these phytochemicals (such as flavonoids and other phenolics) which are located in the cell walls (Landbo and Meyer, 2001). Enzymes cleaving pectic substances are called pectinolytic enzymes, or pectinases, which are of great industrial importance (Fogarty and Kelly, 1983; Saad et al., 2007). Pectinases are classified as pectinesterases, depolymerizing enzymes, and protopectinases based on their role in the degradation of pectin (Jayani et al., 2005). Different pectic substances and their modes of action are illustrated in Fig. 3.1. Pectinesterases, or pectin methyl hydrolases (PE or PMH), catalyze hydrolytic removal of methyl ester groups of pectin, forming pectic acid. Depolymerizing enzymes are hydrolytic enzymes cleaving the glycosidic linkages (Kashyap et al., 2001). Hydrolysis of glycosidic linkages requires use of polymethylgalacturonase (PMG) and polygalacturonase (PG) enzymes. PMG catalyzes the hydrolytic cleavage of α-1,4-glycosidic bonds and is classified as *endo*-PMG that causes random cleavage of α-1,4-glycosidic linkages of pectin, preferentially highly esterified pectin, and *exo*-PMG that causes sequential cleavage of α-1,4-glycosidic

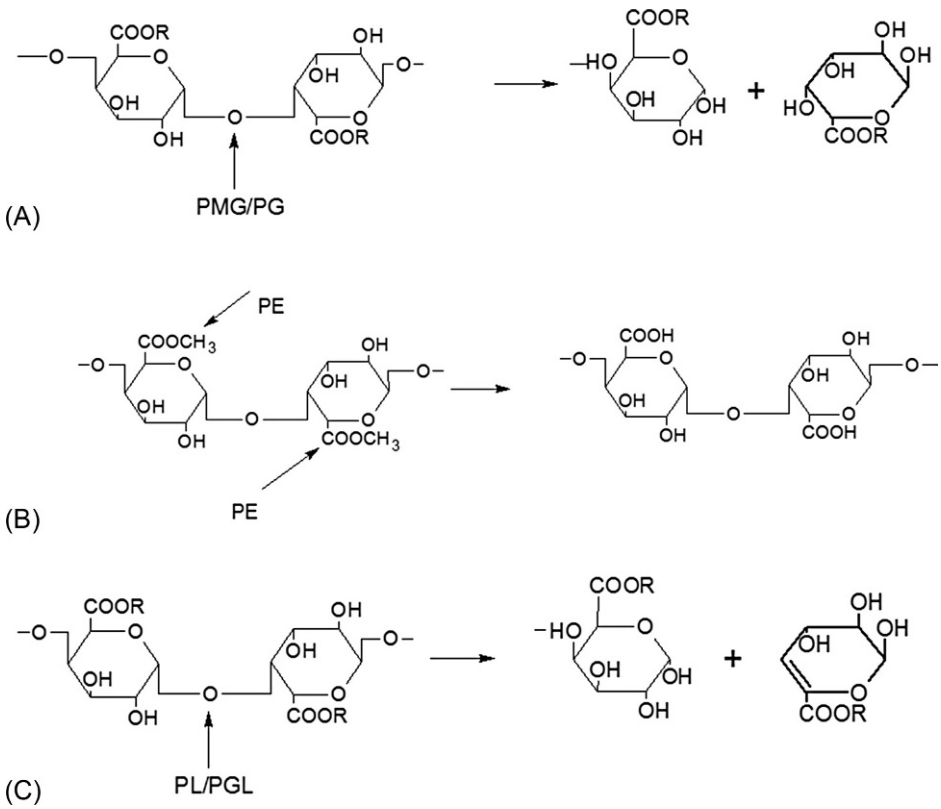

FIG. 3.1 Mode of action of pectinases: (A) R=H for PG and CH_3 for PMG; (B) PE; and (C) R=H for PGL and CH_3 for PL. The *arrow* indicates the place where the pectinase reacts with the pectic substances. *PMG*, polymethylgalacturonases; *PG*, polygalacturonases; *PE*, pectinesterase; *PL*, pectin lyase (Jayani et al., 2005).

linkage of pectin from the nonreducing end of the pectin chain (Kashyap et al., 2001). PG, which catalyzes hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid), are the most abundant pectinolytic enzymes (Jayani et al., 2005). This enzyme is also known as *endo*-PG, or poly (1,4-α-D-galacturonide) glycanohydrolase, and catalyzes random hydrolysis of α-1,4-glycosidic linkages in pectic acid and *exo*-PG, or poly (1,4-α-D-galacturonide) galacturonohydrolase, which catalyzes hydrolysis of α-1,4-glycosidic linkages on pectic acid in a sequential fashion (Kashyap et al., 2001).

Cleavage of α-1,4-glycosidic linkages in pectic acid and pectin by trans-elimination, which forms galacturonide with an unsaturated bond between C4 and C5 at the nonreducing end of the galacturonic acid, is catalyzed by polymethylgalacturonate lyases (PMGL) and polygalacturonate lyases (PGL). PMGL catalyzes the breakdown of pectin by trans-eliminative cleavage and is classified as *endo*-PMGL and *exo*-PMGL. Endo-PMGL, also known as poly (methoxygalacturonide) lyase, catalyzes random cleavage of α-1,4-glycosidic linkages in pectin (Fig. 3.1A). Exo-PMGL catalyzes stepwise breakdown of pectin by trans-eliminative cleavage. On the other hand, PGL catalyzes the cleavage of α-1,4 glycosidic linkage in pectic acid by trans- elimination and is also classified as *endo*-PGL and *exo*-PGL. Endo-PGL, known as poly (1,4-α-D-galacturonide) endolyase, catalyzes random cleavage of α-1,4-glycosidic linkages in pectic acid. Exo-PGL, known as poly (1,4-α-D galacturonide) exolyase, catalyzes sequential cleavage of α-1,4-glycosidic linkages in pectic acid (Fig. 3.1C). Protopectinases solubilizes protopectin and forms highly polymerized soluble pectin (Kashyap et al., 2001).

Pectinases are mainly used for increasing filtration efficiency and clarification of fruit juices (Alkorta et al., 1998) and is used in maceration, liquefaction, and extraction of vegetable tissues (Bohdziewiez and Bodzek, 1994). Several studies have reported that depectinization (called clarification hereafter) of juices with pectinases is an efficient alternative for the food industry to provide a more rapid flow of juice, higher juice yields, facilitating filtration, and greater clarity (Alkorta et al., 1998; Naidu and Panda, 1998; Sandri et al., 2011; Sandri et al., 2013). In the study by Uzuner and Cekmecelioglu (2015), carrot juice was treated with the crude pectinase enzyme (5.60 U/mL) at different concentrations (0.1%–0.5%), pH (4–7), and time (2–6 h), and they reported optimal clarification conditions of 0.5% (*w*/*v*) enzyme load, 7.0 pH and 6 h of time, which corresponds to 100% clarity in final juice samples. In another study, Bhardwaj and Garg (2014) reported 45.3 and 49.9% of juice yields for carrot pulp treated with pectinase from *Bacillus* sp. MBRL576 and commercial pectinase for 2 h at 40°C, respectively. Swain and Ray (2010) illustrated that applications of crude polygalacturonase from *Bacillus subtilis* CM5 resulted in a 13.1% increase in yield of carrot juice compared to the juice extracted with Pectinex. Sin, et al. (2006) investigated the effects of pectinase enzyme concentration (0.03%–0.10%), incubation time (30–120 min) and temperature (30–50°C) on Sapodilla juice in terms of turbidity, clarity, viscosity, and color (L values). According to Sin et al. (2006), optimal conditions were reported as 0.1% enzyme load, 40°C of temperature, and 120 min of time for clarification of sapodilla juice subjected to pectinase enzyme at different incubation times (30–120 min), temperature (30–50°C) and enzyme load (0.03%–0.10%). Moreover, cocktail enzymes including pectinase, cellulase and xylanase enzymes can break down the cell wall and increase the overall juice production. Lien and Man (2010) investigated the effect of commercial sources of enzymes (such pectinase, cellulase, α-amylase) on the production of jicama juice (*Pachyrhizus erosus* L.) and reported increased extraction yield of 92.7% with an

TABLE 3.4 Some Fruit and Vegetable Juice Using Cocktail Enzymes

Fruit/Vegetable	Enzymes	Incubation Time (min)/Temperature (°C)	Juice Recovery (%)	Reference
Date (*Phoenix dactylifera* L.)	Pectinase and cellulase	120 min/50°C	72.4	Abbes et al. (2011)
Kiwi	Pectinase, amylase and cellulase	120 min/50°C	78.5	Vaidya et al. (2009)
Pineapple	Pectinase and cellulase	30 min/27–30°C	73.5	Anastasakis et al. (1987)
Plum	PME and PG	120 min/50°C	96.8	Mieszczakowska-Frac et al. (2012)

Modified from Sharma, H.P., Patel, H., Sugandha, 2017. Enzymatic added extraction and clarification of fruit juices-a review. Crit. Rev. Food Sci. Nutr. 57 (6), 1215–1227.

enzyme cocktail containing pectinase, cellulose, and α-amylase. It has been shown that using pectinase, cellulose and amylase in extraction of fruit juice increases juice yield (Table 3.4). Using a combination of pectinase (0.05 g/kg), amylase (0.025 g/kg) and cellulase (0.025 g/kg) in kiwi showed very high juice yield (78.5%) compared to a controlled sample (58.4%) (Table 3.4). Sreenath et al. (1984) showed that juice recovery of pineapple was found as 74.8% using a combination of pectinase and cellulose.

3.2.1.2 *Cellulase*

Cellulase enzymes degrade cellulose to glucose. Cellulase includes three enzymes such as β-1,4-endoglucanase, cellobiohydrolase, and β-glucosidase (Jecu, 2000). Cellulases are produced by bacteria and fungi (Sharma et al., 2017). Microbial cellulases are produced from fungi especially *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus oryzae* (Sukumaran et al., 2005). Cellulase used in fruit juices and wine processing improves the extraction and clarification of juices and maceration of fruit nectars by breaking the cellulose chains in plant tissues (Juturu and Wu, 2014). Cellulases can aid in the pressing step to increase yields.

3.2.1.3 *Amylase*

Besides pectin, which is responsible for turbid fruit juices, another cause of cloudiness in fruit juice is starch (Dey and Banerjee, 2014). Starch decreases filtration and increase the membrane fouling, haze, and gelling (Carrin et al., 2004). Starch can be degraded by amylase. Amylases comprising about 25%–33% of the enzyme market hydrolyze glycosidic bonds of starch to sugars. For microbial amylase production, bacteria and fungi are used (Sharma et al., 2017). Amylase is used in clarification of fruit juice (Couto and Sanroman, 2006). Cellulase, xylanase, pectinase, and amylase are called macerating enzymes and are used for increasing extraction and clarification yield of fruit and vegetable juices (Galante et al., 1998). α-Amylase is used to degrade starch-containing fruits, especially apples (early stage of harvest), for preventing haze formation (Uhlig, 1998). Dey and Banerjee (2014) showed that starch concentration in unclarified apple juice was 1.143 g/L. After adding an amylase concentration (0.4%), starch content reduced to 0.33 g/L.

3.2.1.4 Xylanase

Fruits and vegetables consist of 15%–19% hemicellulose by dry weight (Luis et al., 2010). The most abundant hemicellulose is xylan (Vicente et al., 2009). Xylanases are glycosidases, which catalyze *endo*-hydrolyis of 1,4-D-xylosidic linkages in xylan and are involved in the production of xylose. Xylanases generally are combined with cellulases for clarifying fruit juices and wine (Diaz et al., 2011). Industrial xylanase is produced from *Aspergillus* sp. and *Trichoderma* sp. as well as *Bacillus* sp. (Sapag et al., 2002). Some fruits and vegetables are xylan-rich such as pineapples, tomatoes, and apples. Pineapple, tomatoes, and apple juice treated with xylanases from *Bacillus pumilus* SV-85S enhanced the clarification by 22.2%, 19.8%, and 14.3%, respectively (Nagar et al., 2012). Adiguzel and Tunçer (2016) also showed that clarity of apple, grape, and orange juice was significantly increased by 17.85%, 19.19%, and 18.36%, respectively.

3.2.2 Enzymes Used in the Beer Industry

Enzymes play an essential role in the production of beer and malted liquor (i.e., whiskey) to provide the formation of sugars during fermentation, viscosity control, and chill-proofing (Kashyap et al., 2001). Enzymes are activated in beer brewing during malting and fermentation. Enzymes are also needed in brewing steps and production of lower-calorie beer. Early stages of brewing involve the release of fermentable sugar from starch. By adding amylase and glucoamylase enzymes, starch is digested, where use of unmalted cereal grains such as corn, rice, and adjuncts is important. Thus maltose and small dextrins are increased in wort to promote the yield of yeast fermentation. Generally, adding amylase promotes gelatinization of starch, and enhances flavor and color characteristics of the final product (Bigelis, 1993). Production of low-carbohydrate beers, known as light beers, involves enzymes usage. Complex carbohydrates in the grains (e.g., barley, rice, and others) are converted to simple sugars using enzymes to achieve the desirable alcohol content with small amounts of grain. Wort from beer consists of 4% (w/v) of unfermentable carbohydrates. Generally, yeast can ferment maltose, but it cannot ferment oligosaccharides such as dextrins. Thus dextrins increase the caloric content of beer. Dextrins found in wort are digested with glucoamylase to eliminate the unfermentable carbohydrates and to obtain a lower calorie beer (Bigelis, 1993). Production of sweet beers needs an amylase supplement. However, glucoamylase from the *Aspergillus* species is directly added in the fermentation step to produce a sweet beer without the need for added sweeteners (Bigelis, 1993). Chillproofing is a common stage of brewing. Addition of papain or protease in chillproofing stage of brewing reduces the colloidal haze, which is composed of polypeptides and a polyphenolic complex (Bigelis, 1993).

3.2.3 Enzymes Used in the Wine Industry

Enzymes in wine making are used for wine clarification, color extraction, and protein stabilization. Red and white wines are fermented beverages having specific compounds such as polyphenols, anthocyanins, polysaccharides, and volatile compounds. Enzymes used in wine production enhance wine quality and stability, maceration, clarification, and filtration steps (Galante et al., 1998). These steps need enzymes such as pectinases, cellulases, and hemicellulases to improve the yield of must after pressing (Rogerson et al., 2000). Moreover, enzymes

TABLE 3.5 Commercial Enzymes Used in Wine Production

Enzymes	Trade Name	Trade Supplier
Polygalacturonase	Novoclair Speed	Novozymes (Denmark)
Pectinase	Vinpflow Max	Novozymes (Denmark)
Glucosidase	Rohavin MX	AB Enzymes GmbH
β-Glucanases	VinoTaste Pro	Novozyme (Denmark)

are also helpful in the production of wine to reduce fermentation time and promote clarification, filtration, and stabilization (Kashyap et al., 2001). The two enzymes used in wine production, β-glucosidase and pectinase, hydrolyze the polysaccharides in cell walls to enhance the wine quality and stability (Table 3.5). Grape skin and pulp consist of pectic substances as well as hemicellulose, cellulose, and lignin (Vidal et al., 2003). During the pressing of grapes for production of white wines, pectic substances are released into juice to form colloidal particles (Klingshirn et al., 1987). Colloidal particles rich in wine increase the alcohol level and hide fruity aromas and the concentration of sulfur compounds causing off-flavors (Lavigne-Cruege, 1996). Therefore, effective enzymatic hydrolysis of pectic substances is needed to break down these colloidal particles. Pectinases are used in the wine industry for improving haze conditions, reducing filtration time, and increasing must volume. Generally, pectic enzymes are added at early stages of fermentation in white wine making since high alcohol levels inhibit enzymatic activity used to enhance the extraction of aroma compounds from skin (Lea, 1995). However, in red wine making, pectinase is combined with cellulose and hemicellulose to improve pressing yields and extraction of color and aroma quality (Ducruet et al., 2000). It was reported that grapes show polygalacturonase and pectinesterase activity (Usseglio-Tomasset, 1978). However, activity of these enzymes is not sufficient. Therefore, commercial enzyme preparation of pectinases can be used to enhance the efficiency of clarification. Some commercial enzymes used in wine production are presented in Table 3.5.

A commercial maceration enzyme (known as Cytolase 219, Danisco-Germany) used in wine production increased extraction and filtration rates by 10% to 35% and 70% to 80%, respectively (Galante et al., 1998). In addition, pectinases increased extraction of phenolic compounds such as anthocyanins and tannins, which are responsible for improving flavor and color intensity in wines (Kashyap et al., 2001; Garg et al., 2016). Li et al. (2015) reported enhancement of the extraction of anthocyanins for wine color stabilization using an enzyme mixture (i.e., commercial pectinase enzyme Vinozym FCEG, Novozymes, yeast VR5, and commercial tannins).

3.2.3.1 Glycosidases (β-Glucosidase)

The cell walls of fruit and vegetables are composed of cellulose by 20% to 35% dry weight. β-Glucosidase hydrolyzes oligosaccharides into simple sugars or glucose (Sindhu et al., 2016). Generally, β-glucosidase is used for aroma improvement in wine making. Aromatic profiles of wines are based on characteristics of grape variety and compounds originated from fermentation (Vilanova and Sieiro, 2006). Terpenes such as linalool, geraniol, β-damascenone, 1,1,6-trimethyl-1,2-dihydronaphtalene, and vinyl guaiacols are present in wine as key aroma compounds (Wirth et al., 2002). Glycosidically-bound volatile compounds are hydrolyzed to produce the wine

aroma characteristics using glycosidase enzyme, which helps to release the bound volatile compounds (Francis et al., 1999). To enhance the extraction of pigments and flavoring substances in grape skins, β-glucosidase is used in the wine industry (Juturu and Wu, 2014).

3.2.4 Enzymes Used in Green Tea and Coffee Processing

For coffee and tea fermentation, pectinases play a significant role. Green tea is the most widely consumed beverage in the world and 1.3 million tons is produced annually (FAO, 2012). The rate of growth of green tea (7.2%) will be four-times higher than that of black tea due to its health benefits (Murugesh and Subramanian, 2014). Tannase (tannin acyl hydrolase EC3.1.1.20) is the most widely used enzyme for tea processing. This enzyme is produced from the *Aspergillus* and *Bacillus* genus (Bajpai and Patil, 1997). Epi-gallocatechin gallate (EGCG) and epi-catechin gallate (ECG) are present in fresh tea leaves as gallated catechins. EGCG is hydrolyzed to epi-gallocatechin (EGC), and gallic acid (GA) and ECG are hydrolyzed to epi-catechin (EC) and GA using tannase with hydrolyzing ester bonds (Murugesh and Subramanian, 2014). One of the major problems is tea haze and cream formation during tea processing, which causes discoloration and precipitation of substances. Generally, caffeine and polyphenols found in tea are responsible for green tea cream (Chandini et al., 2011). Foam forming on instant tea is destroyed using pectinases, which breaks down the pectin (Praveen and Suneetha, 2014). Insoluble forms of tea cream resulting from polyphenols are converted to cold water-soluble forms using tannase to reduce turbidity and increase cold-water solubility (Sanderson and Coggon, 1974).

Polysaccharide-degrading enzymes, such as cellulose, hemicellulose, and pectin, are used for digesting cell wall composition. Enzymes are used in three stages of green tea processing: pre-treatment of green tea leaves, enzymatic extraction of green tea, and enzymatic treatment of the extract (Table 3.6). Enzymatic extraction of green tea aims to recover maximal tea solids,

TABLE 3.6 Overview of Green Tea Processing Based on Enzyme Type and Application

Processing Type	Enzymes	Application
Enzymatic pretreatment of leaves	Polysaccharide degrading enzymes	• Converts flavor ingredients to rich flavor
	Tannase	• Improves cold water extractability of tea leaves
Enzymatic extraction	Polysaccharide degrading enzymes	• Promotes extraction of polyphenols • Improves flavor of extract
	Tannase	• Improves the preparation of cold soluble instant tea
Enzymatic treatment of extract	Polysaccharide degrading enzymes	• Improves flavor of extract
	Tannase	• Hydrolyses gallated catechins • Reduces cream formation

Modified from Murugesh, C.S., Subramanian, R., 2014. Applications of enyzmes in processing green tea beverages: impact on antioxidants. In: Processing and Impact on Antioxidants in Beverages. Elsevier. pp. 99–108.

whereas enzymatic treatment of the extract aims to improve the concentration of catechins without affecting the taste (Murugesh and Subramanian, 2014). Using tannase and polysaccharide degrading enzymes, aroma, flavor, taste, and cold-water solubility are improved during the extraction of green tea processing (Yue et al., 2008; Chen et al., 2012).

It was reported that using commercial pectinase and cellulase enhances tea quality due to increased theaflavin (24.8%, TF), thearubigen (21.5%, TR) high polymerized substances (21.5%, HPS), and total soluble solids (17.5%, TSS) (Marimuthu et al., 1997). Consumption and production of coffee are annually increasing. Generally, coffee is consumed as roasted and ground coffee (RGC) and instant coffee (Baraldi et al., 2016). Green coffee beans are composed of arabinogalacton, mannan, and cellulose (Mussatto et al., 2011). However, the major polysaccharide of coffee extract is mannan (22% to 30% dry weight), which is responsible for high viscosity (Chauhan et al., 2014). Mannan has a linear chain of 1,4-β-D-linked mannose units (Scheller and Ulvskov, 2010). During roasting, 7% to 30% of the glucan, 45% to 60% of the mannan, and up to 75% of the galactan in green coffee beans become soluble (Nunes et al., 2006). Mannan has a high molecular weight and causes high viscosity in coffee extract (Chauhan et al., 2012). Mannan in coffee beans is hydrolyzed by mannanase to reduce the viscosity of the coffee extract (Nicolas et al., 1998). Additionally, pectic enzymes are used for removal of the mucilage coat from the coffee beans. Degradation of the mucilage to sugars helps to improve the quality of the coffee bean. Adding pectinases for degradation of the mucilage increases sugar release and decreases the demucilization time. According to Murthy and Naidu (2011) crude pectinase from *Aspergillus niger* CFR reduced 54% and 71% of the degradation of the mucilaginous layer of coffee beans after 1 and 2 h of the fermentation process, respectively. Mixed enzyme preparation (cellulase and hemicellulase) helps digestion of the mucilage (Murthy and Naidu, 2011).

3.2.5 Conclusion

In recent years, there has been an increase in the consumption of natural fruit juices. To improve color stability and turbidity of juices and alcoholic beverages, specific enzymes are used. Pectinases have a leading role in clarifying fruit and vegetable juices by removing colloidal pectin. In addition to the leading role of pectinases in clarification steps, using a combination of pectinase, cellulose, and amylase in the extraction of fruit juice increases juice yield. β-Gucosidase and pectinase are used in wine production to improve clarification and maceration yield. For coffee and tea fermentation, pectinases, cellulase, and tannase play a significant role. In addition, enzymes in combination enhance juice yield, clarity, and decrease viscosity and turbidity. During the evolution of enzyme technology, higher beverage productivity has been obtained with better quality of the final products.

3.2.6 Future Trends

Approximately 20 enzymes are commercially produced. Generally, enzymes are produced by big companies such as Denmark-based Novozymes, and US-based DuPont. The global industrial enzyme market is highly competitive with small profit margins. However, there is still need to improve novel, sustainable, and economically competitive production processes since naturally occurring enzymes are often not suitable. For industrial processes, microbial

fermentation used to produce enzymes should be cost effective and highly active to compete with the conventional processes. The new technological developments in the enzyme industry offer high productivity with improved quality of final products. Thus, recombinant DNA technology such as microbial genomes and microbial diversity are still used to discover screening and isolation of new enzymes from microbial resources for application in food and beverage processing. Novel enzyme activity and stability should also be designed and developed under challenging processing conditions. In addition, rapid and efficient enzyme purification strategies and techniques should still be needed for further improvement. However, new strategies should be facilitated for commercialization of tropical juices or products.

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CHAPTER

4

Enzymes in Fruit Juice Processing

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4.1 INTRODUCTION

International markets exist for traditional and non-traditional fruits, while recently, the processing of fruits was developed in several countries. Fruit juices (FJ), beverages, and nectars are the most popular fruit products. The consumption of FJ has significantly increased, and this increase has resulted in an upswing in FJ processing in fruit-growing countries, which endeavor to increase FJ production in order to be competitive for export markets. Enzymes are important factors in food processing because they simplify intermediate bio-processes during food production. The commercial utilization of enzymes was first reported in 1930 for FJ preparation (Nisha, 2016; Oslen, 2000). Industrial enzymes fall into various groups, of which, the most important are pectinases, cellulases, and tannases which are used in fruit processing (Nisha, 2016; Sharma et al., 2016; Yao et al., 2014).

Enzymatic treatment of FJ has many advantages over traditional processing. These advantages include an increase in FJ yield, enhanced clarification, increased total soluble solids in FJ, improved pulp liquefaction, and decreased turbidity and viscosity (Kaur and Sharma, 2013; Nadeem et al., 2009; Pal and Khanum, 2011; Sharma et al., 2016). Macerating enzymes are utilized in these FJ processing steps: (1) after fruit crushing, the pulp is macerated, which results in an increase in FJ yield, decreases the processing time, and extracts more bioactive components from the fruits, and (2) after FJ extraction, clarification is carried out, which increases the product stability (Rui et al., 2012). The cloudiness of FJ is due to the presence of pectin, cellulose, starch, proteins, tannins, and lignin (Vaillant et al., 2001). The commercial application of enzyme preparations containing pectinases, cellulases, and tannases benefits the FJ industry (Grassin and Fauquembergue, 1996). These enzymes are known as macerating enzymes, which are used in FJ extraction and clarification (Gailing et al., 2000; Sharma et al., 2014). Pectinases have globally attracted great interest as a biological catalyst in many industrial applications (Rashmi et al., 2008). Pectinase catalyzes degradation of pectic substances through de-esterification (esterases) reactions and depolymerization (hydrolases and lyases) (Kohli and Gupta, 2015; Tariq and Latif, 2012). Cellulases have gained worldwide interest, as they have valuable potential to process cellulosic biomasses and transform them to

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https://doi.org/10.1016/B978-0-12-813280-7.00004-9

useful products. The synergistic effect of cellulases (i.e., exoglucanases, endoglucanases, and β-glucosidases) is needed for cellulose de-polymerization for transformation to useful products using suitable microorganisms (Sharma et al., 2016). Tannases (tannin acylhydrolases) are important groups of enzymes that are utilized in several industrial applications, including the manufacture of FJ, and tea. Tannases act in a wide range of temperatures and pH, and produce microorganisms, including *Aspergillus*, *Paecilomyces*, *Lactobacillus*, and *Bacillus* (Yao et al., 2014). Enzymes are essential tools in FJ processing in terms of cost savings and quality improvement. Development of the FJ industry became strongly connected with the enzyme industry. Over the last years, many steps in FJ processing were improved, optimized, modified, and rationalized using specific enzymes to improve FJ quality (Ramadan and Moersel, 2007). This chapter discusses the application of pectinases, cellulases, and tanninases in FJ processing.

4.2 PECTINASES AND THEIR APPLICATIONS IN FJ PROCESSING

Pectic materials are complex of colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked with α-(1–4) linkage. Carboxyl groups of galacturonic acid are partially or completely neutralized by potassium, sodium, or ammonium ions and partially esterified by methyl groups (Caffall and Mohnen, 2009; Mohnen, 2008; Sieiro et al., 2012). Side chains of pectin consist of arabinose, galactose, L-rhamnose, and xylose. Pectic substances could be classified into four molecules: protopectin (pectic substance in intact tissue), pectic acids (polygalacturonan contains negligible amounts of methoxyl groups), pectinic acids (polygalacturonan that contains >0%–75% methylated galacturonate units), and pectins (pectinic acid with ca. 75% methylated galacturonate units). Protopectines are water insoluble, while other types of pectic substances are partially or wholly soluble in water (Alkorta et al., 1998; Be Miller, 1986; Kertesz, 1951; Tapre and Jain, 2014). Pectin is a mixture of different molecules with pectinic acid as the main constituent. Pectin (Fig. 4.1) is found in the cell wall and pectin might be linked with polysaccharides or proteins to form protopectin. In the cell wall of fruits, pectin is the main constituent (Anuradha et al., 2010; Kohli and Gupta, 2015). Pectin content (%) of some fresh fruits is given in Table 4.1. Among fresh fruits, currants contain the highest percentage of pectin (0.9%–1.5%).

Pectins have many valuable applications in nutraceuticals and food industries. In the food industry, pectin is used as a gelling agent, as nutritional fiber, as well as a replacement for

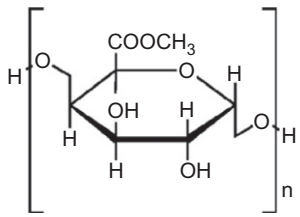

FIG. 4.1 Chemical structure of pectin.

TABLE 4.1 Pectin Content (%) in Some Fresh Fruits (Fogarty and Ward, 1972)

Fruit	Pectin Content (%)
Banana	0.7–1.3
Apricot	0.7–1.3
Apple	0.5–1.6
Currant	0.9–1.5
Grape	0.2–1.0
Guava	0.7–1.5
Tomato	0.2–0.5
Strawberry	0.6–0.7
Peach	0.3–1.2
Pea	0.5–0.8
Pineapple	0.3–0.6

fats and sugars in low-calorie foods (Sakai et al., 1993; Sieiro et al., 2012; Thakur et al., 1997). In addition, pectins contribute to FJ viscosity and turbidity. Mechanical crushing of fruit with high levels of pectin yields a FJ with high viscosity that remains bound to the fruit pulp. It is difficult to extract this FJ using other mechanical methods or by pressing. Pectin is involved in crosslinking cellulose and hemicellulose fibers; thus pectinases helps to enhance the access of cellulases to their substrates (Giacobbe et al., 2014). By treating with pectinases, the viscosity of FJ is decreased, the jelly structure disintegrated, the pressability of the pulp improved, and higher FJ yields are obtained.

4.2.1 Pectolytic Enzymes

Enzymes that hydrolyze pectic materials are known as pectinases, pectic enzymes, or pectinolytic enzymes (Blanco et al., 1999). The first commercial use of pectinolytic enzymes was in 1930 for the preparation of FJ and wine (Oslen, 2000; Tapre and Jain, 2014). In the 1960s, the structure of plant tissues was elucidated, and enzymes began to be used efficiently. Pectinolytic enzymes are very important enzymes in the food industry especially in FJ processing as a prerequisite for obtaining stability and clarification (Girard and Fukumoto, 1999; Lee et al., 2006; Mohnen, 2008; Nisha, 2016; Ribeiro et al., 2010; Tapre and Jain, 2014; Viquez et al., 1981). Pectinases are formed during the fruits' ripening process wherein pectinases split polygalacturonic acid to monogalacturonic acid by breaking glycosidic linkage. Softening of cell walls and increasing the FJ yield from the fruits takes place during this process. Acidic pectinases are mainly utilized in FJ processing (i.e., extraction and clarification), while alkaline pectinases have economic and environmentally friendly industrial applications (Kohli and Gupta, 2015). The detailed

TABLE 4.2 Classification of Pectinases According to Its Mode of Action (Sieiro et al., 2012)

Enzyme	Mode of Action	Main Substrate	Product(s)
Depolymerases			
Lyases			
Endopectate lyase (4.2.2.2)	Transelimination	Pectic acid	Unsaturated oligogalacturonates
Exopectate lyase (4.2.2.9)	Transelimination	Pectic acid	Unsaturated oligogalacturonates
Endopectinlyase (4.2.2.10)	Transelimination	Pectin	Unsaturated methyl-oligogalacturonates
Hydrolases			
Protopectinases	Hydrolysis	Protopectin	Pectine
Endopolygalacturonase (3.2.1.1.5)	Hydrolysis	Pectic acid	Oligogalacturonates
Exopolygalacturonase (3.2.1.6.7)	Hydrolysis	Pectic acid	Monogalacturonates
Esterases			
Pectin methyl esterase (3.1.1.11)	Hydrolysis	Pectin	Pectic acid and methanol
Pectin acyle esterase (3.1.1.6)	Hydrolysis	Pectin	Pectic acid and methanol

classification of pectic enzymes is shown in Table 4.2. Based on its substrate preference and mode of action, pectinases could be classified as:

I. Esterases, that eliminate acetyl and methoxyl residues from pectin giving rise to polygalacturonic acid;
II. Protopectinases, that solubilize protopectin to form soluble pectin;
III. Depolymerases that break the glycosidic linkages between galacturonic residues via either transelimination (pectate lyases and pectin lyases) or hydrolysis (polygalacturonases).

The latter enzymes are further divided into *exo-* if its action pattern is at the terminal end, and *endo-* if its action pattern is random (Fogarty and Kelly, 1983; Sieiro et al., 2012; Whitaker, 1990). Pectinases mainly include pectin esterases, polygalacturonases, pectin lyases (PNL), and pectate lyases with different substrate specificities (Ahlawat et al., 2009; Kohli and Gupta, 2015). Pectin lyases can hydrolyze pectin to oligosaccharides having 4-deoxy-6-*O*-methyl-α-D-galact-D-enuronosyl groups at their ends (Fig. 4.2). Pectinolytic enzymes act on plant cell walls decreasing the intracellular adhesively and tissue rigidity (Pires and Filho, 2005). Pectinases are produced by microbes and plants (Saranraj and Naidu, 2014). There are numerous reports of different types of pectinolytic enzyme production from different pathogenic fungi and bacteria, including pectin methyl esterase, whose isoforms are detected in all higher plants tested so far (Mareck et al., 2012). Pectinolytic microbes were industrially exploited for pectinases that are environmentally friendly enzymes (Kohli and Gupta, 2015). Microorganisms are naturally endowed with the potential

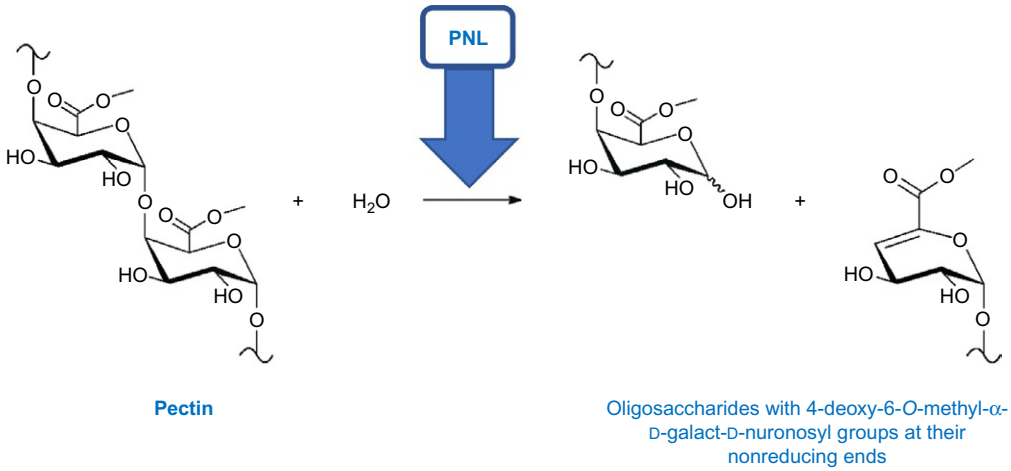

FIG. 4.2 Hydrolysis of pectin using pectin lyase.

to produce enzymes extracellularly, such as Streptomyces GHBA10, which is an efficient producer of pectinases that might be utilized in FJ clarification and extraction processes (Das et al., 2013). Samples for isolation of pectinolytic isolates were from fruit waste processing areas, agro-industry residues, soils rich in pectic waste, sewage of FJ centers of different locations, and waste from the pectin industry (Hoondal et al., 2002; Kohli and Gupta, 2015). Fungal-pectinases are extracellular enzymes, wherein polygalacturonase is the prominent type among them. Pectinases are produced by different fungi, including *Botrytis cinerea*, *Aspergillus* sp., *Fusarium moniliforme*, *Penicillium*, *Rhizopus stolonifer*, *Trichoderma* sp., *Rhizoctonia solani*, *Neurospora crassa*, and *Fusarium* (Joshi et al., 2011; Nisha, 2016).

4.2.1.1 Pectinases in the FJ Industry

Pectinases are important item in the FJ industry and have different food technological applications. Microbial pectinases accounts for about 10% of total global enzyme production. Pectinases act to degrade the complex and long pectin in the fruit pulp, which are found as polysaccharides responsible for pulp turbidity. Applicability of pectinases is pH-dependent, wherein acidic enzymes are mainly used in the beverage industry for clarification and extraction (Alkorta et al., 1998; Blanco et al., 1998) to remove pectic materials responsible for FJ turbidity and consistency (Bonnin et al., 2003). Pectinases are a prerequisite in FJ clarification because they bring down bitterness and cloudiness of FJ, reduce viscosity of fruits, as well as improve pressability of pulp and disintegrate the jelly-like pectin (Kohli and Gupta, 2015). Pectinase treatment was applied with different fruits such as raspberry, strawberry, orange, blackberry, and grape juice and apple pomace, which resulted in improved FJ chromaticity and stability (Kohli and Gupta, 2015; Pasha et al., 2013).

4.2.1.2 Pectinases in the Extraction of FJ

Pectinases are utilized to facilitate FJ extraction and to help in the separation of flocculants precipitated by filtration, sedimentation, and/or centrifugation. If a cloudy FJ is required, it is

pasteurized to inactivate enzymes. Centrifugation removes large-size remains while leaving small particles in the FJ suspension. If a clear FJ is required, those suspended particles should be withdrawn. To perform this, a treatment with commercial enzymes mixtures (cellulases, pectinases, and hemicellulases) is carried out and later the fluid is centrifuged to clarify the FJ (Grassin and Fauquembergue, 1996; Kashyap et al., 2001; Sieiro et al., 2012). Degradation of pectic constituents in the mashed fruit purees is achieved via pectinase treatments resulting in an enhancement in the FJ recovery and its clarification as well as a reduction in the FJ viscosity. In addition, applications with pectinases mixtures could provide filtering (de Gregorio et al., 2002; Fernández-González et al., 2004; Ribeiro et al., 2010; Souza et al., 2003). Tropical pectin-rich fruits are too pulpy to obtain FJ by pressing or centrifugation. Those techniques usually require high amounts of energy and result in meager FJ yield. In fruits like banana, guava, mangoes, and papaya, the expression of FJ is difficult by the traditional techniques. In other fruits like grapes and apples, FJ extraction is usually incomplete because some quantity of FJ is retained in the fruit pomace. In the traditional process of FJ production from soft fruits, the pulp is boiled and the extract is processed (Tapre and Jain, 2014; Waldt and Mahoney, 1967). An enzymatic application enables expression of FJ without drastic processing, and also helps in the FJ clarification (Sreekantiah et al., 1971). The process consists of pulping the fruits and warming them to 65°C for 15 min to inactivate inner enzymes. The pulp is cooled, pectinases are added and left for incubation time, and the FJ is separated by pressing it through cheesecloth or using a centrifuge. The FJ is racked at 3°C to 5°C for 24 to 48 h, and during that time all the suspended particles settle down. The clear supernatant could be clarified and stored after pasteurization.

Pectinases are used in the apple FJ industry to help FJ pressing or FJ extraction and to facilitate the separation of a flocculent precipitate by centrifugation. Schols et al. (1990) reported on rhamnogalacturonase (from *A. aculeatus)* and its effect on the maceration of the apple tissue. Treatment with pectinases takes about 15 to 120 min depending on the enzyme nature and how much is utilized, the reaction temperature, and the apple variety (Kilara, 1982). With pectinase application, there was an increase (up to 30%) in the yield of grape FJ. The FJ yield increased as levels of pectinases increased from 0.05% to 1.50% (Tapre and Jain, 2014; Villettaz, 1993). The FJ recovery of pectinases-treated pulps increased greatly from 38% to 63% in peach, 60% to 72% in pear, 52% to 72% in plums, and 50% to 80% in apricot (Joshi et al., 2011). Ramadan and Moersel (2007) studied the physical and chemical parameters of the *Physalis peruviana* FJ as influenced by enzymatic treatments with enzyme preparations (Rohapect VR-C, Pectinase L 40, and Ultrazym AFP-L). Rohapect VR-C contained a pectinase, protease, and hemicellulose mixture. Pectinase L 40 contains pectinase activity, and a minor polygalacturonase activity. Ultrazym AFP-L contains pectolytic and cellulolytic activities (hemicellulose, pectinase and cellulase). With enzyme treatments, the yield of *P. peruviana* FJ was increased, along with the macro- and micro-constituents. Enzymatic treatments resulted in *P. peruviana* FJ with high pulp content, high total soluble solids, and high acidity. Enzyme-treated *P. peruviana* FJ was characterized by low alcohol-soluble solids and pH. The antioxidant activities of different processed *P. peruviana* FJ were assessed by bleaching of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radicals and the values were correlated with antioxidants found in the FJ. In another study, Sharoba and Ramadan (2011) prepared *P. peruviana* FJ enzymatically-treated with Pectinex Ultra SP-L (300 and 600 ppm), and the FJ was concentrated to 30°Brix and 40°Brix. Rheological characteristics of *P. peruviana* juices were studied

at a shear rate range from 0.3/s to 100/s and at a wide range of temperatures (5°C to 100°C). *Physalis peruviana* FJ concentrates had a definite yield stress and behaved as non-Newtonian fluids. The Bingham and Casson, yield stress, plastic viscosity, consistency index, and flow index were decreased when the temperature and Pectinex Ultra SP-L dose were increased. Arrhenius-type equations described the effect of temperature on the FJ viscosity. The activation energy for viscous flow depended on the TSS. The impact of pectinase obtained from *Paecilomyces variotii* on the extraction and clarification of grapes and pomegranate juices was studied (Nisha, 2016). Different enzyme concentrations (0.5 to 3.5) and incubation times (30 to 160 min) at 50°C to optimize the enzymatic treatment for the yield and clarity of pomegranate juice were examined. Optimum conditions recommended for enzyme treatment for clarification and yield of pomegranate juice were 3.5 mg/20 g pulp for the enzyme concentration, and 180 min for incubation time. There was an increase in the yield of 31.6% and 42.3% of the grape and pomegranate juices when treated with purified enzymes than the untreated juices (Nisha, 2016).

4.2.1.3 Pectinases in the Clarification of FJ

The application of enzymes in FJ clarification was first introduced in Germany and the US in the early 1930s (Neubeck, 1959). The enzymatic-assist clarification is affected by many factors, including the enzyme concentration, incubation time, and temperature (Lanzarini and Pifferi, 1989; Tapre and Jain, 2014). Pectinases hydrolyze pectin and flocculate protein-pectin complexes (Baumann, 1981). The resulting FJ has a much lower level of pectin and a low viscosity, which facilitates the subsequent filtration. Before the technique of enzymatic clarification, heat coagulation or clarification by freezing were techniques adopted to obtain clear FJ. Commercial pectinases were utilized as processing aids for degradation of pectin that settled particles in the suspension. The application of pectinases resulted in higher FJ clarity and yield as well as preserved the nutrients, color, and flavor of the FJ. Traditional clarification methods depend on pectin hydrolysis with pectinases and starch hydrolysis with amylases. Clarifying agents including gelatin, bentonite, or silicasol induce the physical and chemical precipitation of sediments or haze-active components (Cerreti et al., 2016; Mirsaeedghazi et al., 2010; Pinelo et al., 2010; Rinaldi et al., 2013). De-pectinizing actions have two impacts: to form the aggregation of cloud particles and to degrade the viscous-soluble pectin. Pectin carries a negative charge in acidic environments and forms a coat around suspended-proteins which causes them to repel each other. Pectinases degrade the chain of pectin, therefore exposing positively charged proteins. Electrostatic repulsion between the cloud particles is reduced so that they aggregate together (Sorrivas et al., 2006). In the preparation of clarified FJ, cellulase, hemicellulose, and pectinase are effective in the viscosity reduction and filterability enhancement (Jaleel et al., 1978; Koff et al., 1991; Shahadan and Abdullah 1995; Tapre and Jain, 2014). Pectinases have improved the apple FJ clarification with a 35% drop in viscosity (Girard and Fukumoto, 1999; Mondor et al., 2000), pineapple FJ (Carneiro et al., 2002), tangerine FJ (Chamchong and Noomhorm, 1991), as well as peach, plum, pear, and apricot FJ prior to ultrafiltration. Brasil et al. (1995) reported a significant reduction (ca. 63%) in the viscosity of guava FJ when Clarex-L concentrate was used. In addition, Sharma et al. (2005) mentioned that enzyme concentration, temperature, and incubation time affected carrot FJ viscosity (41% reduction) when Pectinex Smash XXL was applied. The viscosity and turbidity of banana FJ are caused mainly by the polysaccharides (pectins) in the banana (Alvarez et al., 1998;

Brasil et al., 1995; Koff et al., 1991; Yusof and Ibrahim, 1994). Pectin makes the clarification step harder due to its fiber-like molecular structure. It was reported that de-pectinization using pectinases might clarify banana pulp (Ceci and Lozano, 1998; Lee et al., 2006; Tapre and Jain, 2014; Vaillant et al., 1999). The commonly utilized enzymes in the apple juice industry are those enzymes that could depolymerize the highly-esterified pectin. Developed techniques for producing apple juice were reported (Jaleel et al., 1978; Tapre and Jain, 2014) whereas by judicious application of pectic enzymes, a sparkling FJ was obtained and 20% of pectin was recovered. Apple juice could be extracted from crushed apple mush with the help of pectic enzymes, followed by pomace liquefaction with a mixture of cellulases and pectinases to complete the extraction and obtain premium FJ (Sieiro et al., 2012; Will et al., 2000).

Kumar and Sharma (2012) studied the impact of enzymatic treatment on pineapple (*Ananas comosus*) juice clarity, yield, and viscosity. The optimized enzymatic treatment conditions were incubation time (446 min), incubation temperature (47°C), and enzyme concentration (0.14 mL/50 g of pulp). The conditions for the enzyme treatment of the same variety of pineapple to improve the FJ recovery and quality were also optimized. The crude enzymes were competitive to the commercial enzymes for the enhancement of pineapple FJ recovery and quality. The comparison was done under optimized conditions using principal component analysis. Saxena et al. (2014) exposed watermelon juice to masazyme enzymes at varying levels (0.01% to 0.1%, *w*/w), different temperatures (30°C to 50°C) and periods (20 to 120 min). Enzymes degraded polysaccharides, resulting in reductions in turbidity, viscosity, and absorbance values, while FJ yield, total dissolved solids (TSS), and lightness were increased. Deshmukh et al. (2015) studied the rheological properties of enzymatic-clarified *Achras sapota* (sapota) FJ at different temperatures (10°C to 85°C) and TSS content (10°brix to 55°brix) corresponding to a water activity (0.865 to 0.986). The effect of TSS content on the viscosity of enzymatic-clarified sapota FJ followed the second order exponential type relationship ($r > 0.99$, rmse % <3.53). The enzymatic-clarified *Achras sapota* FJ behaved like a Newtonian liquid wherein the viscosity (η) values were from 4.34 to 56.41 mPa s depending on the temperature and concentration. The effect of TSS content on flow activation energy was described by using an exponential relationship ($r > 0.95$) and that of water activity, described by using power law relation ($r > 0.99$). An equation representing combined impact of TSS content/water activity and temperature on the viscosity of enzymatic-clarified *A. sapota* FJ was established. Conventional processing of pomegranate into FJ is time-consuming and needs several steps including washing, pressing, clarification, pasteurization, and filtration (Cerreti et al., 2016). Clarification is a basic step in the pomegranate FJ processing to inhibit the substances responsible for FJ turbidity, and to inhibit the development of turbidity during FJ storage, known as haze formation (Cassano et al., 2011; Mirsaeedghazi et al., 2010; Vardin and Fenercioglu, 2003). Removing those particles is an industrial problem, which improves the clarity as well as the color stability. The FJ industry has developed several techniques to solve these problems because consumer interest is driven by FJ quality and appearance (Costell et al., 2010). Cerreti et al. (2016) tested the impacts of pectinolytic and/or proteolytic clarification on the turbidity and the haze from active substances in pomegranate FJ. A synergic impact of the application of protease and pectinase was reported wherein very good results in terms of FJ turbidity and potential haze formation were reached. Although enzymatic treatments with pectic enzymes and proteolytic enzymes did not modify the amounts of protein, pectin, and phenolics, they influenced the haze-forming activity of turbidity-forming molecules. In addition, this kind of enzymatic treatment did not

affect FJ color and levels of anthocyanin. In the orange FJ, where pectin esterases are found, pectins are partially methylated. Polygalacturonases are commonly used for this kind of FJ. During orange FJ extraction, pectinases could be added at the end of the pulp wash to reduce FJ viscosity. This leads to high FJ yield, a better TSS extraction, and a lower viscosity. Pectinases reduce FJ viscosity without attacking the insoluble pectin, which maintains the cloud stability. Enzymes should have the least content of pectin methyl-esterases to avoid clarification of FJ (Kashyap et al., 2001; Sieiro et al., 2012). Manjunatha et al. (2014) evaluated thermos-physical traits of enzymatic-clarified lime (*Citrus aurantifolia*) FJ at moisture levels (30.3% to 89.3%, wet basis). The viscosity of enzymatic-clarified lime FJ and Newtonian viscosity decreased with the increase in the water activity; whereas thermal conductivity and specific heat increased with the increase in the moisture and water activity wherein the thermal diffusivity increased marginally. A correlation between thermos-physical traits and moisture content of enzyme-treated lime FJ was observed. In addition, a significant negative correlation between physical and thermal characteristics was noted.

4.2.2 Cellulases and Their Applications in the FJ Industry

Cellulose is synthesized by microorganisms including some algal species, plants, and also animals (Mohite et al., 2012; Zenga et al., 2011). Cellulose has a multi-level architecture consisting of microfibrils bundles. Each microfibril contains about 36 to 1200 cellulose chains that are linked together by van der Waal forces and hydrogen bonds to form a crystalline structure. A cellulose chain is a non-branched chain of D-glucose monomers that might range from 100 to 20,000 glucose units linked by β-glycosidic bonds (Ioelovich, 2008; Sharma et al., 2016; Zhang and Lynd, 2004). Hydrogen bond interactions in these amorphous regions are sub-optimal, therefore accessible for enzyme attack and water (Lenting and Warmoeskerken, 2001). Generally, cellulose degradation to glucose is achieved by synergistic action of exoglucanases, endoglucanases, and β-glucosidases. Conversion of cellulose polymers using cellulases is a foreseeable approach for the judicial use of abundant agricultural lingo-cellulosic wastes to produce useful products. Cellulases account for an 8% share of the global industrial enzyme demands (Elba and Maria, 2007; Ioelovich, 2008) and the annual globule cellulase market is expected to expand up to $400 million USD (Sharma et al., 2016; Zhang et al., 2006). Cellulases are valuable factors in food technology due to their different applications in several processes. Cellulases are used worldwide due to their useful and promising potential to be exploited in several processes and techniques involved in food technology like FJ clarification and the reduction of nectar viscosity (Bhat, 2000; Efrati et al., 2013; Karmakar and Ray, 2011; Kuhad et al., 2010; Sharma et al., 2014; Singh and Sharma, 2013).

Production of FJ (i.e., apple and pear) includes crushing fruit to pulp mash which is then separated to become clear FJ and pomace (solid phase) by mechanical processing (Galante et al., 1998). The yield and the process performance were increased by using macerating enzymes to clarify the FJ (Sharma et al., 2016; Vieira et al., 2009). There was about 50% decrease in viscosity of passion FJ when a combination of cellulases, pectinases, and amylases were applied (Sandri et al., 2011). The application of *exo*-enzymes in black carrot FJ processing enhanced the antioxidant potential due to the increase in the levels of phenolic compounds and flavonoids (Khandare et al., 2011). Fruit nectars are processed by blending pulpy FJ with sugar syrup and citric acid to form ready-to-drink beverages. The attractive feature of those

beverages to be maintained is cloud stability. Cloud stability of nectars was improved by applying exogenous enzymes. Enzyme preparations like Pectinex Ultra, Rohapect, or preparations containing a combination of pectinases and cellulases, have been found to decrease the nectars viscosity (Kashyap et al., 2001; Sharma et al., 2016). Enzyme preparations were tested in improving the rheological characteristics of mango puree, wherein Rapidase Pomaliq and Rapidase recorded the best results. Those enzyme preparations have high amount of cellulases, pectinases, and xylanases that could reduce puree viscosity in a short time to modify the rheological characteristics to be suitable for commercial use (Brito and Vaillant, 2012; Sharma et al., 2016). Ramadan and Moersel (2007) reported the characteristics of the *P. peruviana* FJ as affected by enzymatic treatments with Ultrazym AFP-L, which contains cellulolytic and pectolytic activities (hemicellulose, cellulase and pectinase). With enzyme treatments, the yield of *P. peruviana* FJ is increased, along with the macro- and micro-components. Fruit juice sensory characteristics include aroma, texture and flavor properties, all of which play an important role in food technology and biotechnology. The fruit sensory traits could be altered with enzyme infusions like cellulases (Baker and Wicker, 1996; Sharma et al., 2016). Enzymatic treatments were found to enhance the nutritional value as well as the aroma of fruits (Shoseyov and Bravdo, 2001). β-Glucosidase, when added to tea beverages, caused aroma enhancement due to the increase in the essential oils content (Contesini et al., 2013; Su et al., 2010). Tea browning could be avoided because immobilized β-glucosidase is able to work at low temperatures when compared to the free enzyme. Enzyme-assisted treatment of FJ was reported to enhance the color, FJ yield, and health-promoting effects. An increase in acidity, total soluble sugar, and β-carotene levels of carrot juice with a decrease in juice viscosity was exhibited due to enzymatic treatment, thus improving the sensory traits of color, flavor, and general acceptability (Kaur and Sharma, 2013; Sharma et al., 2016).

4.2.3 Tannases and Their Applications in the FJ Industry

High levels of tannins in food or feed have negative impacts on nutrition by reducing protein digestibility, inhibiting digestive enzymes, or by systemic toxicity (Curiel et al., 2010). Anti-nutritional effects of tannins might be decreased by treatment with tannase-producing microorganisms, or tannase (Rodríguez-Durán et al., 2011; Yao et al., 2014). Tannase (EC 3.1.1.20, tannin acyl hydrolase) catalyzes the hydrolysis of ester bonds found in gallotannins, ellagitannins, gallic acid esters, and complex tannins (Beniwal et al., 2013). The practical use of tannases is limited due to few reports and knowledge about their traits and purification processes (Yao et al., 2014). They are widely used as clarifying agents in the production of tea, FJ, treating tannin-polluting industrial effluents, and agricultural by-products. Moreover, tannases play an important role in the manufacturing of gallic acid, which is a substrate for the enzymatic or chemical synthesis of propyl gallate (antioxidant). Tannases are produced on tannic carbons such as tannic acid, tea, wheat bran, and coffee husk extract. Microbial tannases are induced extracellular enzymes, produced by solid-state fermentation, and submerged fermentation. Tannase is purified by hydrophobic interaction chromatography and reverse micelle. Most of tannases act in a wide range of temperature and pH, although tannases with acidic pH optima are common. A sequence-based classification spreads tannases in several families, therefore reflecting the molecules' variety (Beniwal et al., 2013; Yao et al., 2014).

Tannases have interesting and useful applications in food and feed industries. The main applications of tannases are instant tea, as well as gallic-acid manufacturing from plant raw materials rich in tannins. Tannases help to reduce the adverse impacts of tannins in FJ and beverages (Yao et al., 2014). Enzymatic treatment of FJ in order to reduce FJ bitterness has advantages like the high quality of FJ due to non-deterioration and the low haze (Beniwal et al., 2013). New FJ (i.e., cranberry, raspberry, pomegranate, and iced tea) were acclaimed for their health-promoting effects and disease-fighting antioxidant traits. High tannin levels in these fruits cause sediment formation, as well as tannin levels responsible for color, bitterness, and astringency of FJ during storage. Because of the inability of traditional FJ debittering processes to effectively eliminate bitterness, enzyme treatments are needed. Oded et al. (1990) reported that enzymes from mutant species CMI CC 324, 626 of *Aspergillus niger* B1 showed activity that endo tannase, β-glucosidase, and anthocyanase can enhance taste and flavor of FJ and the fermented products (Beniwal et al., 2013). Tannase-treated black tea and FJ in high concentrations can be preserved without causing precipitation and clouding, thereby exhibiting excellent quality (Beniwal et al., 2013; Kaoru et al., 2000). Motoichi et al. (2001) reported that FJ could be stored for long periods without showing any precipitation or turbidity when they are treated with tannase and/or chlorogenase (Beniwal et al., 2013). Tannase treatments resulted in 25% degradation of tannin in pomegranate juice, while the combination of gelatin and tannase (1:1, *w*/w) resulted in ca. 49% degradation of tannin (Rout and Banerjee, 2006). Srivastava and Kar (2009) reported the enzymatic treatment of aonla/myrobalan (*Phyllanthus emblica*) juice with 68.8% removal of tannin content resulted in considerable loss of astringency.

4.3 CONCLUSION

Increased health awareness has led to an increase in FJ consumption as an alternative to caffeine-containing beverages. Fruits are usually pectinaceous and pulpy to yield FJ by simple processing techniques. The pectinolytic enzymes cellulases and tanninases are effectively used in FJ biotechnology and FJ processing. Enzymatic processing makes clear FJ by breaking down pectins and allowing the suspended molecules to settle down, as well eliminating undesirable changes in FJ color and stability. Compared with other established processing methods, the costs of producing enzymatically-clarified FJ could be competitive and could have a high production yield.

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CHAPTER

5

Application of Microbial Enzymes in the Dairy Industry

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5.1 INTRODUCTION

Microorganisms from barley yeast have been used in the commercial production of alcoholic beverages by the Sumerians and Babylonians since 6000 BCE. Globally, enzymes of the microbes have been known for their prevalent applications in many industries; for example, agriculture, chemicals, energy, food, and medicine. Microbial enzymes are rapidly attracting attention and mediated processes due to the fact they consume little energy, have a decreased process time, are eco-friendly, nontoxic, and cost effective (Choi et al., 2015; Li et al., 2012). With the emergence of protein engineering and DNA technology, microorganisms could be cultured and manipulated in huge quantities to fulfill needs due to the increased demand of industrial implementations such cost reduction, consumer goods, and depletion of natural resources, (Choi et al., 2015; Liu et al., 2013). The world market for microbial enzymes was constituted in 2014 at nearly $4.2 billion USD and it will be expected to increase by 7% by 2020. Enzymes are biological molecules, are protein in nature, and are considered catalysts that activate the bio-chemical reactions inside the biological system (Cech and Bass, 1986). Enzymes are specific, lowering the activation energy without consuming it (Aldridge, 2013; Fersht, 1985; Piccolino, 2000). Typically, enzymes require mild conditions such as pressure and temperature to catalyze reactions, instead of needing hazardous materials and pollutants (Choi et al., 2015; Illanes et al., 2012). The optimal pH and temperature of mammalian enzymes are 7.4 and 37°C, respectively. In addition, a pH higher than 7.4 and temperatures over 40°C lead the enzymes to denature which limits their use in non-physiologically conditions. Moreover, mammalian enzymes are affected by product and substrate inhibitions and can cause allergies; there is also the higher fee of purification, isolation, and difficulty in reusing them. Enzymes are large macromolecules consisting of a building block made of amino acids; their molecular mass is measured by Dalton (Da). Often the active site of the enzyme is deep within a pocket that causes their specificity of a certain substrate. Large

https://doi.org/10.1016/B978-0-12-813280-7.00005-0

enzyme numbers are characterized and purified and, as a result, the enzyme nomenclature was established. In consultation between the IUBMB and IUPAC, EC was established to guide the systematic classification and naming of enzymes. Microorganisms are considered preferable sources for industrial enzymes because of their fast growth rate and easy availability. Altering genes of microbial cells using recombinant DNA technology could be easily done to elevate the production of enzymes and development of science (Illanes et al., 2012). In Industrial applications, the manufacture of microbial enzymes is an important issue. This importance came from the excellent and high-efficiency achievements of various microbial enzymes, enzymes that work in numerous chemical and physical cases. It has been stated that the enzymes of microorganisms can be used in the treatment of the shortage of human enzymes resulting from genetic disorder (Anbu et al., 2017; Vellard, 2003). As an example, patients who are unable to digest sucrose (sucrase-isomaltase deficiency) were treated orally with sacrosidase (EC 3.2.1.26) to aid sucrose digestion (Treem et al., 1999). It has been reported that the treatment for phenyl ketonuria disorders were treated with ammonia phenylalanine lyase to break down the substrate phenylalanine (Sarkissian et al., 1999).

Enzymes of microorganisms have numerous industrial applications such as in the dairy, pharmaceutical, food, paper, textiles, and leather industries. Moreover, their use is rapidly increasing more than other conventional methods because of their greater efficiency, higher quality, and low impact on the environment (Gurung et al., 2013; Jordon, 1929; Kamini et al., 1999). This chapter will focus on the application of microbial enzymes in the dairy industry.

5.2 THE DAIRY INDUSTRY

In the dairy industry, the microbial enzymes utilized have a significant role, where they are used to improve and enhance organoleptic features like aroma, color, and flavor, as well as giant yield of milk products. There are many types of microbial enzymes used in the dairy industry, such as catalase, aminopeptidase, proteases, lactoperoxidase, lipases, transglutaminase, etc. They are well known in this field and are different from coagulants as they help improve shelf life. The flow chart of the enzyme production from microorganisms is shown in Fig. 5.1. It has been reported that enzymes of microorganisms are used in yogurt and cheese production (Pai, 2003; Qureshi et al., 2015). The mixture of pepsin and chymosin (rennet) is applied to coagulate milk for cheese and whey production. The world requirements of cheese produced by rennet microorganisms constitutes about 33% per day (Van Kampen et al., 2013).

5.3 MICROBIAL ENZYMES IN DAIRY INDUSTRIES

In the dairy industry, microbial enzymes have been utilized to produce diverse products, such as yogurt, cheese, syrup, bread, etc. to enhance their quality. Traditional ancient arts such as brewing, cheese making, and tenderization of meat by papaya leaves were developed before we knew about enzymes. Moreover, early dairy processes included proteolysis, an insensible consequence of enzyme activity in food production. Here are the most important microbial enzymes used in the dairy industry (Table 5.1).

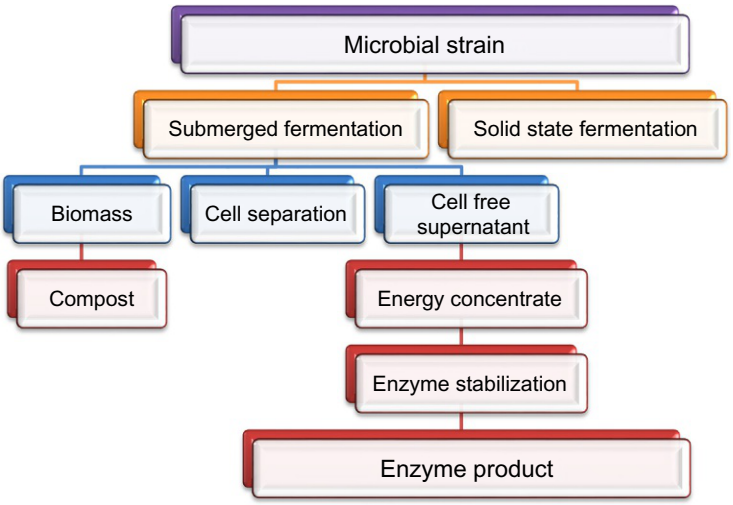

FIG. 5.1 Preparation of enzymes by microorganisms.

TABLE 5.1 Application of Microbial Enzymes in the Dairy Industry

Enzyme	Role(s)	Microorganism(s)	Reference
Aminopeptidase	Faster cheese ripening	*Lactobacillus* sp.	Masoud et al. (2017)
Acid proteinase	Milk coagulation	*Aspergillus* sp.	Qureshi et al. (2015)
Catalase	Cheese processing	*Aspergillus niger*	Perin et al. (2017)
Lipase	Faster cheese ripening	*Aspergillus niger*	Sharma and Sharma (2017)
Neutral proteinase	Faster cheese ripening	*Bacillus* sp., *Aspergillus oryzae*	Palomba et al. (2017)
Transglutaminase	Protein cross linking	*Streptomyces* sp.	Domagała et al. (2016)

5.4 RENNET

Rennet is considered a famous exogenous enzyme used in dairy processing, and has been used since 6000 BCE. The cheese production in the US increased from 8000 to 471,434 metric tons by April 2017 according to (USDA/NASS). This translated to a large demand of the exogenous enzyme rennet from different sources. The rennet enzyme usage by cheese manufacturers is one of the largest application of enzymes in dairy processing. Traditionally, animal rennet is utilized as a milk-coagulant in the dairy manufacturing industry to produce high-quality cheeses with unique features such as good texture and flavor (Fig. 5.2). The demand for cheese production has increased worldwide, while at the same time there has been a reduction in the supply of calf rennet. This led to the search for alternative sources of rennet such as rennet extraction from microorganisms. Worldwide, rennet from microorganism constitutes 30% of the total cheese produced. Rennin has enzymatic and nonenzymatic action that causes the milk to coagulate. The milk transforms to a gel-like structure during

FIG. 5.2 Different types of cheese produced by rennet.

the enzymatic activity due to the temperature and calcium ion effect (Bhoopathy, 1994). There are very familiar microorganisms used to manufacture rennet, such as proteinases, which can replace calf rennet. In cheese production, some microorganisms such as *Aspergillus oryza*e, *R. miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, and *Irpex lactis* are widely used to make rennet. Many researchers have reviewed many studies done so far on rennet substitutes (Farkye, 1995; Fox, 1998; Green, 1993). Predominantly, many manufacturers use different strains of *Mucor* to produce rennet from microorganisms, while the best yields of milk-clotting are done by the protease of *Rhizomucor pusillus* derived from semi-solid media made up of 50% wheat bran. Furthermore, *Endothia parasitica and R. miehei* are shown to be suitable for submerged cultures. Excellent milk-clotting by protease yields could be achieved by utilizing a culture medium composed of 4% potato-starch, 10% barley and 3% soybean-meal. Lipases are excreted with the proteases through the microbial growth. Hence, the activity of lipases becomes wasted by minimizing the pH before the culture preparation. A study was conducted on the effect of incorporating whey protein concentrate (WPC) on the quality characteristics of Mozzarella cheese analogue (MCA) based on rennet casein (RC) (Dhanraj et al., 2017). It is recommended to use a blend of RC and WPC (90:10) as the protein source in the formulation of MCA to obtain a nutritionally superior cheese product that has the desired functional properties for its end use in baking applications. The effects of the size and stability of native fat globules on the kinetics of rennet-induced coagulation were revealed by determining the caseinomacropeptide (CMP) is a 64-amino-acid-residue peptide which is released from kappa-casein by gastric proteinases. Release rates and rheological properties of milk has been studied (Luo et al., 2017). It was concluded that a better understanding of the size of the globules' effect on milk gelation could lead to the development of cheese with specific properties.

5.5 CATALASE

The enzyme catalase could be utilized in a special application in order to produce cheese. In the case of producing some types of cheeses like Swiss, hydrogen peroxide, a strong oxidizer that is toxic to cells, is used in the state of pasteurization. It is used to retain natural milk enzymes that are useful for the finished product and flavor development of the cheese

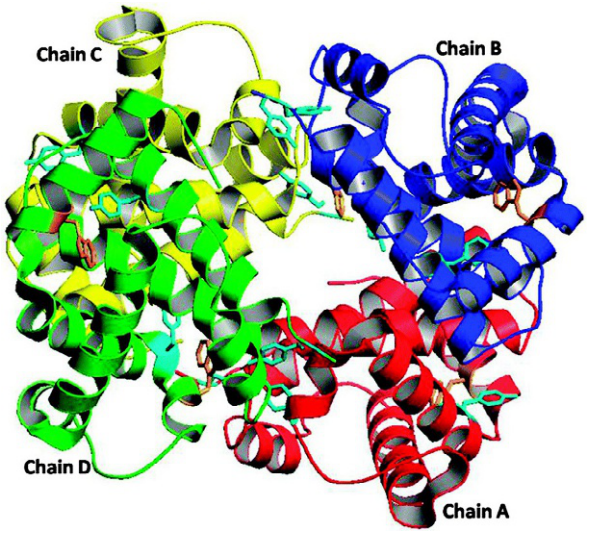

FIG. 5.3 Protein structure of catalase.

(Perin et al., 2017). Even though the high-level heat of pasteurization could break down these enzymes, residues of hydrogen peroxide in the milk prevent the bacterial cultures that are needed for the actual cheese production, so all traces of it must be removed. There are many resources used to get catalase enzymes like bovine livers or microbial sources. To change hydrogen peroxide into water, as well as molecular oxygen, catalase enzymes are added (Fig. 5.3).

5.6 PROTEASES

The proteases of lactate bacteria are necessary for its growth in substrate (milk) and help dramatically in enhancing flavor of fermented milk products. The proteolytic enzyme system constitutes proteinases that first break down the protein of the milk into peptides. Peptidases then breaks down the peptides into amino acids and small peptides, then the transport system takes charge of the uptake of amino acids and small peptides (Fig. 5.4). It has been reported that milk bacteria have a complicated proteolytic activity which can transform milk casein into free peptides and amino acids required for its growth. The proteolytic proteinases involve amino-peptidases, tri-peptidases, *endo*-peptidases, extra cellular proteinases, and proline peptidases (serine proteases) (Qureshi et al., 2015). Other studies have been shown that lactic streptococcal proteinases have many proteinases of a non-lactostreptococcal source. Moreover, proteinases from *L. lactis*, *L. helveticus*, *L. delbrueckiisp*, *L. bulgaricus*, *L. plantarum* and *Lactobacillus acidophillus* are serines type of proteinases. Aminopeptidases play a significant role in enhancing the flavor of fermented milk products because they can secrete single residues of amino acid from large oligopeptides established by activity of extracellular proteinase. They were also used to minimize allergic properties of milk products and accelerate cheese processing. A development of a new laboratory technique to evaluate protease activity

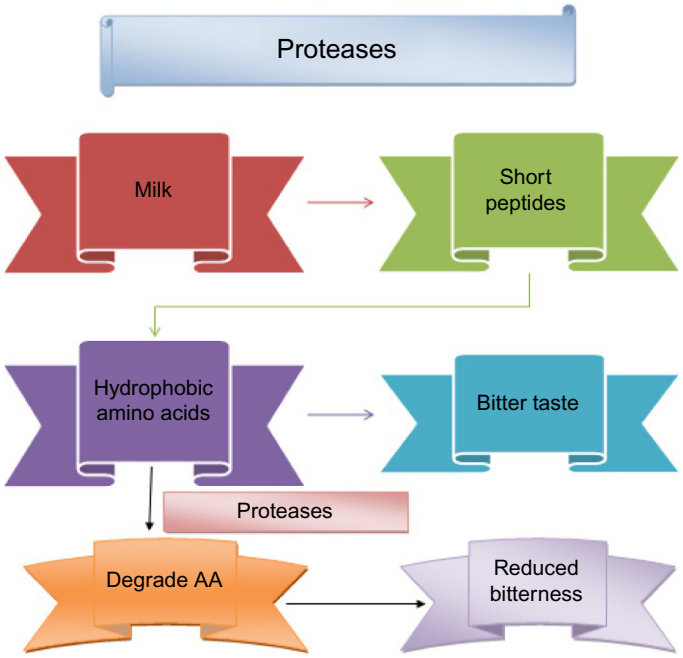

FIG. 5.4 Role of proteases in dairy industries.

in sheep and goat's milk has been done (Palomba et al., 2017). The study showed that the technique is useful for the proteolytic activity in different media and its effectiveness depends on chemical-nutritional characteristics of the sample.

5.7 LIPASES

Lipases are used to enhance flavor, speed the process of cheese, create customized milk products, and break down milk fat. Lipases are water-soluble enzymes, and help catalyze the hydrolysis of ester bonds in substrates (lipids) (Svendsen, 2000). Microbial lipases are produced by microorganisms together or individually with esterases. Examples of lipase-producing microorganisms are *Serratia marcescens*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococococcus aureus*. For the production of free fatty acids, glycerol, and various esters, lipase is used as biocatalyst. Furthermore, lipase is used for the production of fat and glycerides, which are esterified or amended from the cheaper substrates, such as oil of palm. Widely, many industries, such as pharmacological, chemical, and food use those products. Different lipases of animal or microbe origin created clear cheese, low bitterness, improved flavor, and potent malodors, whereas proteinases, in combination with lipases or/and peptidases, developed cheeses with excellent flavor and low bitterness levels. In order to accelerate the ripening of cheese content of peptidases and proteinases we can use attenuate cell-free extract or starter cells to do that (Sharma and Sharma, 2017).

5.8 TRANSGLUTAMINASE

Transglutaminase (Tgase) is a catalysts in the polymerization of milk proteins and enhances the properties of milk products (Fig. 5.5). Human lactose intolerance is due to the inability to digest lactose because of a deficiency in the secretion of the lactase enzyme. Rennet cheese with modified textural and nutritional properties and improved yield could be obtained upon transglutaminase modification but simultaneous addition of rennet and transglutaminase is recommended (Domagała et al., 2016). Moreover, transglutaminase crosslinking and calcium reduction were investigated as ways to improve the texture and storage stability of high-protein nutrition (HPN) bars formulated with milk protein concentrate (MPC) and micellar casein concentrate (MCC). Hardness, crumbliness, moisture content, pH, color, and water activity of the HPN bars were measured during accelerated storage. The HPN bars prepared with MPC were harder and more cohesive than those prepared with MCC. Higher levels of Tgase crosslinking improved HPN bar cohesiveness and decreased hardening during storage (Banach et al., 2016). Also, a study evaluated panela cheeses—made from dairy-plant protein blends, using soybean or peanut protein isolates, supplemented with transglutaminase—has been done. The results showed that panela cheeses can be elaborated following a traditional procedure, but with the addition of soybean or peanut protein to the dairy ingredients. Cheeses containing these protein isolates showed higher protein content than the milk control cheese and had similar textural characteristics (Salinas-Valdes et al., 2015).

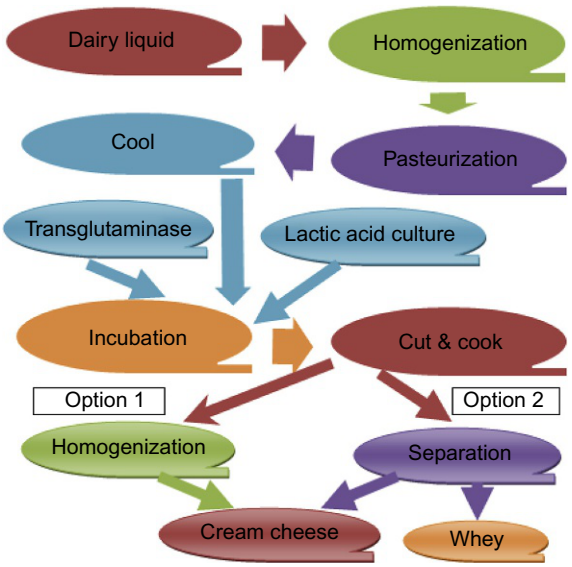

FIG. 5.5 The steps of the cream cheese production by transglutaminas.

5.9 LACTASE

Lactase (EC 3.2.1.23) accelerates the breakdown of lactose into galactose and glucose. It is used to improve the sweetness, solubility, as well as digestive agent, for milk products (Qureshi et al., 2015). Lactases are important in reducing and removing lactose in milk products for lactose-intolerant patients in order to protect them from severe diarrhea, fatal consequences, and tissue dehydration. Among various features of milk treated with lactase is the increased sweetness, hence it can avert the need for adding sugars during the production of flavored milk drinks. Lactase is used by the producers of ice cream, yogurt and frozen desserts to enhance spade and creaminess, sweetness, tastiness, and digestibility, and to decrease sandiness because of crystallization that occurs in lactose converged preparations (Fig. 5.6). Cheese produced from hydrolyzed milk ripens more quickly compared to cheese produced from normal milk. In the last decade, much research has been conducted on lactose existing in whey and milk, and the fatal consequences of β-galactosidase, lactase or hydrolyase (Mehaia and Cheryan, 1987). These are due to the immobilization techniques of enzymes that have provided great and new potential uses for lactose. Due to the incapability of intestinal enzymes for some individuals, there is evidence of sensitivity to lactose—drinking of milk and eating dairy products is difficult. Subsequently, product aids containing small amounts of lactose or food that does not contain lactose is suitable for people who are unable to tolerate lactose. This protects them from tissue dryness, diarrhea, and sometimes death. Scientifically, lactose crystallizes readily and quickly and puts outlines to some operations in the manufacturing of dairy. As a result of high costs, this method of usage of lactase is not viable. Furthermore, environmental damage is considered the major obstacle related to separating high amounts of cheese whey. Moreover, through the fermentation process, the isolated whey can be used to produce lactic acid because it is an inexpensive source of lactose. Due to the fact that it is a derivative product in the production of concentrated whey protein by filtration through a medium, the whey permeate can be fermented effectively by *Lactobacillus bulgaricus* (Mehaia and Cheryan, 1987). Manufacturers could obtain lactose from different sources—plants, animal organs, bacteria, as well as yeasts (intracellular enzyme) and molds, which are utilized to prepare enzyme for commercial purposes. Lactase production from *Aspergillus niger*, *Aspergillus oryzae*, and *Kluyveromyce slactis* are deemed safe due to the safe usage history of those resources. It has been subject to multiple safety examinations, whereas the most developed lactase, rerived from *E. coli* lactase, is not reliable in food treatment due to its high cost and poisoning issues. The enzyme's immobilization, immobilization method, and carrier type can also affect these optima rates. Generally, lactase derived from fungus has a pH in the ideal acidic range of 2.5–4.5, and yeast and lactases derived from bacteria in the neutral area 6–7 and 6.5–7.5,

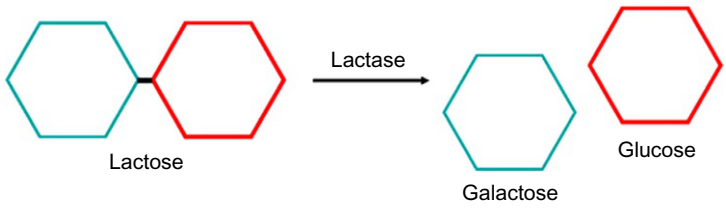

FIG. 5.6 Hydrolysis of lactose milk into glucose and galactose.

consecutively. The differences in ideal pH levels of lactases are the reason for their convenience for various uses. For instance, lactases derived from fungus can be utilized for acid whey decomposition, whereas yeast and lactases derived from bacteria are appropriate for milk with a pH level of 6.6, as well as sweet whey with a pH level of 6.1 decomposition. Galactose inhibition, as a type of product inhibition, is one characteristic that relies on the lactase resource. Galactose can inhibit the enzyme from *Aspergillus niger* more highly than the enzyme from *Aspergillus oryzae*. At minimum, hydrolyzed lactose can solve the product inhibitions through utilizing systems of immobilized enzymes or through improving the enzyme by the process of filtration using a medium after batch hydrolysis. Bacillus species lactases are the top lactases associated with thermo stabilization, the average of pH process, inhibition of product, and the sensitivity toward concentration of high substrate. Enzymes of thermo stabilization can keep their active condition under 60°C or more at protracted times. In this case, they have two distinguishing features: they give higher shorter residence time or higher conversion rates for certain transformation rates. Moreover, the microbial contamination is less because of higher growing temperature.

Bacillus sp. have low inhibition activity by galactose and high activity toward skim milk. For these reasons, *Bacillus* sp. are essential for the production of lactase (Gekas and Lopez-Levia, 1985). Lactose hydrolysis is perhaps accomplished by using the following enzymes: (1) free enzymes, commonly used in the impulse fermentation process, (2) immobilized enzymes, and (3) immobilized total cells manufacturing intracellular enzymes. Many hydrolyzed systems have been examined; few numbers have been successesful and fewer numbers have been used at the semi-industrial or industrial level. Different hydrolysis systems for acids have been improved to serve many industries. By using *K. lactis* lactase, numerous systems that utilize the free-enzyme process have been improved to treat and prepare whey and UHT-milk (Maxilact, Lactozyme).

Many commercially immobilized systems have been improved for large-scale production. In Italy, industrial milk treatment technology operations represent one as a cooperative method. Fiber-entrapped yeast lactase can be beneficial in a batch operation. Additionally UHT is a system used to sterilize the milk used. For leader factories, two other operations have been improved and designed for treating milk by Gist-Brocades (Germany), and Sumitomo (Japan). These are uninterrupted operations having average length of time. The UF-permeate operation of the whey is carried out through this system that was established by Connecticut, Corning Glass, Lehigh, Valio, and Ameracecorp. The process developed by Corning Glass is used in a commercial level in the production of baker's yeast by the use of hydrolyzed-whey (Gekas and Lopez-Levia, 1985). It has been shown that the in-pack addition of lactase after milk sterilization can have a negative sensorial and nutritional consequences, mainly related to the enzyme side of proteolytic activity, especially for prolonged storage times (Troise et al., 2016).

5.10 AMYLASES

Amylases are enzymes that originated from fungi and bacteria. They are playing an important role in food and beverage, baking, brewing, starch, and sugar industries. In order to hydrolyze starch into a water-soluble product, amylase is used for this purpose (Fig. 5.7).

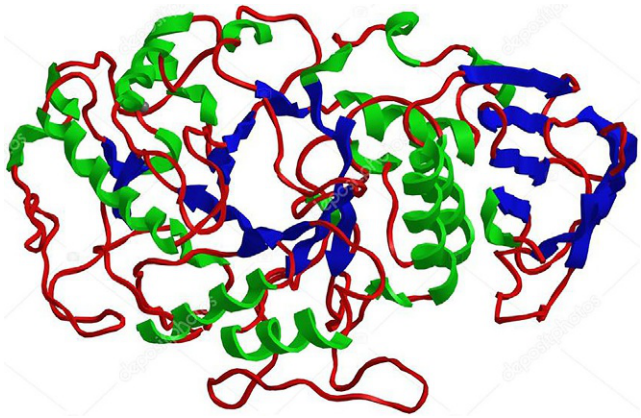

FIG. 5.7 Bacterial α-amylase used in food industry.

It is important to note that amylase is characterized by a glucose of low molecular weight. Widely, amylase enzymes can be applied in both drink and textile industries. In drinking, for instance, it is utilized for the production of High Fructose Syrup (HFS) (Ziegler, 1999). There are many microbial sources that can produce amylases such as *Bacillus*, *Pseudomonas* and *Clostridium* family. Nowadays, and at industrial level *Bacillus licheniformis* and *B stearothermophillus* are the probable bacteria used to produce amylases (Ploss et al., 2016). For the sake of reducing costs, *B stearothermophillus* is widely used because this strain is able to produce thermo-stable enzymes (Sundarram et al., 2014). α-Amylases are characterized by their impact on baked goods. It is important to add α-amylase and sugar to compensate for the grain deficiency. The addition of enzymes provides various advantages on the sugar. At a flour mill, adding enzymes to the flour may be standard so that a same product could be offered. Moreover, enzymes gradually form sugar that meets with the yeast growth requirements (Kulp et al., 1981). While the paste is placed in the bakery, steadily the temperature increases which causes an increase in the reaction rate of the enzyme and more sugars are produced. Malt extract and malt flour may be utilized as an enzyme supplement because malt is rich in α-amylase. However, it is preferable to use fungal α-amylase. The α-amylase hydrolyzes the wheat starch flour to small units of dextrins, hence the yeast is allowed to work constantly during dough fermentation and on the earlier stages of baking (Chi et al., 2009). This causes enhancements in bread volume and crumb texture. Furthermore, when three enzymes produce sugars such as maltose and glucose and small oligosaccharides, it helps in improve the baked flavor and reactions of the crust browning. Supposedly, ß-amylases in cereals are extensively studied as they play a very significant role in the release of facilely fermentable sugars of cereal grain starch and convert it to alcohol (bio-fuel) by yeast. Cereal beta-amylases can be used in many applications including analysis of starch, as well as in the food industry, and they make useful markers in breeding studies and cereal estimation. Finally, amylase splay an important role in degrading milk into monomeric molecules such as fatty acids oligosaccharides, and amino acids; molecules responsible for flavors in cheese (Konkit and Kim, 2016).

5.11 CONCLUSION

Only a relatively small number of microbial enzymes are used commercially in the dairy industry. But the number is increasing day by day, and their field of application will be expanded more and more in near future. The purpose of this chapter is to describe the practical applications of microbial enzymes in the field of the dairy industry.

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CHAPTER

6

Wine Enzymes: Potential and Practices

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6.1 INTRODUCTION

Wine, a word that evokes emotion in millions across the world, is regarded as a form of culture and style in many parts, and claims a major share in the gross revenue of many countries. In 2014, global wine production was 271 billion liters (271 million hectoliters) and consumption was 234 billion liters (http://www.bkwine.com) indicating an oversupply of 15%–20% which led to fierce competition in the marketplace. The wine market does witness some novel changes, like demand for premium, ultra-premium (Pretorius, 2000), and low-alcohol wines. These factors give the wine industry a strong market orientation which in turn opens the scope for research and innovation toward quality improvements and varietal changes to attract consumers. Most of these improvements depend on the application of enzymes of desired types, and their catalytic quality. Enzymes may be applied to facilitate many of the practices of wining such as the release of juice, color and varietal aromas, clarification of juice, and stabilization of wine before bottling. Although native-enzymes from grapes and natural microbes (yeasts, fungi, and bacteria) are naturally added to the must and wine at different stages of production, most of them are hardly effective in harsh physicochemical conditions during wining. Thus, the addition of exogenous enzymes is important. Currently, wine technology is also exposed to applications of cold-active enzymes at different stages to retain and enhance the aromatic profile. However, the most important consideration in the application of enzymes is the ability of individual enzyme to resist the harsh physicochemical conditions at specific stages of application during winemaking.

https://doi.org/10.1016/B978-0-12-813280-7.00006-2

6.2 PHYSICOCHEMICAL CONDITIONS DURING IMPORTANT STAGES OF WINING

6.2.1 Red Wine

Red wine is produced from red grapes. The first step, maceration, involves the crushing of fruits to get a product called must, which contains pulp, skin, and broken seeds. The style of the wine developed from this must thus has the features of a variety of specific inherent characteristics of all three parts of the fruit. A recommended dose of free SO_2 is added at this stage to inhibit growth of undesirable microbes. A one- to two-day incubation period of must at cold temperatures (15–20°C) called cold sock, is generally followed to delay the onset of fermentation so as to facilitate release of color (mainly anthocyanins and tannins) from skins (the skin contact phase) before ethanol production. The color and tannins continue to be extracted from skins during fermentation. The amount of color also depends on pH and SO_2 concentration—a lower pH means more color, while a higher SO_2 means less color as the latter bleaches monomeric anthocyanins. Tannins, which are the astringent compounds of variable sizes, can condense and polymerize to form bigger molecules of lesser astringency and yellow-brown color. Tannins may now form complexes with pigments, which are less sensitive to changes in pH and SO_2 concentrations and able to cause color stability in wine.

6.2.2 White Wine

Clean grape juice without skin and seeds (sources of anthocyanins and tannins) is used to make white wine. Grape juice is then allowed to settle for some period to facilitate settling down of any solids. The clear juice on the top is then taken in another tank (racking) to initiate fermentation. Typically, fermentation is done at low temperatures to prevent spoilage as juice from white grapes does not contain preservative substances. The common aging practices for white wines include batonnage, that is, the mixing of dead yeast cells with wine to add a creamy and nutty characteristic to the wine, and barrel (oak made) aging in small barriques to impart a range of flavors in the wine. Finally, the removal of haze-causing proteins typically by the addition of bentonites, and tartaric acid and/or by keeping the temperature consistently low (cold stabilization) may be followed. Therefore, it may be concluded that:

(a) The pH at which red wines are produced is lower than that at which white wines are produced. Thus, although acidic enzymes would be desirable for both the wines, they are essential for red wine.
(b) White wine fermentation is done at lower temperatures and hence cold-active enzymes are more desirable for white wine, though they are also good for red wine.
(c) SO_2 is added at most stages of white winemaking, although it is used less liberally during red winemaking. It is always used following the completion of the malolactic fermentation of these wines. SO_2 resistant/tolerant enzymes would thus be desirable in general but more specifically for white wine.
(d) Unstable proteins that precipitate in bottles are commercially undesirable, and the problem exists more with white wine. These proteins are generally removed with bentonite. Because bentonite precipitates proteins, it would also precipitate enzyme(s) to

be used during wining thus reducing activity substantially. Enzymes are therefore used before the addition of bentonite.

(e) Because tannins react with proteins, wines containing higher amounts of tannins have to be treated with gelatin to remove excess tannins prior to adding enzymes.

(f) Cinnamyl esterase (CE) activity, whose source may be contaminating microbes (*Brettanomyces bruxellensis*) or wine enzymes themselves, catalyzes the first step of the two-step process for the synthesis of vinyl-phenols; the latter reduces the fruity character and in extreme case, adds unpleasant smells in white wines. CE free enzymes are thus desirable.

6.3 ENZYMES USED IN WINE MAKING

6.3.1 Pectinases

Pectinases are the generic name given to an assemblage of enzymes, each catalyzing hydrolysis of specific forms of pectin.

6.3.1.1 Classification

As pectinases are the most important group of food processing enzymes, they are also the most extensively studied. They are classified on the basis of mode of action, substrate preference, and nature of products, as seen in Table 6.1.

6.3.1.2 Roles

Hydrolysis of pectic substances: The cell walls of plant cells contain pectins that need to be modified during certain growth stages (Whitaker, 1990) and during ripening of fruits. To achieve this, plant cells are capable of synthesizing pectinases. In addition to the endogenous enzymes, the grape obtains various pectinases produced by associated microflora that enter the must and the wine with the grape berries. Among microflora, *Botrytis cinerea*, which causes gray rot in grape, is of prime importance. Juice extraction and clarification: pectinases disrupt cell walls and thus facilitate the release of free-running juice and color. If applied before pressing, the enzymes yield a pulp with good pressing characteristics. Post-pressing application causes reduction in grape juice viscosity and aggregation of cloud particles into bigger molecules, thus facilitating their settling and removal. Often, the combination of pectinases, cellulases, and hemicellulases (together called the macerating enzymes) is found more efficient than pure pectinolytic enzymes in the extraction of juice and degree of settling. Extraction of pigments and phenols: the treatment of red grape must with pectinases is generally found to accelerate the release of pigments and phenols. The enzyme has also been reported to increase the intensity of certain varietal fragrance and flavors and magnify astringency features (Pretorius, 2000). Methanol formation: one undesirable effect of the use of pectinases in winemaking is the enhancement of methanol levels in the product. This is to note that methanol is also naturally contributed by endogenous pectinases in wine due to the action of pectin methyl esterases (PME), a component of pectinases complex. Because heavily methylated pectin is a poor substrate for polygalacturonases, the activity of PME to demethylate the pectin for its subsequent degradation is essential. PME in this way seems to

TABLE 6.1 Classification of Pectinases Based on Mode of Action, Substrate Preferences and Products

Enzyme	EC No.	Substrate	Mode of Action	Product(s)
Protopectinase	3.2.1.-	Protopectin	Hydrolyze glycosidic bond at water-soluble pectin sites with >3 nonmethylated GA*	Water-soluble pectin
De-esterifyer				
Polymethylesterase (PME)	3.1.1.11	Pectin	Random cleavage of methyl ester	Pectic acid + methanol
Polyethylesterase (PEE)	3.1.1.6	Pectin	Random cleavage of ethyl ester	Pectic acid + ethanol
Depolymerizer				
(a) Hydrolases	NF	Pectate	Cleave α-1,4-glycosidic bond	NF
i. Poly GAses (PG)				
EndoPG	3.2.1.15	Pectate	Random cleavage	Oligo-GA
ExoPG	13.2.1.67	Pectate	Terminal cleavage	Mono-GA
ii. Polymethyl GAses (PMG)				
EndoPMG	NF	Pectin	Random cleavage	Oligomethyl-GA
ExoPMG	NF	Pectin	Terminal cleavage	Methylmono-GA
(b) Lyases	NF	Pectin/ Pectate	Cleave α-1,4-glycosidic bonds by transelimination	Unsaturated GA
i. Poly GAlyases (PGL)				
EndoPGL	4.2.2.2	Pectate	Random cleavage	Unsaturated GA
ExoPGL	4.2.2.9	Pectate	Cleave penultimate bonds	Unsaturated di-GA
Oligo GL	4.2.2.6	Oligo-GA	NF	Mono-GA
ii. Polymethyl GAlyases (PMGL)				
EndoPMGL	4.2.2.1	Pectin	Random cleavage	Unsaturated methyl-GA
ExoPMGL	NA	Pectin	Terminal cleavage	Mono-GA

GA*, galacturonic acid; NF, not found.

be a "necessary evil." It has been suggested that certain factors like grape variety, yeast strain, and wining practices used may affect methanol formation (Nicolini et al., 1994). This is thus a challenge for wine makers to ensure PME activity occurs without letting methanol levels to be raised above permissible limits (400 mg/L is the maximum methanol limit as recommended by the International Organization of Vine and Wine, OIV).

Though pH conditions (3–4) maintained throughout wining process are generally favorable for the endogenous pectolytic enzymes (optimum pH varies between pH2 and 8), the temperatures that are maintained during winemaking and other factors such as sulfur dioxide (SO_2), tannins, alcohol, and bentonite, are inhibitory to the same. The application of commercial pectinase preparations is therefore generally felt necessary.

6.3.1.3 Desired Characteristics

(a) Low optimum pH (3–5), and temperature (10–20°C)
(b) Resistant to SO_2, tannin and bentonite
(c) Ideally, able to act on methlylated pectin with negligible PME activity

6.3.1.4 Commercial Pectinases and Their Characteristics

The pectinases are the most important and commonly used enzyme preparations applied in wining to facilitate extraction of juice, color, tannin and flavor compounds. Fungus (*Aspergillus* sp.) is the main source of available commercial pectinases preparations (Alkorta et al., 1994); some of them are given in Table 6.2.

6.3.1.5 Genetic Engineering to Generate Pectinolytic Yeast

Overexpression of the polygalacturonase gene in *Saccharomyces cerevisiae*: the gene *PGU1* encoding polygalacturonase was successfully cloned, sequenced from *S. cerevisiae* IMI-8b (Blanco et al., 1998) and expressed as additional copy in many strains of *S. cerevisiae* with the result of increased enzyme activity in the recombinant strains. Plasmids were used, and genetic backgrounds of the strains were found to be of vital importance for the expression of *PGU1*. Heterologous expression of pectinase genes in *S. cerevisiae*: the genes *pelE* and *peh1* encoding pectate lyase and polygalacturonase from *Erwinia chrysanthemi*, and *Erwinia carotovora*,

TABLE 6.2 Some Commercial Pectinases for Winemaking

Enzyme	Company	Activities	Time of Addition
(A) Purpose: To improve the clarification, filtration and yield of juice and wine			
Rapidase Vino Super	Gist brocades	Pectolytic	To juice before settling
Endozym A	AFB Africa	Pectolytic	To juice before settling
Pectinex superpress	Novo Nordisk	Pectolytic + hemicellulose	Directly into mill
Rapidase filteration	Gist-brocades Yeast Anchor	Pectolytic + glucanase Glucanase	End of fermentation
(B) Purpose: Improve extraction and stabilization of color			
Enzym' color plus	Darleon	Pectolytic + proteolytic	To juice or must
Endozyme contact	AFB Africa	Pectolytic	To juice or must
Vinozym EC	Novo Nordisk	Pectolytic + arabinose + cellulase	Into crusher or mash tank
Rapidase color	Gist-brocades	Pectolytic + side activities	Before maceration

Modified from van Rensburg, P., Pretorius, I.S., 2000. Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations—a review. S. Afr. J. Enol. Vitic. 21 (Special Issue), 52–71.

respectively, were expressed as inserts in various expression cassettes. These cassettes contained promoter, secretion signals, and terminator sequences from bacterial and yeast sources (Laing and Pretorius, 1993a). For example, sources of transcription initiation signals were promoters of the yeast alcohol dehydrogenase I gene (*ADH1*) and mating α-factor (*MFαS*) and bacterial (*Bacillus amyloliquefaciens*) α-amylase (*AMYl*) genes, whereas the source of transcription termination sequence was the yeast tryptophan synthase (*TRP5*) gene. The leader sequences controlling secretion from yeast *MFαS*, *B. amyloliquefaciens* α-amylase, *E. chrysanthemi* pectate lyase (*pel1*), and *E. carotovora* polygalacturonase (*pehE*) genes were used as sources for secretion signals. Thus, constructed cassettes ADHlp-MFαS-peIE-TRP5T (*PEL5*) and ADHlp-MFαS-pehl-TRP5T (*PEH1*) were used to express (Laing and Pretorius, 1993b) and co-express in *S. cerevisiae*. The co-expression was found to increase pectin hydrolysis synergistically. The effort was furthered by the construction of another construct (*END1*) harboring the *endo*-β-1,4-glucanase (*Endl*) gene and its successful co-expression with *PEL5* and *PEH1* in wine yeast strains of *S. cerevisiae* (Van Rensburg et al., 1994). Likewise, the endopolygalacturonase gene from fungus (*Aspergillus niger*) under the control of the *ADH1* promoter was also successfully expressed in *S. cerevisiae* (Lang and Looman, 1995). The recombinant pectolytic wine yeast was also constructed by introducing expression cassettes containing the *PelA* gene from *Fusarium solani* F. sp. pisi fused to *S. cerevisiae* actin gene promoter in wine yeast strains (Gonzalez-Candelas et al., 1995). Another pectinolytic strain of *S. cerevisiae* was generated using *S. cerevisiae PGU1* under constitutive promoter *PGK1* (of 3-phosphoglycerate kinase gene) in an integrative process targeting a dispensable upstream region of the acetolactate synthase locus (*ILV2*), which determines sulfometuron methyl resistance. Though application of this recombinant strain was found to enhance the yield of red wine by 7% and color intensity to some extent, the performance was not on par with what was achieved with enzymes (Fernández-González et al., 2005).

6.3.1.6 Cold-Active Pectinases

Application of a cold-process (15–20°C) is believed to be advantageous in the wine industry as it increases the production and retention of volatile compounds, thereby improving the aromatic profile of wines (Sahay et al., 2013). Pectinases suitable for the cold wining process need to be cold-active and acid-tolerant apart from tolerance to other factors. Therefore, cold-active pectinolytic enzymes are required for the wine industry for both extraction (juice and color), and clarification (Sahay et al., 2013). Despite these advantages, a few reports on isolation and characterization of cold-active pectinases are available (Singh et al., 2012; Martín and de Ambrosini, 2013; Merín and de Ambrosini, 2015; Sahay et al., 2013). Cold-active pectinases from bacterium *Bacillus* sp. has been found to have predominantly polymethylgalacturonase activity under acidic and low-temperature growth conditions, whereas the highest levels of pectate lyase activity were found at 60°C. The enzymes were active at oenological conditions; that is, at 20°C, with a pH of 3.6, and showed 15% and 30% of the maximum activity at 5 and 10°C, respectively (Martín and de Ambrosini, 2013). Under similar conditions, pectinases (PME, exoPG and endoPG) from the psychrotrophic yeasts *Cystofilobasidium capitatum* SPY11 and *Rhodotorula mucilaginosa* PT1 showed 50%–80% activity (Sahay et al., 2013). The cold-active pectinases from *Aureobasidium pullulans* strains have been reported to remain active at glucose, ethanol, and SO_2 concentrations usually found in vinification (Merín and de Ambrosini, 2015).

6.3.2 Glucanases

Glucanases are a group of enzymes that catalyze the hydrolysis of glucans. Glucans constitute a wide variety of carbohydrates mainly because of the rich diversity of monosaccharide building blocks and possibility of their many stereo and regiospecific isomers (Laine, 1994), giving rise to simple and linear complex and branched molecules (Varki, 2009). There is also flexibility in linkages between the building blocks that may be of α (α-glucans) or β (β-glucans) types. The participating carbon may also vary, thus there is the possibility of further linkages, namely, α-1,3, α-1,4, α-1,6 or β-1,3, β-1,4, β-1,6. There is also the possibility of mixed types of linkages, for example, β-1,3–1,4, and the building blocks may be arranged in a linear (e.g., paramylon consisting of β-1,3 linkage only hence called β-1,3-glucan), or branched pattern (e.g., scleroglucan containing β-1,3–1,6-linkage). Cellulose is the most abundant glucan consisting of D-glucose bound by β-1,4 glycosidic bonds.

6.3.2.1 Classification

As there are various types of glucans, different enzymes are found in nature to hydrolyze these glucans. In traditional systems, enzymes are classified on the basis of the reaction-type they catalyze. The enzyme commission also provides a code, called the EC number, based on these criteria. If any enzyme catalyzes more than one type of reaction, it will bear more than one EC number. Likewise, if more than one enzyme exhibits similar reactions, they would share same EC number. Such a conflicting situation exists in the case of glucanases, as many of them exhibit broad substrate specificity and some exhibit more than one type of enzymatic activity, for example, hydrolase (EC 3.2.1) and transglycosidase activity (EC 2.4.1). Therefore, a computer program has been developed to apply a sequence-based classification of glucanases (www.cazy.org). The families are differentiated by the similarities of amino acid sequences and corollary folds. Usually, family members exhibit similar mechanisms (retaining or inverting) except the members of GH97, which exhibit both retaining and inverting mechanisms. The GH4 and GH109 family of enzymes that exhibit NAD-dependent catalysis could hydrolyze both α- and β-glycosides. Altogether, there are 120 families (GH1-GH125), and the enzymes are named as CAZyme (carbohydrate active enzyme). For practical purposes, the traditional classifications may also be useful whereby the glucanases are grouped into three categories, namely, (1) endoglucanases (EC3.2.1.4), which hydrolyze internal glycosidic linkages, randomly produce oligosaccharides, (2) exoglucanases, also known as cellobiohydrolases (EC 3.2.1.91), hydrolyze glycosidic linkages sequentially from nonreducing end producing glucose residues and, (3) β-glucosidases (EC 3.2.1.21) cleave linkage between glucoses in cellobiose. Furthermore, in the former two groups, the type of linkages the enzymes cleave may also be used to characterize a glucanase. Some of the characterized glucanases on that basis are given in Table 6.3.

6.3.2.2 Roles

For a wine maker, the most important glucan is β-1,3–1,6 glucan (Dubourdieu et al., 1981), secreted by *Botrytis cinerea* when it infects grape berries and the yeast during fermentation and aging. This *B. cinerea* glucan shows a very high molecular mass and a chemical structure composed of β-D-l,3-linked glucose chain, with short β-D-l,6 linked side branches (Dubourdieu et al., 1981). Yeast releases glucans along with many desirable compounds such as

TABLE 6.3 Some of the Glucanases Identified on the Bases of Substrates Used (Bonds Cleaved)

Enzyme	EC Number	Glycosidic Bonds Attacked
α-1,3 Glucanases	3.2.1.84	α-1,3 bond
α-1,4 Glucanases	3.2.1.3	α-1,4 bond
α-1,6 Glucanases	NF	α-1,6 bond
α-1,3–1,4 Glucanases	NF	α-1,4 bonds adjacent to α-1,3 bond
α-1,3–1,4 Glucanases (Mycodextranase)	NF	α-1,4 bonds with alternating α-1,3 and α-1,4 bonds
Endo-β-1,3 Glucanases	3.2.1.4	Internal β-1,3 bonds
Exo-β-1,3 Glucanases	3.2.1.58	Terminal β-1,3
Endo-β-1,6 Glucanases	3,2.1.75	Internal β-1,3 bonds
Endo-β-1,3–1,2 Glucanases	3,2.1.75	β-1,2 bonds adjacent to β-1,3 bonds
Endo-β-1,3–1,4 Glucanases	3.2.1.73	β-1,4 bonds adjacent to β-1,3 bonds
Endo-β-1,3–1,6 Glucanases	3.2.1.73	β-1,6 bonds adjacent to β-1,3 bonds

NF, not found.
Adapted from Nombela, C., Molina, M., Cenamor, R., Sanchez, M., 1988. Yeast β-glucanases: a complex system of secreted enzymes. Microbiol. Sci. 5, 328–332.

mannoproteins, amino acids, low-molecular-mass peptides, and nucleotides, which have a positive effect on wine mouthfeel. Another glucan of interest is β-1,3–1,2 a glucan released by spoilage lactic acid bacteria, such as *Pediococcus.* These glucans are highly viscous in nature which negatively affects wine filterability and clarification.

6.3.2.3 Desired Characteristics

(a) Low optimum pH (3–5), and temperature (10–20°C)
(b) Resistant to SO_2 and tannins; preferably to bentonite
(c) High β-1,3–1,6 glucanase activity

6.3.2.4 Commercial Glucanases

Wine glucanases (β-1,3–1,6 glucanase) are produced by applying *Trichoderma harzianum*. Commercial preparations of glucanases for winemaking are given in Table 6.4.

6.3.2.5 Genetic Engineering to Generate Glucanolytic Yeast

Attempts have been made to generate a glucanolytic strain of *S. cerevisiae* by the heterologous expression of the *endo*-β-D-1,3–1,4 glucanase gene (*beg1*) from *B. subtilis* under its own promoter and signal sequences (Hunchliffe, 1985). Higher intracellular levels of β-glucanase expression was however found when the gene was ligated to 2.1 μ-based vector under *ADH1* promoter and transformed in the yeast (Cantwell et al., 1986). In another attempt, the *endo*-β-D-1,4 glucanase gene (carboxymethylcellulase), from the bacterium *Cellulomonas fimi*, was expressed in wine yeast (Skipper et al., 1985). The recombinant yeast showed a higher level of secretion if the upstream region of the coding sequence of this enzyme contained leader

TABLE 6.4 Commercial Preparation of Some Wine Enzymes

Enzyme/Trade Name	Company	Activity	Comments
Glucansaes			
Extralyse	Laffort	β-1,3–1,6 Glucanase + pectinases. Improve filterability	Releases more N_2 compounds from autolytic yeast cells
Lallzyme MMX	Lallemand	β-Glucanase + Pectinases	Clarification, filterability, aging of botrytized grapes
Rapidase Filtration	DSM	Pectolytic + β-glucanase	Add at the end of fermentation
Glucanex	Novo Nordisk	β-Glucanase	Improve filterability/clarification
β-Glycosidases			
Expression[20]	Darleon	Pectinase + β-glucosidase	Add to fermenting wines with residual sugar below 10 g/L
Trenolin Bukett	Erbsloh	Pectinase + β-glycosidase	At the end of alcoholic fermentation
lafase® HE Grand Cru	Laffort	Pectinase + β-glycosidase	For high stable color and elegant tannins with easier clarification in red wine
lafase® fruit	Laffort	Pectinase + β-glycosidase	Improve color, fruity aroma in red wine
Lafazym® Arom	Laffort	Pectinase + β-glycosidase	Improve free run & aroma in white wines
lafazym® Cl	Laffort	–	Facilitate must & wine clarification
lafazym® Extract	Laffort	Pectinases + side effects	Add during cold maceration, improve juice yield and aroma extraction
lafazym® Press	Laffort	Pectinases + side effects	Facilitate skin contact in white wine, juice yield and aroma extraction
Glucose oxidases (GOXs)			
Gluzyme Mono®	Novozymes	GOX	Reduce glucose content in must
Acid urease			
Enzeco®	EDC, NY	Acid urease	Hydrolyzes residual urea in wines
Acid Protease			
Proctase	AWRI	Aspergillopepsins I & II	Add to grape juice, prior to fermentation and heat for 1 min

Modified from van Rensburg, P., Pretorius, I.S., 2000. Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations—a review. S. Afr. J. Enol. Vitic. 21 (Special Issue), 52–71.

signals from the yeast K1 killer toxin gene. On the other hand, co-expression of endoglucanase- and exoglucanase-encoding genes from *C. fimi* in *S. cerevisiae* was found to enable the recombinant yeast to degrade filter paper and pretreated aspen wood (Wong et al., 1988). The objective of all these attempts, however, does not seem to specifically overcome the β-1,3–1,6 glucan effect during winemaking.

6.3.2.6 Cold-Active Glucanases

A metagenomic library of microbes isolated from the surface of pheophycean alga *Ascophyllum nodosum* has been reported to yield cold-active *endo*-β-1,4-glucanase (Martin et al., 2014). Genes coding for basic β-1,3-glucanases and acidic β-1,3–1,4-glucanase, cloned from ruminal bacterium *Fibrobacter succinogenes* S85, have been found to yield enzymes with 14%–35% residual activity at −4°C (Yaish et al., 2006). Another gastrointestinal bacterium *Paenibacillus* sp. YD236 strain, isolated from *Bos frontalis* feces, yielded gene *pglue8* encoding cold-active endoglucanses that could hydrolyze barley-β-D-glucan, CMC-Na, soluble starch, laminarin, and glucan from black yeast, optimally at pH 5.5 and 50°C, and retain 78.6%, 41.6%, and 34.5% maximum activity when assayed at 20, 10, and 0°C, respectively (Dong et al., 2016). Cold-adapted *endo*-1,4-β-glucanase, with 42 kD molecular mass, has also been purified from *Citrobacter farmeri* A1, a symbiotic bacterium of *Reticulitermes labralis*, showing activity at pH 6.5–8.0 and temperatures 30–40°C with >50% residual activity even at 5°C (Bai et al., 2016). A promising cold-active *endo*-β-1,4-glucanase gene (CaCel) has been cloned from Antarctic springtail *Cryptopygus antarcticus* coding for enzymes that work optimally at pH 3.5 and retain 80% of their activity at 0–10°C (Song et al., 2017).

6.3.3 Glycosidases

Glycosidases (EC 3.2.1 *O*-glycoside hydrolases) are key enzymes of carbohydrate metabolism. They catalyze cleavage of *O*-glycoside bonds in carbohydrates and carbohydrate-containing biopolymers (Sova et al., 2013).

6.3.3.1 Classification

For a biochemist, the words glucanases and glycosidases are confusing as both these categories of enzymes are included in the broad class of CAZyme (carbohydrate active enzymes). The author follows modern systems and hence suggests referring to CAZy (www.cazy.org) for details. Of various glycosidases, β-glycosidases (also named β-D-glucoside glucohydrolase) and EC 3.2.1.21, catalyze the hydrolysis of the β-glucosidic linkages, such as alkyl and aryl β-glucosides, β-linked oligosaccharides, as well as several oligosaccharides, with the release of glucose (Béguin, 1990), important for winemaking.

6.3.3.2 Roles

The aroma precursors of many varieties of grapes remain unexpressed due to its bondage with sugar molecules. These can be naturally and slowly released during wine aging, or intentionally released by using oenological enzymes during winemaking. The volatile compounds that can be released from glycosidic aroma precursors are mainly terpenes, C13 norisoprenoids, benzenic derivatives, volatile phenols, and C6 compounds. The sugar components in these compounds are either glucosides or diglucosides or less frequently

triglucosides. They all contain a glucosyl moiety, but in di- or triglucosides, and in addition to glucose, other monosaccharides, such as α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, or β-apiofuranose, may be present. The glycosidases, which are involved with the enzymatic cleavage of the disaccharide glycosides, include α-L-arabinofuranosidase, α-L-rhamnopyranosidase, β-D-xylopyranosyl, β-apiofuranosyl, and β-D-glucopyranosidase (also called β-D-glucosidase). The disaccharide-linked flavor compounds are hydrolyzed in two steps: first, depending on the precursors, the glycosidic linkages are cleaved by either an α-L-arabinofuranosidase, α-L-rhamnosidase, or a β-D-apiosidase; the second involves the liberation of the monoterpenols by β-glucosidase (Sanchez-Torres et al., 1996). The aroma precursors themselves are slowly converted to the free aromatic form over time through hydrolysis ensuring longevity of these styles of wine. Wines with short shelf lives, however, require exogenous enzyme addition for faster release of aromas. Because the strains of *S. cerevisiae* possess little or no β-glycosidase activity (Dubourdieu et al., 1988), and aroma compounds in grapes may be linked to various types of sugar components, hence, diverse types of exogenous glycosidases are required to release the aroma compounds. Prompted by this, many nonsaccharomyces yeasts such as *Hanseniaspora valbyensis*, *Brettanomyces anomalus* (Fla et al., 2005), and *Candida* species (Gunata et al., 1990) have been explored for glycosidases that may be suitable for varietal-specific aroma release (Laffort et al., 1989). Glycosidases are inhibited by glucose and can therefore only be used on finished wines with a residual sugar of <20 g/L. Some β-glycosidase activities can cleave the sugar from anthocyanin, leaving the unstable aglycon that spontaneously transforms into a colorless form. It is important that any enzyme used for the production of red or rosé wines should therefore not have any anthocyanase activity. Table 6.4 depicts some commercially available glycosidases and their characteristics.

6.3.3.3 Desired Characteristics

(a) Low optimum pH (3–5), and temperature (10–20°C)
(b) Resistant to SO_2, tannins, and ethanol (9%–16%)
(c) Possess β-glycosidase plus other desirable glycosidase activities
(d) Free from anthocyanase activity
(e) Resistant to glucose inhibition

6.3.3.4 Genetic Engineering

Heterologous expression of *Trichoderma longibratum* gene encoding β-1,4 glucanase in *S. cerevisiae* has been found to result in increased aroma intensity of the wine produced by recombinant yeast (Perez-Gonzalez et al., 1993). Recombinant aroma-producing *S. cerevisiae* strains containing heterologous genes *ABFI* (α-L-arabinofuranosidase) and *BXLI* (β-D-xylosidase) have been developed subsequently (Margolles-Clark et al., 1996). Another recombinant strain of wine yeast has been developed by the co-expression of α-L-arabinofuranosidase and a β-glucosidase from *Aspergillus awamori* and *Saccharomycopsis fibuligera*, respectively, that could produce wine with higher levels of monoterpenes such as citronellol, linalool, nerol, and α-terpineol as compared to the wine produced with commercial enzyme treatment (Zietsman et al., 2011). A yeast strain expressing a monoterpene synthase gene from the plant *Vitis vinifera* was reported to produce aroma by catalytic conversion of a universal precursor geranyl diphosphate to monoterpenes (Cordente et al., 2012). A comprehensive effort to construct two yeast expression/secretion gene cassettes namely, (1) a pectinase gene cassette (pPPK)

comprising the *endo*-polygalacturonase gene (*pelE*) from *E. chrysanthemi,* and the pectate lyase gene (*peh1*) from *E. carotovora* and, (2) a glucanase/xylanase gene cassette (pEXS) comprising the *endo*-b-1,4-glucanase gene (*end1*) from *Butyrivibrio fibrisolvens* and the *endo*-b-1,4-xylanase gene (*xynC*) from *A. niger* was made. The transformed wine yeasts produced pectinase, glucanase, and xylanase activities during the fermentation of Pinot Noir, Cinsaut, and Muscat d'Alexandria grape juices, thereby showing potential to improve the clarity, color intensity, and stability and aroma of wine concurrently (van Rensburg et al., 2007).

6.3.3.5 Cold-Active Glycosidases

Cold-active glycosidase showing 5% residual activity at 0°C, and over a broad pH range (5.5–10.9), with seven times greater activity with glucoside than with galactoside, has been purified from psychrophilic isolate *Paenibacillus* sp. (Shipkowski and Brenchley, 2005). β-Glycosidase from bacterial *Shewnella* sp. G5, isolated from intestinal content of the marine benthoic organism *Munida subrrugosa,* has been reported to exhibit high activity at 4 and 20°C on plate (Cristóbal et al., 2008). Gene encoding cold-active β-glycosidase (*bgl1C*), cloned from deep sea bacterium *Exiguobacterium oxidotolerans* A011, has been found to yield enzymes that retain 61% activity at 10°C (Chen et al., 2010). A glucose-tolerant β-glucosidase has been reported from psychrotolerant *Pseudomonas lutea* BG8 exhibiting activity over a broad pH range (5–10) (Tiwari et al., 2014).

6.3.4 Glucose Oxidases

Glucose oxidase (GOX, EC 1.1.3.4) is the enzyme that catalyzes the oxidative conversion of glucose into gluconic acid.

6.3.4.1 Roles

A growing trend toward low-alcohol wines has been seen in the global market. The factors leading to such a trend may be attributed to stricter drinking-and-driving legislation and the awareness of health risks arising from excessive alcohol intake (Room et al., 2005). Low-alcohol wines are said to have all the benefits (Stockley and Høj, 2005) without the toxicity; thus the demand for such wines have increased at the cost of high alcohol wines. Low-alcohol wines are classified as reduced-alcohol (1.2% to 5.5%–6.5% v/v), low-alcohol (0.5%–1.2% v/v), and de-alcoholized (not above 0.5% v/v) wines (Gladstones, 2000). Techniques to produce low-alcohol wines include various physical treatments such as reverse osmosis, distillation and osmotic distillation, thermal gradient processing, membrane separation and membrane extraction, pervaporation. Thin-film evaporation under reduced pressure removes excess alcohol from the product or redirects more of the grape sugars to glycerol or to other products when enzymes such as GOX and catalase are applied so that less sugar is available for fermentation to ethanol (Malherbe et al., 2003). All the physicochemical methods for de-alcoholization were, however, met with the criticism that they reduce sensory quality of the finished wine (d'Hauteville, 1993) and raise the overall production cost (Pickering et al., 1998). An initial experiment of the possible application of GOX in low-alcohol wine production showed some encouraging results such as the reduction of alcohol by 36%–40% (Pickering et al., 1999a) and enhanced stability against browning after 6 months of bottle aging, vis-à-vis, control wine (Pickering et al., 1999b).

The GOX-treated wine, though showed modification in taste, but the mouth feel and aroma features in general were found to be unaltered (Pickering et al., 1999c), barring reduction in the intensity of some specific aromas as a consequence of exposure to a mix of enzyme juice-to-air. Another similar experiment was performed with limited success (Biyela et al., 2009). The important concerns raised against GOX-treated wines are (1) the production of a higher level of carbonyl compounds that enhance their sulfur dioxide-binding attributes (Pickering et al., 1999b), thus necessitating the use of more sulfur dioxide to stabilize microbes (Pickering et al., 1999a), and (2) poorer aromatic profiles in the finished wines. The GOX application to get low-alcohol wines would thus require a refinement of techniques and GOXs with suitable catalytic properties. Table 6.4 depicts commercially available GOXs and their characteristics.

6.3.4.2 Desired Characteristics

(a) Low optimum pH (3–5), and temperature (10–20°C)
(b) High glucose oxidase activity
(c) Little or no effect on taste and aroma of wine

6.3.4.3 Cold-Active Glucose Oxidase

There is hardly any report on cold-active glucose oxidase.

6.3.5 Acid Urease

Acid urease (EC 3.5.1.5) is an enzyme which hydrolyzes urea into ammonia and carbon dioxide under acidic conditions, as follows:

$$NH_2CONH_2 + 2H_2O \rightarrow CO_2 + 2NH_3$$

6.3.5.1 Roles

Urea gives rise to ethyl carbamate after reacting with ethanol. Ethyl carbamate is a known carcinogenic compound for some animals, and probably for humans, as well. It is therefore recommended that the urea concentration should not exceed 1 mg/mL limit in the wine to avoid the formation of ethyl carbamate (Varga et al., 2011). Wine via various means receives urea at variable concentrations. Some of the important ways include (1) the metabolic conversion of arginine, a major amino acid present in grape juice, to various products by the wine yeast *S. cerevisiae* (Monteiro et al., 1989); urea being the intermediate product secreted in the must, (2) nonjudicious use of fertilizers containing urea shortly before harvest, (3) adding nutrient-containing urea to the wine fermentation medium to avoid lick or sluggish fermentations, and (4) use of *S. cerevisiae* strains showing higher urea-forming activity. Although one of the mild alternatives to reducing urea levels in wine is the use of a low-urea-producing yeast strain; a more effectively controllable method is the use of a suitable enzyme.

6.3.5.2 Desired Characteristics

(a) Low optimum pH (3–5) and temperature (10–20°C)
(b) High enzyme activity

6.3.5.3 Commercial Acid Urease

Application of commercial preparations of the acid urease in must to control urea concentration in wine has been advocated earlier (Ough and Trioli, 1988). Recently, the OIV has permitted this practice to reduce ethyl carbamate levels in wines, paving way for intense research to explore enzymes with better catalytic features. To comply with the regulatory regimes, whole-cell acid urease techniques (Esti et al., 2007) or immobilization techniques applying a suitable matrix (Eupergit C250L or chitopearls BCW-30003 and BCW-3010) and purified enzymes to remove urea from wines have also been developed (Andrich et al., 2010). Table 6.4 depicts commercially available acid urease.

6.3.5.4 Genetic Engineering

Urease-producing wine yeast strain: Because the arginase knock-out strain produced through sequential deletion of *CARI* gene in wine yeast turned out to be commercially nonviable due to its stunted growth (Kitamoto et al., 1991), efforts were made to engineer urease-secreting strains. A fusion product, consisting of the three subunits (α, β and γ) of urease operon from *Lactobacillus fermentum,* and linker sequences from jack bean urease, inserted between the three subunits (Visser, 1999), was constructed and expressed under the *S. cerevisiae PGKl* promoter and terminator signals in two yeasts. The construct was able to express in *S. cerevisiae* and *Schizosaccharomyces pombe*, in *S. cerevisiae,* however, the secretion of protein was very low and that too did not show any enzyme activity (Visser, 1999). The absence of urease activity in transformed-*S. cerevisiae* cells was attributed to the lack of accessory gene products; the latter are present only in urease-producing species such as *S. pombe*. In another approach, the urea importing strain 522^{DUR3}, constructed by the constitutive expression of the *DUR3* gene encoding integral membrane protein responsible for the import of urea at low concentrations, under the control of the *S. cerevisiae PGK1* promoter and terminator signals in *S. cerevisiae* strain 522, was found to reduce EC by 81% in Chardonnay wine (Dahabieh et al., 2009).

6.3.6 Acid Proteases

Proteases (protein hydrolases) catalyze amide (peptide) bond hydrolysis in protein or peptide substrates. Acid proteases are enzymes that catalyze degradation of proteins under acidic conditions.

6.3.6.1 Classification

(a) Exopeptidases (that may be aminopeptidases or carboxypeptidases) and endopeptidases, on the basis of site of action
(b) Aspartyl proteases, cysteine proteases, serine proteases, threonine proteases and zinc (metallo) proteases, on the basis of nature of active site residues involved

6.3.6.2 Roles

Wines, especially white wines, face protein-denaturation-led haze formation problems encountered any time during winemaking or after bottling that ultimately reduces the aesthetic quality of the finished product. The sources of such proteins may be grape, pathogens infecting grape (Pathogenesis related protein, PRP), or yeasts. The main responsible proteins

are thaumatin-like proteins (TLP), Chitinases, Invertases, and β-glucanases. In white wine production, therefore, an additional practice to prevent protein haze formation is required. The addition of bentonite, which is a chemically complex compound consisting of hydrated aluminum silicate and associated exchangeable cations, is generally used to deal with this problem. The method however has attracted criticisms like, (1) loss of about 10% of wine as removed with bentonite precipitate, (2) prolonged processing or settling period, (3) quality reduction due to thinning of color and flavor compounds, (4) compulsion to manual handling, and (5) requirement of additional expenditure on bentonite waste disposal. Therefore, alternative methods have been evaluated for the removal of proteins from wine, but with limited success to date. Recently, zirconium dioxide alone, or in combination with bentonite, has been reported to be effective in managing wine haze (Marangon et al., 2011; Salazar et al., 2006). Likewise, specific yeast mannoproteins called haze protection factors (hpfs) have been found to reduce the aggregate of haze-causing particles, and subsequently, the genes for hpfs (*YOL155*c and *YDR055*w) have been identified and over-expressed in wine yeast (Brown et al., 2007). The recombinant strains thus engineered showed reduced haze formation. While the recombinant approach needs more refinement, other techniques, such as ultrafiltration, is becoming unattractive due to problems like presence of residual protein in filtrate, reportedly high costs, and possible loss of organoleptic compounds (Høj et al., 2003). Similarly, flash pasteurization or short periods of heating at 90°C, have been found detrimental for wine quality (Tattersall et al., 2001). Hence, suitable acid protease tolerant to wining conditions and causing the least loss of wine volume and aroma stripping provides an alternative biological solution to this problem. Because many wine proteins originate in the grape in response to attacks by fungal pathogens (defense proteins or PRPs), they are inherently resistant to proteolysis. The addition of sturdy proteases like Aspergillopepsin II or Aspergilloglutamic peptidase (AGP or Proctase) to clarified grape juice prior to flash pasteurization and fermentation has been found effective in producing stable wine (Marangon et al., 2012). In another experiment, pre-heat treatment at 70°C, in combination with protease treatments at optimal temperatures, and treatment with Aspartyl protease from *B. cinerea* (BcAP8), have also been found effective in haze reduction in white wines (Van Sluyter et al., 2015). Table 6.4 depicts commercially available acid proteases for winemaking.

6.3.6.3 Desired Characteristics

(a) Tolerant to wining conditions such as low temperature (20°C), pH (3–5), and ethanol
(b) Robust enough to degrade even otherwise-protease-resistant proteins.

6.3.6.4 Cold-Active Protease

There are very limited reports available on cold-active acid proteases. A cold-active serine protease from the *Chrysosporium strain* (A6) has been found to cause significant degradation of both chitinase and thaumatin at 60°C (Nevalainen, 2011).

6.3.7 Laccase

Laccases (EC 1.10.3.2, para-benzenediol:dioxygen oxidoreductases) are multicopper proteins that use molecular oxygen to oxidize various aromatic and nonaromatic compounds, including phenolic ones by a radical-catalyzed reaction mechanism (Strong and Claus, 2011).

6.3.7.1 Roles

Wine grapes are rich in phenolic compounds that find their way to wines. Red grapes are even richer than white grapes—total polyphenol contents of red wines is (300–5000 mg/L) about ten times higher than that of white wines (60–200 mg/L) (Eder and Wendelin, 2002). The phenolic compounds affect physicochemical properties such as color, odor, and taste; exhibit antioxidant effects, and some polyphenols possess anti-carcinogenic, anti-inflammatory, anti-bacterial, and anti-hepatotoxic properties (Strong and Claus, 2011). The polyphenols, especially resveratrol in red wines, and possibly the nonflavonoid phenolics tyrosol and hydroxytyrosol in white wines (Zinnai et al., 2013), are believed to serve as effective free-radical scavengers. They retard the removal of nitric oxide from the blood and thus lower blood pressure and reduce the risk of heart problems and strokes. Despite the various health benefits phenolic compounds exert, they themselves are extremely sensitive to oxygen and oxidize easily when exposed to oxygen or the oxidizing enzymes polyphenoloxidases, including tyrosinase, and laccase to quinines, which in turn react to dark-colored polymers. These polymers are insoluble in water and precipitate out from must and wine. *Botrytis* laccase, received from *Botrytis cinerea*-infected grapes, is extremely stable and continues to alter phenolic compounds from the must stage to the bottled wine. Moreover, the oxidation of phenolic compounds may adversely affect the sensory and nutritional properties of wine (Zinnai et al., 2013). This phenomenon of oxidation that phenolic compounds undergoes in musts and wines leading to flavor alterations and color distortions is known as maderization. This is a challenge for the oenologists as to how to deal with this phenolic paradox. Solutions include the removal of phenolic compounds with polyvinylpolypyrrolidone (PVPP), blocking oxidizers by applying sulfur dioxide, avoiding contact of must and wine with air, etc. A biological solution to this challenge is the use of phenoloxidases that selectively target specific polyphenols, causing oxidative browning (Minussi et al., 2002), polymerizing them, and then removing them by clarification without eroding antioxidant properties of the wine. For example, the treatment of a red must with *Trametes versicolor* laccase reduces the must antioxidant properties, but the treatment of white musts with the same laccase does not, and so the treatment of white musts with this laccase seems feasible (Minussi et al., 2007). A previous assessment with respect to the polyphenol content, color, haze stability, and sensorial quality of Riesling wines also suggests that the wines made with laccase-treated must was better than untreated must, and that the former wine needed little or no addition of SO_2 to make it stable and a higher quality (Maier et al., 1990). The finding that the laccase of *Pyricularia oryzae* oxidized resveratrol but did not change its antiradical capacity (Espn and Wichers, 2000) makes it a potentially anti-maderization enzyme. Laccases have also shown their potential to be applied to the subsidiary activities in wine production such as (1) the treatment of cork stoppers for oxidative removal of phenols so as to avoid evolvement of the off-flavor tetra-chloroanisol in the bottled wine (Sponholz, 2000), (2) use in biosensors for a rapid and reliable amperometric estimation of the polyphenolic contents in wines (Claus and Strong, 2010), (3) oxidative removal of unhealthy aromatic amines like tyramine (Claus and Strong, 2010), (4) treatment of waste water from distilleries with acidic pH values, high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and dark color due to the presence of phenolic compounds or nitrogenous polymers (melanoidins) (Strong and Claus, 2011).

6.3.7.2 Desired Characteristics

(a) Low optimum pH (3–5) and temperature (10–20°C)
(b) Oxidizes the polyphenols without eroding antioxidant properties

6.3.7.3 Cold-Active Laccase

No reports of cold-active laccase are available in literature (Mukhopadhyay et al., 2015).

6.4 CONCLUSIONS

Commercial preparations of grape/must processing enzymes like pectinases, glucanases, and glycosidases are available in pure and various combinations. Other enzymes like acid ureases, glucose oxidases, acid proteases, and laccases are still largely at the study level. Current trends in wine industries emphasize variety which, in turn, depends largely on the judicious use of various enzymes. The quality issues like methyl esterase activity in pectinases, efficient sources of diverse types of β-1,3–1,6 and β-1,3–1,2 glucanases, and glucose inhibition in glycosidases, need a concerted effort to find effective solutions. Major constraints in large-scale commercial application of other potential enzymes include loss of mouthfeel, aromas, and microbial resistance, as in the case of glucose oxidase-treated wines, low-efficiency in the degradation of PRP as in the case of acid proteases and the oxidation of polyphenols without reducing their antioxidant properties as in the case of laccases. Genetically engineered strains encoding various enzymes, especially pectolytic enzymes, look to be attractive ways to ensure the availability of enzymes during winemaking. But their application goes hand in hand with certain issues like the stability of the strains and scheduling of the expression of enzymes so as to avoid their contact with inhibitory substances added/produced in the must/wine. Searching for suitable enzyme-producing strains from environmental biodiversity applying microbiological or metagenomic approaches seems to be more important and effective way to find solution to many of these constraints.

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CHAPTER

7

Enzymes in the Animal Feed Industry

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7.1 INTRODUCTION

All animals use endogenous enzymes for digestion of feed. Endogenous enzymes are either produced by the animal's system, or by the microorganisms that naturally inhabit the gut. Though, the animal's digestive system is not fully efficient. Swine and poultry cannot digest approximately one fourth of the diet they are fed because the feed ingredients contain indigestible deleterious factors that hinder the digestive process, and/or the animal is devoid of the necessary enzymes to degrade certain components in the feed. The inclusion of exogenous feed enzymes in animal feed is becoming a custom to conquer the undesirable effects of deleterious factors and improve digestion of feed and animal performance. Supplementation of feed with definite enzymes enhances its nutritive value, thereby increasing the effectiveness of digestion. The appropriate use of enzymes in livestock feed allows it to retain maximum nutrition not only for the animals, but also for its surroundings. Enzymes are the most important and useful additives in the animal feed industry. There are four different categories of marketable enzyme products applied by the animal feed industry: (1) enzymes targeting phytates, (2) enzymes targeting viscous cereals (e.g., triticum), (3) enzymes targeting nonviscous cereals (e.g., maize, jowar), and (4) enzymes targeting noncereals/legumes (e.g., linseed meal). Studies suggest that multienzyme preparations may provide a feasible approach to develop nutrient utilization in livestock/poultry diets (Cowieson et al., 2006; Selle and Ravindran, 2007). A multienzyme preparation with cellulolytic and proteolytic activity can degrade the structural polysaccharides and proteins. The collective appliance of enzymes may result in additive, subadditive, or synergistic effects on nutrient utilization and livestock growth/production (Juanpere et al., 2005; Ravindran et al., 1999). A multienzyme preparation, rather than a single enzyme, corresponds to the next generation of feed enzymes, because feed ingredients are structurally complex. Naturally, nutrients in feed and feed ingredients are presented as complexes, with various bonds to protein, fatty acid, crude fiber, and

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https://doi.org/10.1016/B978-0-12-813280-7.00007-4

other complex carbohydrates/polysaccharides. For instance, in wheat-based diets, simply targeting the arabinoxylans complex with xylanases may not give the complete benefits. It has been reported that the combination of a carbohydrase enzyme with predominantly xylanase activity and a microbial phytase in wheat-based broiler rations provide complete benefits in terms of nutrients (protein and energy), utilization, and growth performance (Ravindran et al., 1999; Selle et al., 2009; Zyla et al., 1999). In multienzyme preparations, the activity of one type of feed enzyme may be increased by the other, probably in a mutual approach, by providing a larger substrate contact, and by lowering the deleterious effects of the substrates (nonstarch polysaccharides and phytate) on the utilization of nutrients (protein and energy). The multienzyme-containing cocktail of phytase with α-galactosidases, protease, β-glucanase, and xylanase enzymes in maize, barley, or triticum-based diets, has been studied (Cowieson and Adeola, 2005; Juanpere et al., 2005) and it was found that phytase enzymes, when mixed with protease and carbohydrase, had additive effects in nutritionally marginal broiler diets.

The exogenous enzymes used mainly in the animal feed industry as feed additives are cellulase, β-glucanases, xylanases, and associated enzymes phytases, proteases, lipases, and galactosidases (Table 7.1). In the animal feed industry exogenous enzymes are mainly used in monogastrics, like poultry and swine, to neutralize the effects of the viscous, nonstarchy polysaccharides (NSP) in cereals such as barley, wheat, and triticale. These carbohydrates are undesired, as they decrease digestion and absorption of the nutrients present in the diet. There is an increasing trend of supplementing phytase as a feed additive, as it not only enhances the accessibility of phosphorus in plants but also reduces environmental pollution. Other enzyme products are being evaluated in the livestock feed industry, including protease to improve protein digestion, lipases to improve fat digestion, β-galactosidases to neutralize certain anti nutritional factors in noncereal feedstuffs, and amylase to aid in the digestion of starch in early-weaned animals (Adeola and Cowieson, 2011). The worldwide feed enzyme market is valued at over $550 million, and saves the global feed market an expected $3–5 billion per year. This market can be roughly separated into phytase (about 60%) and nonphytase (40%) enzyme segments. In the last decade the feed enzyme industry has developed so fast,

TABLE 7.1 Type of Exogenous Enzymes Used in Animal Feed Industry and Their Substrates

Enzyme	Substrate	Feedstuff
Xylanases	Arabinoxylans	Wheat, triticale, fibrous plant complexes
β-Glucanases	β-Glucan	Barley, oats, rye
α-Galactosidases	Oligosaccharides	Linseed meal, leguminous grain
Mannanases, cellulases, hemicellulases, pectinases	Cell wall matrix (crude fiber)	Plant-origin fibrous ingredients
Amylase	Starch	Cereal grains, leguminous grain
Proteases	Proteins	Plant derived protein ingredients
Lipases	Lipids	Fats and oils in feed sources
Phytases	Phytate	Plant based feed sources

mainly due to adoption of phytase supplementation technology and wider use of carbohydrate digesting enzyme in corn-based diets; both apparently in response to rising input costs (Adeola and Cowieson, 2011).

7.2 BENEFITS OF ENZYME SUPPLEMENTATION

The final mean of supplementing enzymes is to advance animal performance and productivity through improved digestion of nutrients (proteins, carbohydrates and fats) in feed ingredients. Increase in the range of feedstuffs that can be used, and increased flexibility in ration formulations by dropping or removing the constraint on the inclusion limit of poorly digested ingredients, are the other benefits of enzyme supplementation in animal feed. Motivations for enzyme use in animal feed thus include degradation of otherwise-indigestible carbohydrates (Bedford, 1995), and decrease in viscosity which might otherwise negatively affect energy uptake by forming unstirred layers on the surface of the epithelium (Rainbird et al., 1984). Supplementation with enzymes increases the value of nutritionally poor samples and lessens the difference between high- and low-quality samples of a specified feed ingredient. This outcome, consecutively, improves the degree of accuracy of the ration formulation (Ravindran, 2013). Another incentive for enzyme additions to feed is the release of micronutrients, such as the phosphates and other nutrients from phytic acid with phytase (Greiner and Konietzny, 2006). Research has shown that enzyme supplementation certainly may have an effect on nutrient utilization (Létourneau-Montminy et al., 2012). Because digestion has become more efficient and fewer amounts of undigested nutrients are reaching the lower gut, as well as beneficial microbes altering gut flora, gut health has improved (Bedford and Cowieson, 2012). The result is that gut flora has a protective effect on the overall health of poultry due to improved immune status (Yegani and Korver, 2008). Improved intestinal morphology (Jaroni et al., 1999) and integrity results in improved digestion and absorption of nutrients. Decreased manure yield in terms of the quantity and nutrient stack (excreta nitrogen and phosphorus levels) are consequences of improved nutrient utilization. The ecological effect of these benefits is relevant to global intensive rearing of livestock/poultry. By elevating the growth of poorly growing/performing animals, their uniformity at marketable ages improves. In summary, the profit of supplementing feed with enzymes is not only improving nutrient utilization, but it has implications in the ongoing changes in the worldwide animal production in terms of environment, gut fitness, animal wellbeing, and sustainability.

7.3 MODE(S) OF ENZYME ACTION

Enzymes used as additives in the animal feed industry have diverse modes of action. Regardless of their escalating recognition as feed additives, the accurate mode(s) of action of animal feed enzymes remains to be seen. One or more of the following mechanisms are accountable for the experiential advantages (Bedford and Partridge, 2011; Ravindran, 2013).

(1) Exogenous feed enzymes helps degrade exact bonds in feed ingredients/components that are usually not hydrolyzed by endogenous enzymes.

(2) These help in degrading the deleterios factors present in feed which are responsible for reducing digestion and increasing viscosity of gut, thus achieving better nutrient utilization.
(3) They may be interfere with the integrity of endosperm and cause the discharge of nutrients that are bound to or trapped by the plant cell wall.
(4) Feed enzymes modify the digestion process to further aid digestion sites.
(5) Feed enzymes reduce internal secretions and protein loss from the digestive tract, thus reducing animal maintenance requirements (Cowieson et al., 2009).
(6) Feed enzymes reduce intestinal load and modify intestinal morphology (Wu et al., 2004).
(7) Feed enzymes change the microbial populations in the gastrointestinal (GI) tract directly by manipulating the amounts and appearance of substrate present within the GI tract due to the increase of beneficial bacteria while harmful microbes decreases in the gut (Apajalahti et al., 2004; Bedford and Cowieson, 2012).

7.4 TYPES OF FEED ENZYMES

Enzymes are categorized in accordance with the substrates they operate upon. In livestock nutrition the types of enzymes used are those that degrade fiber, proteins, starch, and phytates.

7.4.1 Carbohydrases

Carbohydrases degrade complex carbohydrates into simpler sugars. Studies show that carbohydrase supplementation improves the digestibility of dry matter (Nortey et al., 2007), organic matter, and energy (Yin et al., 2000) in monogastric animal nutrition. They can be generally divided into those that target either fiber (NSP) or soluble sugar (starch) in nutritional science.

7.4.1.1 Fiber-Degrading Enzymes

The entire plant-origin of feedstuffs enclose fiber. Cellulose, hemicellulose, and lignin (complex carbohydrates) are the main components of fiber present in the cell wall of plants. Fibers are mainly of two types: soluble and insoluble. Fiber might be harmful to animals and acts like an antinutritional factor in so many ways. First, some nutrients, like soluble sugars and crude protein present in plant cell walls, get trapped by insoluble fiber. These trapped nutrients cannot be utilized by monogastric animals such as pigs and poultry, as they lack the enzymes needed to break down fiber. Secondly, soluble fiber forms a sticky gel after dissolving in the gut of monogastrics; this viscous gel traps nutrients, diminishes the digestion process and the course of ingesta through the alimentary canal. Thirdly, fiber can trap water-soluble nutrients by retaining water. Lastly, fiber forms bulk in the gut, which reduces the movement of ingesta, lowering feed ingestion and successive development. Xylanase and β-glucanase are the two main fiber-digesting enzymes used in the animal feed industry. They break down Arabinoxylans which are chiefly ubiquitous in cereals and their by-products degraded by xylanases. β-Glucans are widespread in barley, oats, and their by-products are degraded by β-glucanases. β-Mannanase, pectinase, and α-galactosidase are the other fiber-degrading

enzymes presently applied in animal feed science, but to a minor level. There are numerous mechanisms to explain the encouraging effects of exogenous enzymes supplementation in animal feed. It was established that soluble β-glucans and arabinoxylans ("pentosans") present in viscous cereals (wheat, barley, rye, oats, and triticale) raised enteric thickness which is linked with antinutritional properties of those cereals (Bedford and Classen, 1992; Bedford and Morgan, 1996; Choct and Annison, 1992). These enzymes grasp considerable amounts of moisture and owing to the consequential elevated viscosity, their nutrient absorption becomes restricted. In an expedient situation this can be seen as a decreased feed conversion ratio (FCR), increased body weight, and wet droppings in poultry. By adding enzymes β-glucanases and xylanases to feed, these problems can be resolved, resulting in enhanced livestock performance. Other benefits of the addition of enzymes in poultry feed linked to decreased digesta viscosity include decrease in the quantity of dirty eggs and improved yolk color. Outcomes from a number of experiments specify that enzymes are capable of developing livestock performance as well with "nonviscous cereals," for instance, corn and jowar (Choct, 2006), and in swine, where differences in the digestive systems' mode of action differs from that of poultry (Dierick and Decuypere, 1994). Consequently, it is extensively implicit that the capability of xylanases and β-glucanases used to digest vegetative cell walls make nutrients free from grain endosperm and aleurone layer cells. Thus this system can be well regarded as significant for developing the assessment of feed energy.

Xylanases (endo-1,4-β-xylanase, EC 3.2.1.8) slice the xylan backbone erratically, producing branched or nonsubstituted xylooligosaccharides (Collins et al., 2005; Polizeli et al., 2005). As far as feed application is concerned, merely a fractional hydrolysis of xylan is required for decreasing viscosity and therefore adding xylanase to feed is very useful. However, a synergistic act of numerous hemicellulases is required for complete hydrolysis of the composite xylan structure (Coughlan et al., 1993). The "accessory" enzymes, which can cleave the side chain used for removing the substituent groups, and the 1,4-β-D-xylosidase enzyme (EC 3.2.1.37) breaks xylobiose and xylooligosaccharides into monomers of xylose (Coughlan et al., 1993; Shallom and Shoham, 2003; Sunna and Antranikian, 1997). Hydrolysis of cereal xylans by xylanases releases oligosaccharides consisting of xylose or xylose and arabinose residues. Mathlouthi et al. (2002) studied the effect of exogenous enzymes (containing xylanase and β-glucanase) activities in rye- or maize-based rations. They observed that feeding the rye-based diet decreased performance by 43% compared with the maize-based diet, and enzyme addition, at 560 and 2800 IU of xylanase and β-glucanase, respectively, restored performance to levels similar with the maize-based diet. Cowieson and Ravindran (2008) found both increased body weight (BW) and feed intake in response to supplementation of feed with a mixture of enzymes xylanase, amylase, and protease. Similarly, Olukosi et al. (2007) fed a xylanase-supplemented wheat and rye-based diet to broiler birds and reported a dose-related increase in BW gain, feed intake, and feed conversion efficiency. Narasimpha et al. (2016) reported that supplementing low-energy diets with NSP enzymes (xylanase, cellulase, and β-D-glucanase) can sustain egg production, concomitantly reducing the cost of production.

7.4.1.2 Starch-Digesting Enzymes

The extent of starch degradability in plant-origin feed ingredients will differ in accordance with the levels of resistant starch, starch particle size, chemical composition, and starch encapsulation. Differences in crop genetics, cultivating conditions, cutting conditions,

conservation, and processing are all important factors responsible for inconsistency in starch digestibility. Amylases degrade starch present in grains and grain by-products during the digestive process. Amylases potentially permit swine and poultry to derive more energy from the feed by improving starch digestibility, which is capable of improving pork/chicken and egg production. In young piglet diets, amylases afford benefits by supplementing an undeveloped digestive system where, in postweaning, a lower intake of feed is linked with a sluggish maturation of amylase secretion. Additionally, amylase allows the utilization of half-cooked grain in the rations, with consequential benefits in feed cost reduction without affecting the performance of postweaned piglets. The major amylase enzyme commonly supplemented exogenously in livestock rations is α-amylase, which is extracted from the bacteria *Bacillus amyloliquefaciens* (BAA). It degrades starch quickly into short oligosaccharides. The end products obtained by hydrolysis are maltotriose (DP 3) and maltohexose (DP 6) (Robyt, 2009). This amylase can also survive in feed pellets due to having a comparatively high thermostability. Conversely, when a starch molecule is hydrolyzed by porcine pancreatic α-amylase (PPA), glucose to maltotetraose (DP 1–4) products are chiefly formed (Robyt, 2009). The degradation of amylopectin by BAA and PPA is dissimilar, as BAA has a higher tendency than PPA to split the bonds of the inner chain (Goesaert et al., 2010). So BAA quickly degrades the amylopectin to lower molecules than PPA, while PPA breaks down the chains of amylopectin in a more consistent manner. It has been reported that 10% of the hydrolysis enzyme BAA was established to collect primarily DP 6–10 molecules, while enzyme PPA collected DP 2–4 primarily (Bijttebier et al., 2010). On the basis of these differences in mechanisms, it might be concluded that if the enzyme BAA is added exogenously to PPA, it enhances the rate of amylopectin and amylose degradation to short maltooligosaccharides that can readily be digested by the enzymes maltase and isomaltase to glucose for assimilation by the epithelial cells and thus increase the soluble sugar utilization. The effectiveness of exogenous amylases in monogasric animal nutrition has not been explicitly established. Though, numerous theories exist to explain that exogenous amylase might have a role in augmenting undeveloped pancreatic secretions in neonates (Noy and Sklan, 1999a,b) or in supporting animals in instances when starches are refractory to digestion. Gracia et al. (2003) verified that exogenous amylase is capable of improving the performance of broiler birds fed corn/soy-based rations. Moreover, exogenous amylase supplementation enhanced the digestion of starch and organic matter, and was linked with better apparent metabolizable energy (AME). These advantageous effects were independent of bird age, which suggests that it is not exclusively the neonate that saw advantages from the supplementation of exogenous amylases. Even though improved AME and starch degradability was reported by Gracia and coworkers, the great improvements in performance (about 9% for body weight gain and 5% for feed conversion efficiency) cannot be explained only through an improvement in the digestibility of dietary nutrients. Definitely, the effect of amylase on AME was a comparatively modest 50–80 kcal/kg in this meticulous study (Gracia et al., 2003). This positive effect on metabolizable energy contributed to the increase of the birds' body weight and feed efficiency. The lack of relationship between bird age and enzyme supplementation, and the noticeable inconsistency between birds performance and digestibility improvements, indicate that the exogenous feed amylase may have physiological effects not readily detected via conventional nutrient recovery assays.

7.4.1.3 *Proteases*

Proteases are protein-degrading enzymes that are supplemented in swine and poultry diets to degrade proteins stored in diverse plant feeds and proteinaceous antinutrients in plant proteins. Seeds of legumes, such as peas, contain large amounts of storage proteins. During the seed development stage, plants reserve the seed protein where it is used by the budding embryo as a nitrogen source during germination. These are storage proteins, which bind to starches and carbohydrates present in the feed. Exogenous protease supplementation is helpful in degrading storage proteins, thus making the bound energy-rich starch available to the animal for digestion. Protease inhibitors and lectins (hemagglutinins) are the two most important proteinaceous antinutritional factors. Protease inhibitors found in raw plant proteins, such as soybeans, and are concentrated in the outer part of the cotyledon. They can inhibit digestion as they block the enzyme trypsin and/or chymotrypsin, both of which are secreted by the pancreas to help digest protein in the small intestine. Lectins are sugar-binding proteins that have also been shown to lessen digestibility. When combined with the glycoprotein components of red blood cells they cause agglutination of the cells. Heat treatment is commonly used to destroy these antinutrient substances present in the feed; excessive heating may diminish the accessibility of free amino acids, mainly lysine. Proteases can be supplemented in the feed to reduce the levels of protease inhibitors and lectins, therefore improving protein digestibility. Mahagna et al. (1995) reported encouraging effects of adding protease (and amylase) to sorghum-based diets for broiler chicks, and this was linked with a decrease in chymotrypsin secretion by the pancreas. The effect of a protease-containing cocktail of enzymes has been comprehensively reported, but only some research has been available where the consequence of protease supplementation has been recognized separately from an enzyme admixture. Odetallah et al. (2003) reported enhanced performance of broiler starters when a maize/soy-based diet was supplemented with a keratinase from *Bacillus licheniformis*. Yu et al. (2007) did a trial on broilers and studied the effect of exogenous protease supplementation, where they fed a conventional as well as a low-crude, protein-containing corn/soy diet. The enzyme protease improved degradation of soy protein in an in vitro model that mimicked the GI tract, while fish meal and maize was not influenced by protease supplementation. These findings were established in digestion trials, where broilers fed exogenous protease supplemented diets showed arithmetical improvement in weight gain through the entire growth phase (0–38 days) and a significant decrease in FCR. Despite this, no improvements were seen in apparent digestibility of protein and DM in the GI tract. Thacker (2005) conducted an experiment by supplementing exogenous protease in a wheat-based diet and reported that FCR was significantly improved but there was neither any significant effect observed on digestibility of DM and energy nor retention of nitrogen. These two trials could explain that other than merely improving protein digestion in the GI tract, there may be a similar "sparing" effect, as recommended for amylase supplementation. But this argument is not supported directly, partially due to the small number of animal trials where protease has been supplemented separately. Peek et al. (2009) conducted an experiment on broiler birds exposed to *Eimeria* spp. The birds were fed a protease-supplemented corn-wheat-soy diet during trial and it was observed that adding exogenous protease decreased the harmful effect of a coccidial infection on BW gain. The results of this study remain imprecise, though instructively coccidial lesions and oocyst emission remained unaltered, and the mucin coating was considerably thicker

in the protease-supplemented broiler birds. Ghazi et al. (2002) fed protease-supplemented soybean meal to broilers and cockerels and studied the effect supplementation had on the nutritive value of feed. In this case, different types of proteases were used. The most consistent effects were observed when acid fungal protease was used compared to alkaline subtilisin. O'Doherty and Forde (1999) established that adding a neutral protease to barley/wheat/soy-based pig diets showed an enhanced efficiency in feed conversion. To explain the advantages of supplementing poultry rations with exogenous proteases, a number of probable outcomes have been recommended. Proteases may enhance endogenous peptidase production, reducing the need for amino acids and energy, or increase the digestibility of dietary protein. Moreover, exogenous proteases may degrade proteinaceous antinutritional factors present in feed such as hemagglutinins or protease inhibitors (Ghazi et al., 2002; Huo et al., 1993; Marsman et al., 1997), which enhance the effectiveness of the birds' amino acid utilization and decreasing protein turnover. Still, more research is needed to understand the effects of exogenous proteases, variations among dissimilar protease classes (e.g., optimum pH, kinetics, and favored substrate) and as well their efficacy in livestock feeding, either fed in isolation, or as part of a cocktail of enzymes (e.g., cellulase, amylase, and phytase).

7.4.2 Phytases

Phosphorus is an essential mineral for bone maturity and metabolic functions in monogastric animals. In plant-based ingredients phosphorus is mainly present as phytate phosphorus. Phytate phosphorus bonds are formed for storage of phosphorus in plants. It reduces absorption of minerals, proteins, and carbohydrates by forming complexes with them. In grains and oilseeds, phytase dephosphorylates insoluble phytic acid into orthophosphate and inositol phosphates. On the site of initial dephosphorylation on the phytic acid molecule, phytases are broadly classified as 3- and 6-phytase (Adeola and Cowieson, 2011). Monogastric animals do not generate the phytase required for degrading phytate bonds. Adding exogenous phytase enzymes to feed degrades the phytate bond and releases phytate-bound nutrients. These nutrients then can undergo digestion and absorption by the animal's digestive system. Thus phytase helps improve the efficiency of livestock. Phytase increases the absorption of phosphorus and thus decreases the chance of soil and water pollution by surplus phosphorus excreted by monogastrics. Monogastric animals have a very limited capacity to hydrolyze phytate in their small intestine due to the short but significant endogenous phytase activity and few microbial inhabitants in the upper digestive tract (Iqbal et al., 1994). This helps to understand why phytate phosphorus is inadequately accessible to swine and poultry (Walz and Pallauf, 2002). Phosphorus is absorbed by animals as orthophosphate, and thus utilization of phytate phosphorus by monogastric animals will basically depend on their ability to degrade the phytate complex. Animal research has revealed the efficiency of supplemental microbial origin phytase in enhancing the exploitation of phosphate phosphorus from phytate phosphorus (Adeola et al., 2006; Augspurger et al., 2003; Esteve-Garcia et al., 2005; Simons et al., 1990). Thus, simple-stomach animals reduce the need for phosphorus as orthophosphate supplementation of the feed by including adequate amounts of dietary phytase. As an effect, phosphate excretion can be reduced by as much as 50%, which is obviously valuable from an environmental point of view. Thus, adding dietary phytase has proved to be the most efficient implement for the livestock industry to diminish phosphate emission from animal excreta,

enabling observance with ecological convention. Additionally, the addition of phytase might increase amino acid availability. A harmful effect of phytate on the biological value of protein, however, was not evidently established in experiments with monogastric animals (Sebastian et al., 1998). Some researchers reported that phytate does not influence protein degradability (Peter and Baker, 2001), while others have established enhanced availability of amino acids with diminishing levels of phytate (Cowieson et al., 2006). This disparity might be due, in part, by the use of dissimilar protein sources (feed) in the different experiments. Phytase supplementation proved to advance the use of minerals by monogastric animals (Adeola et al., 1995; Debnath et al., 2005; Lei et al., 1993; Lei and Stahl, 2001). Additionally, it was assumed that adding phytase resulted in improved energy consumption in pigs and poultry (Selle and Ravindran, 2007). The capability of a phytase enzyme to break down phytate complex in the gastrointestinal tract is resolute by its enzymatic properties. In the GI tract of animals phytate dephosphorylation occurs which is influenced by pH of the compartment. It is essential to consider the low pH in the crop of a bird (pH 4.1–5.0), the proventriculus and gizzard of poultry, and the stomach of pigs and fish (pH 2.0–4.0) (Simon and Igbasan, 2002). Conversely, the small intestine of animals presents a nearly neutral pH atmosphere (pH 6.5–7.5). So, pH optima and pH activity profiles of added phytase usually decides their capacity to extend catalytic action in the aforementioned GI compartments. So far, two types of phytase enzymes have been identified: acid phytases which show a maximum phytate dephosphorylation of around pH 5.0, and alkaline phytases with an optimum pH of around 8.0 (Konietzny and Greiner, 2002). All exogenous phytase used as additives in the animal feed industry belong to the group of histidine acid phytases. Thus they are estimated to perform most efficiently in the fore stomach or the stomach of the monogastric animal. Feeding trials of monogastric animals have established that the major functional site of exogenous phytase in swine and fish is the stomach (Jongbloed et al., 1992; Yan et al., 2002; Yi and Kornegay, 1996). In the GI tract of poultry, the site of action of enzyme phytase is not very clear. Though, the crop was suggested to be almost certainly the main site of phytate dephosphorylation by exogenous phytase (Selle and Ravindran, 2007). For elevated phytase action in the small intestine, a sufficiently high pH in the stomach and intestine is required, in addition to a high resistance to protein splitting actions, mainly of pepsin in the stomach and trypsin and/or chymotrypsin in the small intestine. For better utilization of exogenous phytase supplementation to feed the prerequisites are steadiness in an acid environment and resistance to proteolytic enzymes. These conditions may assure an efficient phytate dephosphorylation in the crop and stomach of poultry/animals.

7.5 FACTORS AFFECTING ENZYMES RESPONSES

The important objective of this chapter is to evaluate the extent that feed enzymes, being protein molecules, can "survive" the primary digestion themselves and retain some activity in the gastrointestinal tract. Because enzymes are protein catalysts their activity is susceptible to variations in pH, and the enzyme proteins may be attacked by proteases acting inside the GI tract. Hence, for successful enzyme use it is important to assess the factors affecting enzyme activity and stability during passage. The biological actuality is that there are physiological restrictions, imposed by the circumstances in the GI tract, to enzyme responses.

Based on previous studies and existing literature, it is practical to anticipate the digestion of probably 25%–35% of the undigested portion by supplementation of exogenous enzymes.

7.5.1 Digestive Physiology

In monogastric animals, including pigs, digestion can be divided into four different stages: firstly, in the mouth, mastication provides mechanical degradation, the addition of water, as well as amylase (EC 3.2.1.1) (Arkhipovets, 1962). This step is not considered detrimental to feed enzymes. Next, the feed enters the stomach, where it encounters hydrochloric acid (HCl) and pepsin (EC 3.4.23.1), both of which may compromise exogenous enzyme stability and, in turn, activity, via acid denaturation and proteolytic digestion, respectively. Afterwards the food bolus is slowly released into the small intestine, where the feed is neutralized by a pancreatic secretion containing bicarbonate and digestive enzymes including amylase, lipases, and proteases (Corring, 1982). The main digestion and uptake of nutrients such as sugars, fats, minerals and amino acids, take place in the small intestine, and feed enzymes, being proteins, are then digested by the natural digestion processes. Eventually, the food bolus continues into the large intestine, where bacteria inactivate endogenous enzymes (Gibson et al., 1989) and digest the remnants of the feed, mainly NSP and residual protein.

7.5.2 Retention Time

As the various parts of the GI tract present different challenges for preserving exogenous enzyme activity, the retention time in each segment is of utmost importance. The relevance of retention times is twofold: on the one hand, relatively long reaction times are required for the enzymes to accomplish a sufficient extent of substrate degradation; in particular, several hours may be required for degradation of nonstarch polysaccharide fibers (Rasmussen and Meyer, 2010; Rasmussen et al., 2012) and for the full de-phosphorylation of phytic acid (Bohn et al., 2007). On the other hand, the longer the enzyme is exposed to a hostile environment, the lower the chances for it to retain its catalytic activity, due to acid denaturation and proteolytic digestion. In addition, most commonly used exogenous enzymes have limited activity outside of a neutral or slightly acidic pH, and only then can low substrate conversion occur in the acidic stomach. The retention time in the stomach is thus a decisive characteristic for retaining enzyme action. In the digestive tract of poultry, the passage time of feed is comparatively short. In the digestive tract, excluding the caeca, the average retention time is approximately 3–4 h (Svihus, 2011b). Enzymes get very limited time to act on digesta as feed probably spends only 60–90 min in the anterior digestive tract. In poultry farming birds are generally fed finely ground pelleted feeds ad libitum but this practice does not support general functions of crop and gizzard. The role of the crop is to act as a storage organ under discontinuous feeding systems, whereas in continuous feeding systems this role appears to be missing. Likewise, feeding finely ground feed does not improve the gizzard because it is meant for grinding. An immature gizzard serves as a passage rather than a grinding organ, which means reduced retention time. Management of retention time in the anterior digestive tract is an important tactic to prevail over the physiological restrictions of residence time, and to further improve the efficiency of exogenous supplemental feed enzymes by increasing their time to act with digesta. There is confirmation that feeding, as an alternative to ad libitum access to feed, may

noticeably enhance the retention time in the crop, together with a quick moisturization and a decline in pH ranges between 4 and 5. Svihus et al. (2010) conducted studies on broilers in which they were fed a wheat-based diet containing exogenous phytase. It was reported that phytate was steadily dephosphorylated, with a 50% decrease in inositol-6-phosphate subsequent to a retention time of 100 min. The feeding of coarse particles or whole grains has been revealed to arouse development of the gizzard, ensuing improved grinding capacity, improved reverse peristalsis of digesta, and superior gizzard volume, thus increasing the retention time (Amerah et al., 2007; Svihus, 2011a). For the adult pig, reported retention times in the stomach range from 1 h, independent of the fiber content of the diet (Wilfart et al., 2007), to up to 13 h on a high-fiber, high-water-retaining diet as reported by van Leeuwen and Jansman (2007). A main reason for the differences is likely the feeding frequency, namely every fourth hour in the Wilfart et al. (2007) compared to the twice-daily regimen in the Van Leeuwen and Jansman (2007). The data thus suggests that a lower feeding frequency will cause retention in the stomach as the animal will eat larger portions on a limited-feeding frequency. In another study, approximately 50% of the feed was emptied from the stomach after 3 or 5 h for a small and large meal, respectively (Gregory et al., 2007). Johansen et al. (1996) used four dissimilar diets with different levels of fiber, and reported gastric retention times of 3–5 h with 3× daily feeding, but no effect on diet type. The retention time in the small intestine is reported to be as short as 4 h in the high frequency feeding study of Wilfart et al. (2007), and up to 21 h with the high fiber, high-water-retaining diet of van Leeuwen and Jansman (2007).

7.5.3 pH

Animal feed generally has a pH near to neutral while the digestive tract of animals/poultry have an acidic pH in the stomach/crop and a slightly acidic pH at the proximal end of the intestine. In the distal part of intestine, this changes to neutral to slightly alkaline in nature. Svihus, 2011b reported that a pH range of 4–6 is optimum for exogenous feed enzyme activity but substantial distinctions exists between diverse enzymes sources, which may result in degrading action of some sources at both lower and higher pH. It is probable that exogenous feed enzymes are active and catalyze their substrates in the anterior tract (crop, proventriculus, gizzard) before they are digested by endogenous enzymes (Selle and Ravindran, 2007). However, if an exogenous enzyme is resistant to the protein-splitting act of pepsin in stomach, it can stay active in the small intestine and be further effective. For example, *E. coli* phytases have been revealed to be refractory to pepsin and pancreatic enzymes and have a high stability from protein-degrading enzymes (Igbasan et al., 2000), which makes them ideal supplements to augment the discharge of phytate-linked phosphorus. A variety of pH is found along the GI tract and pH becomes the first physiological restriction that determines the action and steadiness of enzymes. The porcine stomach is acidic due to an influx of hydrochloric acid. Although the acidity of the stomach is believed to be highly acidic, the actual pH is fairly variable, and may range from 1 to 5, due to a rapid buffering effect of immediate feeding behavior (Iraki et al., 1997; Karamanolis et al., 2008). Gastric pH has been found to be generally above 4.0 during feeding on an ad libitum corn-based diet, but dropped to 2.5 at night and to 2.1 when feed was removed (Ange et al., 2000). According to Potkins et al. (2007) pH increased to 5.0 upon feeding with a large barley-based meal then dropped below 3.0 after 7.5 h, independent of bran or oat addition. If the barley meal was finely ground, however,

it resulted in a significant drop to 2.5 after 7.5 h (Potkins et al., 2007). On a barley-based diets, pigs in a study by Baas and Thacker (1996) produced gastric digesta with a pH of 4.8, which dropped to 4.0 after 4 h, although they sampled the digesta in the duodenum. In the small intestine, pH-levels increased due to an influx of bicarbonate from the pancreas. It has recently been documented that the pH in the terminal ileum of barrows was 5.7–6.0, regardless of whether the diet was high in fiber and/or supplemented with organic acids or not (Partanen et al., 2007).

7.5.4 Endogenous Digestive Proteases

The porcine GI-tract produces several proteases; the most important are pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1). Because these enzymes actively digest proteins, they are a major obstacle for the use of exogenous enzymes, and proper estimation of proteolytic resistance of putative feed enzymes is warranted to predict in vivo performance (Strube et al., 2013). In vitro testing of proteolytic resistance is usually done by incubation of exogenous feed enzymes for up to 2 h with different ratios of endogenous proteases usually at a 0.001–0.1 ratio of endogenous protease (pepsin or trypsin) to exogenous feed enzymes (Pandee et al., 2011; Rodriguez et al., 1999, 2000). Enzymes are also strongly affected by proteolytic activities in the stomach as well as in the small intestine, apparently mostly trypsin and other small-intestinal enzymes.

7.5.5 Water Content and Ionic Strength

Enzymes are often dependent on ionic strength; in as much as they often exhibit limited activity at low-salt concentrations, a definite peak at certain concentrations and then a decrease at higher concentrations. This, as an example, occurs at around 0.1 M NaCl for a fungal pectinase (Dahodwala et al., 1974) and at 0.2 mM NaCl for a glucanase (Sena et al., 2011). Naturally, halophilic enzymes do not function optimally until NaCl concentrations reach 5%–15% (Wainø and Ingvorsen, 2003). To initiate enzyme activity an aqueous environment is needed. When the exogenous-enzyme-supplemented feed is fed to the animal, the condition for an aqueous environment is completed rapidly and the feed becomes more and more moistened as it goes along the GI tract (Svihus, 2011b). The concentrations of ions in the GI tract of pigs do not appear to be an issue for enzyme stability, but rather, the ionic conditions may be more favorable in the small intestine as compared to stomach. Additionally, because the lower water content in the stomach results in higher viscosity, which, in turn, results in lower enzyme activity (Uribe and Sampedro, 2003), the small intestine would be a more active site of action. Measurement of divalent cations depends on the method as they are usually bound by phytate and are usually not soluble and hence less detectable in the liquid phase on a high-phytate diet (Woyengo et al., 2010).

7.6 CONCLUSIONS

The possible benefits of supplementation of exogenous enzymes to feed are improving nutrient utilization and animal performance is well accepted. But feed enzymes can enhance digestive performance up to a certain limit beyond those physiological barriers like pH and

retention time within the GI tract where they cannot perform. Suitable use of exogenous enzymes in feeds requires strategic reductions in dietary energy and nutrient content, as well as cautious selection of feed ingredients to confine cost-effective profits of the different feed enzymes. Animal feed enzyme expertise is a dynamic field of research and development, and one can be convinced that enzymes better modified to the environment of the GI tract of animals may be developed in the future. These developments will promote improvement and effectiveness of enzyme supplementation under practical situations. Since retention time, endogenous proteases and pH are inherent properties of animal physiology, novel ways to protect enzymes warrant attention.

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CHAPTER

8

Enzymes in the Meat Industry

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8.1 INTRODUCTION

Enzymes have been used for a long time, even before documented history. For many years, traditional food products like bread, cheese, and wine have been processed with enzymes. Hence enzymes have been applied in many different food processes and the practice continues in modern food technology, but with more precision. The controlled use of enzymes in the food industry has been harnessed to develop quality food products (Choi et al., 2015). The meat industry is going through a revolution where quality is the primary concern, as consumers are aware and quality conscious. The quality and economics of meat products have been the major attraction. The major issue with meat products is the post mortem changes in muscle, which affects tenderness and textural properties, and the role of enzymes plays an important role.

The postmortem changes influenced by enzymes involve the glycolytic activity where glycogen is metabolized to produce lactic acid which lowers muscle pH. The rate of fall in muscle pH can affect meat quality, and the preslaughter handling of animals may play a role. The depleted energy subsequently leads to changes in membranes, sarcoplasm, sarcolemma, and muscle fiber. Concomitantly, the contractile protein (actin and myosin), complexes get irreversibly bound together resulting in rigor mortis, the stiffening of muscles, which leads to toughness of meat. Quality parameters like meat tenderness is influenced by a number of factors, which may be preslaughter or post slaughter in nature (Destefanis et al., 2008). This toughness may be resolved by the enzymes present in the muscle cell or from exogenous sources to obtain a tender meat. The meat industry's use of enzymes improves the manufacturing process and upgrades poor quality meat. The major approaches to achieve quality meat products are tenderization of tough cuts and restructuring of low-value meat pieces to higher ones. Meat tenderness has been marked as the most important characteristic quality meat according to consumers (Destefanis et al., 2008; Zork et al., 2009). The tenderness of

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https://doi.org/10.1016/B978-0-12-813280-7.00008-6

a meat product involves three aspects: ease of penetration by the teeth, ease of fragment breakdown, and the residue that remains after chewing (Wier, 1960). In meat acquired from young animals, myofibrillar elements contribute to toughness due to actomyosin formation during post mortem changes. In old animals, the toughness of meat is due to connective tissue, considered background toughness. The toughening and tenderization take place in the postmortem storage phase especially during the storage period. Background toughness does not change during the storage period and it exists even at the time of slaughter (Koohmaraie and Geesink, 2006). It is the myofibrillar toughness caused by actomyosin in meat that improves the tenderness in meat and meat products. The endogenous calpain system has been found to play an important role in post mortem proteolysis of proteins, leading to a more tender product (Bilak et al., 1998). The calpain attacks proteins and degrades them into large fragments, which enhances the susceptibility of the proteins to other enzymes (Kolodziejska and Sikorski, 1996; Ladrat et al., 2000). The activity of calpain and calpastatin in postmortem muscle has been linked to significant differences in meat tenderness (Goll et al., 2003). The lysosomal enzymes, or cathepsins, and other enzymatic elements have been indicated in meat tenderness, but their roles are not well defined and still need further study. It has been found that meat with extensive toughness, enzymes from external sources are also required. There are numerous fruits and vegetables that contain naturally occurring proteolytic enzymes which have potential to improve the tenderness of tough meat. Among these plant proteolytic enzymes most commonly discussed are papain, zingibain, and ficin. Similarly, some microbial enzymes have been found to have a tenderizing effect.

Meat has been found to be a source of several bioactive peptides, and researchers have reported different health-promoting applications of these peptides. They are obtained after strategic use of enzymes on the meat as substrate. Bioactive peptides exhibiting antihypertensive, antioxidant, and antimicrobial effects have been observed in the meat protein hydrolysates (Kim et al., 2009). In addition, the enzymes can be used for binding small meat pieces to improve their market value. This also enhances the efficiency of carcass utilization. The protein cross-linking enzymes, like transglutaminases (TGase), have been used extensively in several food products to improve the texture. In raw meat products, the enzymes can create uniform shapes and sizes as it catalyzes the cross-linking between glutamine and lysine residues (Kumazawa et al., 1996). In another prominent role enzymes act as a flavor enhancer in meat products, especially in the ripening or aging process.

8.2 POSTMORTEM-CHANGES

A plethora of enzymes acts in concord in living muscle tissue for the contractile activity, maintenance, and growth of muscle. For this, homeostasis is achieved by the body where all organs and systems work in harmony to maintain an efficient internal environment. Maintenance of the physiologically balanced internal environment with respect to pH, temperature, oxygen, and energy is known as homeostasis which is performed at the expense of energy. The energy for physiological activity is obtained from ATP (adenosine triphoshate), and in fact the majority of the enzymes in muscle are involved in the energy metabolism of the fiber. Immediately after slaughter there is loss of homeostasis and a number of physicochemical reactions and other changes follow in the body and affect the quality of meat

produced. The conversion of muscle to meat is a complex process mediated by several enzymatic reactions which are affected by a number of preslaughter and post slaughter practices. Stress or handling prior to slaughter are indicated as one of the major factors affecting the post mortem glycolysis; hence the ultimate pH and consequently the meat quality. Though researchers have worked to understand the physiology and biochemistry of muscle, we still find novel features that affect the meat quality (Lonergan et al., 2010). The fibers continue their metabolism, even after sticking of the animal, as they would in an anaerobic situation in a living condition. Degradation of muscle glycogen by anaerobic glycolysis is followed to meet the basic need of ATP in the absence of oxygen. The glycolysis in meat, which is an outcome of a chain of enzymatic reactions, leads to the production of lactic acid with a concomitant decrease in pH from about 7.2 in a living muscle to approximately 5.5 in meat. The reduction in pH and temperature results in a decrease of the glycolytic enzymes' activity, which in turn, leads to a gradual decrease in the ATP level. Hence, the main contractile proteins like actin and myosin, get irreversibly bound together, in a phenomenon known as rigor mortis. Rigor mortis contributes to meat toughness as the sarcomere, being a contractile unit of muscle, undergoes a shortening phase during the post mortem changes. Meat with very short sarcomeres tend to be tougher (Koohmaraie et al., 1996; Locker and Hagyard, 1963), and endogenous proteases lead to resolution of shortening-induced toughness during post mortem storage of meat (aging). These changes can have a prominent effect on several proteins of the muscle cell, primarily the proteolytic enzymes that play an important role in meat tenderization (Lonergan et al., 2010).

8.3 TENDERNESS

Meat tenderness is determined by factors like background toughness, the toughening phase, and tenderization. Meat toughness depends on the nature, structure, and amount of different connective tissues like collagen and elastin (Lepetit, 2008). In general, the organization of the perimysium in muscle tissue seems to have an effect on the background toughness, as there exist a correlation between the perimysium and the tenderness of muscles (Strandine et al., 1949). It has been found that the toughening pattern is similar in all carcasses under similar processing conditions, however, there is a variation in the tenderization phase. Consumers are well aware of tenderness variability in different cuts of meat and relate it with economic value. The meat industry is aware of the problem of toughness in cuts and its financial implications (Koohmaraie and Geesink, 2006). Polkinghorne et al. (2008) is of the view that prime grilling cuts are less than 10% of a carcass and the remaining 90% can be improved by the use of tenderizing techniques. Meat tenderness has been one of the most important criteria in deciding quality attributes, and there are several techniques to evaluate it. Many sensitive tools have been used to analyze the tenderness of meat, such as enzyme activity estimation (Koohmaraie et al., 1988), the myofibrillar fragmentation index, (Olson and Parrish, 1977), hydroxyproline measurement (Ashie et al., 2002), scanning electron microscopic studies, etc.

The postmortem results in subsequent changes in membranes and sarcolemma of muscle tissue leads to another event of importance—tenderization during aging, as a lower pH favors the activity of proteolytic enzymes in meat. The cooling process during aging causes a substantial variation in meat tenderness which depends on the animals' stress prior to slaughter,

and time/temperature/pH combinations postmortem. During tenderization, proteolysis attacks all muscle proteins including connective tissue (Hwang et al., 2003). The postmortem proteolysis of myofibrillar and myofibrillar-associated proteins is a key factor in tenderization (Koohmaraie and Geesink, 2006). The biochemical changes during the postmortem phase influence meat tenderization, which is a well-established concept (Herrera-Mendez et al., 2006). A number of endogenous enzyme system have been identified in the postmortem tenderization process during the aging of meat. In addition to endogenous enzymes, several exogenous enzymes from plants and other sources have been found to have an appreciable tenderizing effect on meat.

8.4 ENDOGENOUS ENZYMES

Findings revealed proteolytic mechanisms present in a muscle: the system of calpain, lysosomal cathepsins, and proteasomes (MPC—multicatalytic proteinase complex) (Dransfield et al., 1992a,b; Kemp et al., 2010; Koohmaraie and Geesink, 2006; Korzeniowski et al., 1998). However, the system of calpains is thought to be the prime factor for the postmortem-proteolysis of proteins resulting in meat tenderization (Bernard et al., 2007; Neath et al., 2007). Cathepsin-treated myofibrillar proteins have different degradation patterns in comparison to those occurring during postmortem storage of muscle. Additionally, it has been suggested that MPC does not play a significant role in tenderization, as myofibrils are very poor substrates for this type of protease (Koohmaraie, 1992). Wheeler et al. (1992) reported that injection of calcium (calpain activator), in muscles accelerates postmortem proteolysis. and injection with calpain inhibitors prevents postmortem proteolysis and hence tenderization. Many studies confirm the importance of calpains in tenderization, and the same proteolytic patterns have been observed in postmortem muscle and the incubation of calpains with myofibrils (Geesink and Koohmaraie, 1999).

8.4.1 Calpains

Calpains (EC 3.4.22.17) present in muscles have been characterized as neutral cysteine endopeptidases, activated by calcium ions and thiol compounds. The calpain system in a skeletal muscle is said to consists of at least three proteases: calpain I (μ), calpain II (m) and calpain 3, as well as calpastatin, which is found to be a calpain inhibitor (Moudilou et al., 2010). Calpain II is mainly located in the cytosol, whereas calpain I bonds to myofibrils in the muscle cell (Xian-Xing et al., 2009). The majority of calpain 3 is situated in sarcomere near the Z- and M-line of myofibrillar structure (Ilian et al., 2004). It has been observed that the calpains 1 and 2 are negatively regulated by the calpastatin, a specific endogenous inhibitor (Koohmaraie and Geesink, 2006; Moudilou et al., 2010). The activity of the calpain system depends on several factors like pH, temperature, and more importantly, the concentration of calcium ions (Goll et al., 2003). Calpains are calcium-activated proteases, and according to Koohmaraie and Geesink (2006), among calpains, μ-calpain appears to be the enzyme that has an effective role on postmortem proteolysis. In some of the findings it has been suggested that the calpain I and calpain II need 3–50 μmol/L and 0.4–0.8 mmol/L calcium ions, respectively, for their activation. However, the concentration of calcium ions

in a living muscle reaches up to 0.2 μmol/L, which is lower than the required (Goll et al., 2003; Kurebayashi et al., 1993). In another study, Hopkins and Thompson (2001) reported the post slaughter concentration of calcium ions in *longissimus lumborum* (LL) and *longissimus thoracis* (LT) muscles of sheep reached 110 μmol/L and was sufficient to activate the calpain I. Application of calcium ions in a muscle can improve the tenderness of meat as the calpain system is activated (Rees et al., 2002). Biotechnological tools have been used to identify markers of the calpain I and calpastatin to segregate beef cattle, having potential for tender meat (Casas et al., 2006; Kemp et al., 2010).

It has been found that electric stimulation (ES) can result in enhanced inflow of calcium ions to cytoplasm and may lead to activation of μ-calpain (Veeramuthu and Sams, 1999). In another finding, ES was shown to improve the tenderness by lowering the activity of calpastatin (Hope-Jones et al., 2010). In vitro findings revealed the optimum condition of calpains, where the pH was 7.2–7.8 and temperature 25°C (Dransfield, 1999; Kanawa et al., 2002). Another study showed that μ-calpain can effectively degrade the myofibrils at the temperature of 4°C, when the pH was 5.6 in the presence of 100 μmol/L of $CaCl_2$ (Huff-Lonergan et al., 1996). Proteolysis of muscle proteins through calpains is caused by the degradation of the Z-line, titan, and costameric proteins (Taylor et al., 1995). During the tenderization process experienced in meat aging, a number of changes occur at the structural level. The most commonly observed are fragmentation of myofibrils, changes in the area of I-band and Z-line, and degradation of myofibrillar proteins like T troponins, I troponins, titins, nebulins, desmins, etc. (Kolczak, 2000; Koohmaraie and Geesink, 2006).

8.4.2 Cathepsins and Other Enzymes

Cathepsins are a group of enzymes and have been classified into: cysteine (cathepsins B, H, L, X), serine (cathepsin G), and aspartic (cathepsins D, E) peptidase (Kemp et al., 2010). The use of cathepsins in meat tenderization is doubtful, as there is dearth of evidence that they are released from lysosomes during the postmortem storage of meat. Moreover, cathepsins have been found to have an effect on myofibrillar proteins like myosin, actin, and α-actinin, and during normal aging of muscle, only a small amount of these proteins is affected (Purslow et al., 2001). Many types of cathepsins have been identified, and out of them cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) are said to be the key factors for deterioration of muscle proteins (Jamdar and Harikumar, 2002). Effective degradation patterns of myofibrillar proteins were observed in salmon muscle tissue which was almost reproduced upon treating myofibrils with purified cathepsin L (Yamashita and Konagaya, 1990). Studies reveal that the myofibrillar proteins are least affected during postmortem storage and the softening of muscle is due to proteolytic digestion of minor cell components that link the major structural units (Hernandez-Herrero et al., 2003). Endogenous collagenases and proteases may cause the degradation of collagenous fibrils in the meat of blue grenadier fish (*Macruronus novaezelandiae*) (Bremner and Hallett, 1985).

Some researchers suggest that meat tenderness may be affected by proteasomes, while others are of the view that caspases have a similar importance in reducing final meat toughness (Kemp et al., 2010). Sarcoplasm is where proteasomes are present in the form of large protein units. It has been explored that proteins destined for destruction by the ubiquitin system are actually identified and attacked by the 26S proteasome. The 26S proteasome

consists of two subunits where 19S is a regulatory part and 20S is a multicatalytic structure of proteolytic-enzyme activities identified as MPC (multicatalytic proteinase complex) (Dahlmann et al., 2001). Another group of enzymes are caspases, which are highly specialized in nature and can lead to proteolysis through an intracellular network signaling system. Proteolytic changes and caspases activation is said to be caused by enzymes like granzyme B, cathepsin G, cathepsin D, etc. (Korzeniewska-Dyl, 2007). Kemp et al. (2006) reported that activity of caspase 3 was higher during the initial phases of postmortem, however it decreased with time and was inversely related to Warner-Bratzler shear force. Hence, the calpain system alone is not responsible for the postmortem tenderization and caspase-3 may have a role in proteolysis and muscle degradation, resulting in a reduction in meat toughness (Chen et al., 2011).

8.5 EXOGENOUS ENZYMES

There are numerous naturally occurring proteolytic enzymes of plant origin that have the potential to improve tenderness of tough meat. The most commonly used are papain, zingibain, cucumin, and ficin. Papain-treated meat was very tender, but also scored high for bitterness (Gerelt et al., 2000). Kang and Warner (1974) reported that meat tenderization via papaya latex preparations is achieved due to the presence of the enzyme papain found in raw papaya. Research has also shown that zingibain in the ginger rhizome has proved to be an effective tenderizing agent for meat and meat products (Naveena and Mendiratta, 2001). Cormier et al. (1989) reported that the protease enzyme ficin, present in figs, could be used as a meat tenderizer. It has been observed that several proteases sourced from microorganisms can be used to tenderize meat and similarly some of the enzymes from the animals can be used for this process.

Figs, pineapple, and papaya have been used as food for a long time, hence the proteases from these fruits have been given the Generally Regarded As Safe (GRAS) status by the US Food and Drug Administration. Commercial papain has a toxicity level of 10 g/kg body weight (mice), and in the subsequent report the enzyme was granted GRAS status in 1977 (Denner, 1983; FDA, 1999, 2009). In accordance with this ficin and bromelain have also been granted GRAS status (Denner, 1983). Similarly, the microorganisms used for the production of these proteases are safe if they comply with the required safety rules set by the Food and Drug Administration, outlined in FAO/WHO (2003). The Food and Drug Administration (FDA, 1995, 1997, 1999) also mentions the GRAS status of several proteases from different sources, including plants (ficin, papain, bromelain), microbes (*Bacillus subtilis*; *Aspergillus niger*; *Rhizopus oryzae*), and animals (pepsin, trypsin). Most of the enzymes exogenously used in meat tenderization have an optimal temperature of 50–70°C, hence maximum activity occurs during high-temperature processing or cooking.

8.5.1 Plant Enzymes

8.5.1.1 Papain

Papain (EC 3.4.22.2) is a cysteine protease acquired from the latex of the papaya plant (*Carica papaya*) and has been used for protecting plants against insects (Konno et al., 2004). The enzyme has been reported to have a high optimal temperature (65°C) and a wide pH

range (5–8) for its activity (Smith and Hong-Shum, 2003). Commercial use of enzymes from varied industries have a different ratio of papain, chymopapain, and papaya peptidase A, resulting in distinctive physical, chemical, and biological characteristics leading to variations in performance (Kang and Warner, 1974). Tenderization of meat can be improved by application of papain which acts on the structural component of muscle (Gracey and Thronton, 1985). Mapping of the active site revealed that papain has specificity for amino acids with aromatic side chains such as Phe (Phenylalanine) and Tyr (Tyrosine) at the P2 position (Berger and Schechter, 1970).

Papain is a highly efficient enzyme causing significant degradation of myofibrillar as well as collagen proteins (Ashie et al., 2002). Grzonka et al. (2007) found that papain had an optimum activity at a wide range of pH levels (5.8–7.0) and temperatures (50–57°C) specially when the substrate used was casein. However, Landmann (1963), found that although papain is active over a broad range of pH levels, its maximum activity appears to be in the range of 4.0–6.0. In another finding, Tappel et al. (1956) reported that maximum tenderizing activity for meat takes place during the cooking process. Kang and Rice (1970) concluded that papain showed higher activity for myofibrillar fraction with stronger solubilizing activity on connective tissue. It has been reported that oxidation of the active site (thiol group) can lead to inactivation of enzymes which can be reversed to some extent by thiol reagents like cysteine or sodium metabisulfite (Grzonka et al., 2007). The enzyme activity is affected by factors like pressure (800 kPa) and temperature (60°C), as they may have impact on the active site due to oxidation of thiolate ions (Gomes et al., 1997).

Swift and Company Ltd. developed a process of effective tenderization ("ProTen") by injecting the enzyme 10–30 min prior to the slaughter of the animal to achieve a uniform distribution in the carcass (Kang et al., 1974, 1982). As active papain may lead to severe shock and stress symptoms (Dransfield and Etherington, 1981; Kang and Warner, 1974), an alternative approach was followed of injecting inactive papain (oxidation of cysteine by hydrogen peroxide) which got reactivated in the carcass due to the anoxic conditions (reduction of cysteine), and protein degradation took place leading to tenderization (Rhodes and Dransfield, 1973). Khanna (1995) suggested that papain infusion plus forking technology were more suitable methods for tenderizing spent hen meat cuts than the injection method. Grover et al. (2005) also showed the synergistic effect of papain and sodium tripolyphosphate in increasing the tenderness of chicken gizzard.

8.5.1.2 Ficin

Ficin (EC3.4.22.3, MW = 26 kDa) is obtained from the latex of *Ficus glabrata*, *Ficus anthelmintica*, etc. (Gaughran, 1976), which contains about 10 proteases (Kramer and Whitaker, 1964) with the half-life of 1.5 h at 60°C (Whitaker, 1957). In fact, the ficin is most commonly obtained from the fig fruit and reported to have a meat tenderizing effect. Cormier et al. (1989) conducted an experiment on the cell culture of figs and evaluated the cell culture as a source of protease enzyme that could be used successfully as a meat tenderizer. Ramezani et al. (2003) investigated the water-holding capacity of ficin-tenderized meat and evaluated the effect of ficin on meat protein by gel electrophoresis and concluded that solubility of meat protein increased when ficin was used. Ficin can attack elastin at lower temperatures like 20°C, however it has little activity against collagen and myofibrillar proteins if subjected to a temperature lower than 40°C. Some workers (El-Gharbawi and Whitaker, 1963;

Foegeding and Larick, 1986) reported that the enzyme is having an optimal activity between temperatures of 60°C and 70°C. In another finding, El-Gharbawi and Whitaker (1963) found that the optimal pH for enzyme activity is around 7 for collagen and myofibrillar proteins, and about 5.0–5.5 for elastin. Hence the pH of 7 is found to have an optimal activity but the enzyme results in extensive breakdown of elastin at pH of 5 (Gaughran, 1976).

8.5.1.3 Cucumin

Among the widely used plant proteolytic enzymes, cucumin, which is obtained from kachri (*Cucumis pubescens*) has been reported to have proteolytic activity and coarsely ground and dried kachri fruits are traditionally used as a food-tenderizing agents (Hajjatullah and Baloch, 1970). Naveena et al. (2004) concluded that cucumin could be used as a better alternative to papain for tenderizing tough buffalo meat. Mendiratta et al. (2003) reported that tough sheep meat was effectively tenderized within 4 h at room temperature (25°C) by treatment with 5% extract of cucumis fruits. Kumar and Berwal (1998) reported that kachri could be used successfully to improve the tenderness of spent hen meat.

8.5.1.4 Bromelain

The pineapple (*Ananas comosus*) has been found to have an enzyme, bromelain, which contains cysteine proteases obtained from the stem (EC 3.4.22.32, 24.5 kDa) as well as fruit (EC 3.4.22.33, 25 kDa). Out of the two sources, the fruit bromelain is said to have higher proteolytic activity and a greater specificity in comparison to stem bromelain (Barrett et al., 2004; Grzonka et al., 2007). The enzymatic activity scale is slightly smaller than that of papain and reveals an elaborate proteolytic activity on synthetic peptides at pH levels of 5.0–7.0 and a temperature of 50°C (Napper et al., 1994; Rowan et al., 1990), although a wider range (optimum at pH 6–8.5 and a temperature range of 50–60°C) has been reported by Grzonka et al. (2007). Pure bromelain is stable when stored at −20°C (Rowan et al., 1988) and the most effective compound to activate the enzyme is cysteine (Murachi et al., 1964). Bromelain first attacks and degrades around 40% of the collagen present in the sarcolemma, then the degradation of myosin is followed in the myofibrillar region (Kang and Rice, 1970;Wang et al., 1958). This enzyme has a low, but significant, activity at 0°C which dramatically increases at 50°C to 70°C and is unaffected up to 80°C (El-Gharbawi and Whitaker, 1963; Tappel et al., 1956).

8.5.1.5 Zingibain

A powerful proteolytic enzyme has been found in ginger, which can be utilized as tenderizing agent (Lee et al., 1986; Mansour and Khalil, 2000). Zingibains are obtained from ginger rhizomes, a new source of plant proteolytic enzyme (Thompson et al., 1973). The enzyme has a greater proteolytic activity when heated, a desirable feature and an advantage over the other enzymatic tenderizers (Naveena and Mendiratta, 2001). Naveena et al. (2004) observed that the inexpensive and easily found ginger rhizome could effectively be used for meat tenderization. Lee et al. (1986) described that extensive degradation of the myofibrils is possible with a higher concentration of ginger extract; the degradation appears to begin at the I band of each sarcomere which progresses toward the M line. Su et al. (2009) found that ginger extract contains 2 cysteine proteases with a molecular mass of 29 and 31 kDa, however, Ohtsuki et al. (1995) reported three enzymes with proteolytic activity, each having a molecular mass of 29 kDa. The proteolytic activity of the enzymes were reduced by 15% with a NaCl (2%)

treatment, and only 25% of the activity was retained when heated at 70°C, however, it had a higher specificity toward collagen in comparison to actomyosin (Thompson et al., 1973).

8.5.1.6 Actinidin

Actinidin, or actinidain (EC 3.4.22.14), is obtained from the kiwi fruit (*Actinidai deliciosa*). The enzyme varies greatly among different cultivars of fruit and it ranges from non-detectable to 10.7 mg/mL juice (Nishiyama, 2007). The actinidin was stable at a pH range of 7–10, however, it had an optimal activity at 58–62°C (Yamaguchi et al., 1982). The best activity was found to be in the pH range of 7.3–7.6 (Yamaguchi et al., 1982), but a pH range of 5–7, reported by Boyes et al. (1997), reflects the variation in the cultivar and assay used in estimation of proteolytic activity. Actinidin hydrolyzes both myofibrillar proteins and connective tissue proteins (Christensen et al., 2009; Han et al., 2009) but appears to have higher proteolytic activity toward collagen (Wada et al., 2002). When porcine muscles were subjected to a brine solution containing actinidin, a decreased Warner-Blatzer shear force and improved sensory feature was observed. Restricted degradation of connective tissue proteins and myofibrils were observed when the enzyme was added to the raw meat and hence is considered a prime agent for improving meat tenderness.

8.5.2 Microbial Enzymes

In line with plant proteases, several enzymes have been isolated from microorganisms and bring a desired change in meat characteristics. With respect to meat tenderization, alkaline elastase from alkalophilic *Bacillus* sp. strain Ya-B demonstrated an optimum activity in the pH range of 5.5–6.0 and temperature range of 10–50°C (Yeh et al., 2002). In another study, it was found that *Bacillus subtilis* and *Bacillus subtilis* var. a*myloliquefaciens* are the prime source of proteases like subtilisin (EC3.4.21.62) and neutral protease (EC3.4.24.28), each of which also have a GRAS status (FDA, 1999). McConn et al. (1964) reported that neutral protease activity increases at 50°C but drastically drops at temperatures above 65°C. However, the enzyme is active at wide pH range of 5.0–9.0, with optimal activity at 7.0. Qihe, et al. (2006) found that an elastase from *Bacillus* sp. (EL31410) on beef led to a 30% decrease in relative hardness. Similarly, it was reported that some microorganisms (e.g., *Bacillus*) play a very important role in protein degradation in fermented meat and fish products (Bekhit, 2010). Collagenases from *Clostridium histolyticum* (Foegeding and Larick, 1986) and *Vibrio* B-30 (Miller et al., 1989), and elastases from alkalophilic *Bacillus* spp. (Takagi et al., 1992) have been used in meat products. However, it has been found that collagenases and elastases found in fish and aquatic invertebrates have very different specificities compared to those in mammalian muscles (Shahidi and Kamil, 2001).

Several fungal enzymes have also been found to have an appreciable effect on the meat system*; Aspergillus oryzae* produces an aspartic protease and has an effective proteolytic activity in meat (Ashie et al., 2002). The enzyme possesses minimal activity under refrigerated storage but increases to an optimum at 55°C before drastically dropping at 60°C. *Aspergillus oryzae* is a source of several proteolytic preparations available commercially which contain several GRAS (FDA, 1995, 1999, 2009) proteases like neutral protease (EC 3.4.24.4), alkaline protease (EC 3.4.21.14), and aspartic protease (EC3.4.23.6), indicating a wide pH range for activity. Both collagen and elastin have good proteolytic action against connective tissue

proteins (Ashie et al., 2002). Benito et al. (2003) tested the use of a fungal protease on pork, and observed the effect of EPg222, a protease from *Penicillium chrysogenum*, on products like dry-cured ham. Proteases having potential to act against both myofibrillar and connective tissue proteins from *Penicillium chrysogenum* Pg222 (Benito et al., 2003), *Aspergillus sojae* (Gerelt et al., 2000), *Aspergillus oryzae* (Ashie et al., 2002; Gerelt et al., 2000), have been explored for meat and other food items.

8.6 TRANSGLUTAMINASE: MEAT RESTRUCTURING

Transglutaminase (TGase) is used for the improvement of textural characteristics (Muguruma et al., 2003) of food products. The enzyme is calcium-dependent and catalyzes acyl transfer reactions with the ε-amino group lysine, where γ-carboxamide groups of glutamine act as acyl donors and lysyl residues act as acyl acceptors (Kumazawa et al., 1996). The reaction involves the intermolecular linking between the glutamine and lysine component of proteins by creating a complex network. Hence, in raw meat products, it provides an enzymatic way of producing uniform shapes and sizes. The enzyme results in an effect on soluble proteins in such a manner that a gel network is produced and combines pieces of meat together. TGase is present in many animal tissues, and is a component of several biological processes: blood clotting, wound healing, epidermal keratinization, etc. (Aeschlimann and Paulsson, 1994). The first approach is to extract and purify the enzyme from the body fluids or tissues of the animal followed by an attempt to obtain the enzyme by means of genetic manipulation using host microorganisms such as *Escherichia coli*, *Bacillus* spp., *Aspergillus* spp., etc. Several workers, like Takehana et al. (1994) (Streptomyces TGase in *E. coli*), Washizu et al. (1994) (Streptomyces TGase in *Streptomyces lividans*), Yokoyama et al. (2000) (Streptomyces TGase in *E. coli*) have attempted to obtain TGase at economic cost. However, some workers adopted a method to screen out the TGase-producing microorganisms in nature and go for mass production of TGase by fermentation technology from select organisms. The pH for MTGase (Microbial-transglutaminase) activity ranged between 5 and 8, however, some activity was observed at a pH of 4 or 9, and hence is active at wide pH range. The enzymatic activity was found to be optimum at a temperature of 55°C (10 min at pH 6.0) and fully active for 10 min at 40°C. However the activity was lost when it was subjected to 70°C (Ando et al., 1989).

Tseng et al. (2000) suggested that high-quality, low-salt chicken meatballs could be formed with the help of TGase and the binding strength of the balls increased with the elevated concentration of TGase in the mixture. In another report, Nielsen et al. (1995) observed that cross-linking meat proteins by TGase depended on salt, phosphate, and temperature levels. In order to develop the enzymes, including the one obtained from guinea pig liver, calcium is required (Aeschlimann and Paulsson, 1994). However, MTGase is totally independent of calcium, and is different from the mammalian enzymes in some attributes and hence has been widely used to improve the textural quality of several products such as ham, and sausage (Motoki and Seguro, 1998). The enzyme of microbial origin is capable of gelling concentrated solutions of proteins such as soybean proteins, milk proteins, and meat proteins like myosin (Nonaka et al., 1997). This system can be used to produce large pieces of restructured meat from smaller fragments (Tsukamasa et al., 2002).

Although a lot of research has been done on the use of transglutaminase, other cross-linking enzymes are also there which have the potential to modify the texture of meat and meat products. Enzymes like polyphenol oxidases (PPO) and lipoxygenases can help in binding by acting on sulfhydryl groups and disulphide bonds. Enzymes like tyrosinase are an important example of polyphenol oxidases that help in binding by oxidizing tyrosine to quinines. Hence adding compounds to meat proteins that tyrosinase can cross-link offers textural improvement to meat products. It has been found that milk proteins like β-casein are promising substrates in tyrosinase-induced binding (Hiller and Lorenzen, 2009; Monogioudi et al., 2009). In a similar way, another enzyme, laccase (a polyphenol oxidase), has not been explored properly for its potential use in meat products. However, it has been found that when adding milk proteins to meat products, it can be of use to modify texture (Minussi et al., 2002).

8.7 BIOACTIVE PEPTIDES

Bioactive peptides can be produced from meat proteins by the action of various digestive enzymes which have been reported to have some functional properties. ACE (angiotensin converting enzyme) inhibitory peptides are produced by in the vitro gastric digestion of pork, where a group of enzymes like pepsin and pancreatin are in action (Escudero et al., 2010). Similarly, another finding observed that antihypertensive peptides can be released from porcine skeletal muscle when crude myosin B was subjected to hydrolysis by enzymes like pepsin (Muguruma et al., 2009). In another report, Arihara et al. (2001) reported that thermolysin digestion of porcine myosin resulted in production of ACE inhibitory pentapeptides and the antihypertensive activities of myopentapeptides were found to be significant (Nakashima et al., 2002). It was also reported that action of five proteases viz alcalase, α chymotrypsin, neutrase, pronase E, and trypsin on bovine skin gelatine, resulted in the release of ACE inhibitory peptides (Kim et al., 2001). Similarly, the hydrolysates of chicken collagen produced by an *Aspergillus oryzae* protease, resulted in production of ACE inhibitory peptides, and when subjected to analysis, the results indicated a reduction of blood pressure after administration of the peptides (Saiga et al., 2003b).

Further investigations revealed that peptides obtained from pork myofibrillar proteins after the hydrolysis by proteases like papain and actinase E had antioxidant activity (Saiga et al., 2003a). The antioxidant peptide QGAR was released from the porcine collagen when a concoction of enzymes like pepsin, papain, and proteases from the bovine pancreas and bacterial proteases from *Streptomyces* and *Bacillus polymyxa*, were used for hydrolysis (Li et al., 2007). Alcalase-treated plasma hydrolysates were observed to be effective antioxidants; they inhibited the lipid oxidation, effectively scavenged DPPH (1,1-diphenyl-2-picrylhydrazyl), and chelated metal (Liu et al., 2010). Similarly, digestion of venison with papain lead to the production of hydrolysate displaying strong antioxidant activity (Kim et al., 2009).

8.8 FLAVOR

Flavor design by using the enzymes like lipase, glutaminase, protease, and peptidase as a tool is an example of emerging technology in the industry. During the ripening of sausages, the enzyme-mediated protein breakdown reactions yield nitrogenous compounds such as small

peptides and free amino acids which contribute to the taste of product. They may act as precursors of volatile compounds and strong flavor contributors. Products like free amino acids may be further converted to other metabolites by the decarboxylation and deamination leading to amines and organic acids, respectively. The plenty of end products produced here play an important role in flavor imparting effect or a precursor for the same. Selgas et al. (1993) found the proteolytic activity in several strains of *Micrococci*, isolated from fermented sausages, and suggested microbial enzymes play a role in flavor production. The proteolytic phenomena have been indicated in the enzymes like proteases from micrococci and lactic acid bacteria in dry fermented sausages (Guo and Chen, 1991). However, Montel et al. (1992) found that several species of lactic acid bacteria, like *Lactobacillus* and *Pediococcus*, have a limited role in protein hydrolysis, but the intracellular peptidasic activities could contribute to the increased levels of free amino acids. In another finding, Toldra et al. (1993) has observed an intense proteolysis due to cathepsin activities during the ripening process of ham and dry sausage.

Demeyer et al. (1974) observed that lipases from lactobacilli attacking the lipids with short chain fatty acids may be one of the major flavor contributors; lipids in dry fermented sausages, ranging from 25% to 55% of crude matter, could be the reason for extensive lipolytic activities and the marked flavor. Similarly, Garcia et al. (1992) also found an increase of free fatty acids in products such as dry fermented sausages when they were inoculated with the lactobacilli or micrococci organisms. In another work it was revealed that lipids are affected by lipolytic activity and oxidative phenomena which result in the release of compounds like fatty acids and low-molecular weight substances responsible for the flavor of the final product (Cantoni et al., 1966). However, it was found that lipolytic activity can also be attributed to endogenous enzymes, as only low levels can be accessed by microorganisms in the inner part of cured ham (Francisco et al., 1981). Lipases are found commonly in nature and are present in different organisms like animals, plants, fungi and bacteria. They are in fact a type of esterase responsible for fat digestion by attacking insoluble triacylglycerols and converting them into soluble fatty acids as well as di- and mono-acylglycerols. Peak activity of maximum microbial lipases is exhibited in the pH range of 5.6–8.5; however, maximum stability is observed at neutral pH (Malcata et al., 1992) and the enzyme is commercially available from different suppliers in food-grade form.

Glutaminase (L-glutamine aminohydrolase, EC 3.5.1.2), an enzyme produced by starter cultures to impart flavor, is important in products like meat sausage. Incorporating the enzyme to fermented seasoning agents leads to an increase in the glutamic acid content, and enhancing the "umami" taste in foods. The enzyme is responsible for hydrolytic deamidation of L-glutamine to produce L-glutamic acid, the element indicated as a flavor enhancer, and ammonia, another component which acts as an acid neutralizer. It is a ubiquitous enzyme in bacteria and eukaryote, but its presence in archaea, thermophiles, and plants is doubtful (Nandakumar et al., 2003). The majority of organisms having glutaminase activity have been explored from sources like soil. Amano Enzyme, Inc. markets enzymes extracted from *Bacillus amyloliquefaciens*, which has also been isolated from *Aspergillus oryzae* (Thammarongtham et al., 2001).

8.9 CONCLUSION

The enzymes which are known to us are being harnessed to have a positive effect on the meat industry and product quality. The quality attribute of tenderness is rated as the most important sensory parameter of meat. To achieve meat tenderness there is a need to explore

more effective enzyme use from the nature to have desired effect. The majority of meat produced is not naturally tender, especially red meat, as it is produced from the spent animals of the dairy industry. In addition to tender meat, the aware consumer demands fresh meat products without excessive fat and with a desired texture. This is achievable when we redesign or restructure the products and remove the extra fat. Moreover, restructuring also helps improve lower quality meat and the efficient utilization of the carcass. Meat cuts, especially the lower valued cuts and trimmings, can be bound together with TGase to create products with a designer content, shape and size, that are stable in cold storage, and stand up to cooking conditions. There is boundless potential for developing this type of meat product, and many enzymes can be further researched to achieve better results. Similarly, new approaches are required to design the microorganisms for desired enzyme production and application in the industry. The recombinant technology is the best tool of the future to have the organisms on demand for the desired enzymatic changes like tenderization, flavor production, bioactive peptide production, etc. Bioactive peptides may become the most demanded product for the future of the functional food industry. Hence a more scientific approach is needed to address these quality issues of the meat industry and explore the novel areas of application.

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CHAPTER

9

Enzymes for Use in Functional Foods

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9.1 INTRODUCTION

Enzymes are indispensable for life. In living body systems, they are responsible for catalyzing a host of biochemical reactions that make life possible. Enzymes are also used as natural catalysts in several industrial processes and in many food industries. Life cycle assessment (LCA) studies have shown that, overall, enzymatic processing reduces environmental footprints, and enzymes provide a viable and sustainable alternative to the use of hazardous and toxic catalysts in industrial processes (Andreaus et al., 2016; Jegannathan and Nielsen, 2013). The catalytic powers of enzymes therefore continue to be the subject of intense research and industrial exploitation in products such as pulp and paper, textile and detergents, cosmetics and pharmaceuticals, and food. In many industrial sectors, as in the food and beverage industry, there is a growing worldwide interest in renewable, eco-friendly, economic, and cleaner or "green" strategies for the production of foods (Andreaus et al., 2016; Jegannathan and Nielsen, 2013; Wageningen University and Research Centre, 2011). It is well accepted that industrial biotechnology (i.e., the production of useful compounds from biological materials via bio-based processes such as enzyme catalysis) meets several of the Principles of Green Chemistry, namely waste minimization and prevention, use of catalysts rather than stoichiometric reagents, avoidance or reduction in use of hazardous and toxic chemicals, safer product and processes, and use of renewable materials (Anastas and Warner, 1998; Andreaus et al., 2016). The ability of enzymes to catalyze reactions at high efficiency and specificity with little or no waste products, and at mild conditions, is particularly attractive in the food industry, and makes enzymes a promising alternative to replace or supplement conventional catalysts (Wohlgemuth, 2009). Enzymes are therefore poised to play a significant role in food manufacturing operations in the coming years (Daiha et al., 2016). In fact, the global enzyme market is forecasted to grow at an average of 4.6% to $7.2 billion by the year 2020 with the food and beverage sector being the largest enzyme market by value (Freedonia Inc., 2016). Within the food and beverage sector, functional foods, or

https://doi.org/10.1016/B978-0-12-813280-7.00009-8

health and wellbeing foods, are also growing at a high rate. A 15% growth is anticipated by 2021 (Euromonitor International, 2017).

Globally, the human population is growing, as is the market for health and wellbeing foods. As such, production technologies that are sustainable and economically feasible for preparing safe and potent functional foods need to be investigated. This chapter discusses the role of enzymes in the development of functional foods. The release of dietary and bioactive compounds from proteins, lipids, and carbohydrates through the catalytic actions, respectively, of proteases, lipases, and glycosidases, is explored.

9.2 DEFINITIONS, MARKET VALUE AND CONSUMER ACCEPTANCE OF FUNCTIONAL FOODS

Hippocrates is reputed with the quote "*Let food be thy medicine, and thy medicine food.*" In modern times, consumer awareness of the influence of diet on health has grown rapidly. The demand for "foods with health functions" continues to increase and this is attended with a blurring of the interface between food and medicine (Lang, 2007). All foods perform one or more functional roles—from sensory to nutritional properties; as such, all foods are functional in that sense. However, the ability of food components to perform hormone-like bioactive or physiological roles has warranted the term "functional foods" as a new food category that describes the role of food in health (Hasler, 2002). The physiological health function of foods is an observation with worldwide consensus, and in various parts of the world, there is knowledge about certain indigenous food products that offer health benefits in addition to providing nutrition (Ozen et al., 2012).

There is no universal accepted definition for "functional foods." Food regulatory institutes and some research organizations have therefore adopted their own definitions, some of which have been highlighted below:

- US FDA: "Natural or processed foods that contain known or unknown biologically active compounds, which, in defined, effective, nontoxic amounts, provide a clinically proven and documented health benefit for the prevention, management or treatment of chronic disease" (Hunter and Hegele, 2017).
- Institute of Food Technologists (IFT): "Foods and food components that provide a health benefit beyond basic nutrition (for the intended population). Examples may include conventional foods; fortified, enriched or enhanced foods; and dietary supplements. These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects" (Clydesdale, 2005).
- The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE): "A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern" (FUFOSE, 2007).

- The United States Food and Nutrition Board (USNFB): "Any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (USFNB, 1994).
- International Life Sciences Institute (ILSI): "Foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition" (ILSI, 1999).
- American Dietetic Association (ADA): "Functional foods … include whole foods and fortified, enriched, or enhanced foods, [that] have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels" (AMA, 1999).
- Functional Food Center: "Natural or processed foods that contain known or unknown biologically-active compounds; which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease" (Martirosyan and Singh, 2015).

A common element in all the above definitions is that functional foods should have their origin in food material, must be present in quantities sufficient enough to be bioavailable and bioaccessible after the food is consumed, and must show some bioactive properties with physiological health benefits. Some examples of functional food classes are nutraceuticals, probiotics, prebiotics, psychobiotics, and foods for specific health uses (FOSHU).

The growing trend in the promotion of foods with health functions is as a result of factors such as a rapidly aging society, increased interest in health-consciousness and beauty, rapid advances in science and technology, increasing healthcare costs, and a growing market (Hasler, 2002). There has also been a switch in consumer priorities toward the consumption of natural foods and beverages which has almost become synonymous with functional foods. These products are often tagged as "naturally healthy," "free from (allergens, dairy, gluten, lactose, meat)," "organic," "fortified/functional packaged," or "better for you" (Euromonitor International, 2017). These recent trends are key drivers for the manufacturing of functional foods and health and wellbeing foods in the 21st Century. The global functional foods market is expected to reach $255.1 billion by 2024 (Grand View Research, 2016a) providing an opportunity for the food and beverage manufacturing industry. To satisfy this huge and growing market, food manufacturers have to develop techniques for processing food materials in order to maintain their functional and bioactive components. Enzymes are anticipated to play a significant role in the processing of functional foods.

9.3 ROLE OF ENZYMES IN THE PRODUCTION OF FUNCTIONAL FOODS

9.3.1 The Use of Proteases for Bioactive Peptides Production From Proteins

9.3.1.1 Classification and Catalytic Activity of Proteases

Since the beginning of the 20th century when the first study on proteases was published by (Levene, 1905), the biochemical characteristics and applications of this unique class of

enzymes continue to be researched. Consequently, a Google search on "proteases" yields over 9,400,000 results in 0.54 s. Proteolytic enzymes are present in all life forms and constitute the largest single family of enzymes (Li et al., 2013). In vivo, proteases are responsible for regulating several biochemical processes such as nutrient digestion, molecular signaling, the creation of bioactive molecules, and the control of homeostasis (Ciechanover, 2005; Li et al., 2013; Lopez-Otin and Overall, 2002; Oikonomopoulou et al., 2006).

Proteases play a unique role in the hydrolysis of peptide bonds in proteins to give protein hydrolysates, peptides, or amino acids. During protein hydrolysis, the position of attack is one basis for the classification of proteases. Some enzymes cleave terminal amino acids from the N-terminus (i.e., aminopeptidases) or C-terminus (i.e., carboxypeptidases); others cleave the internal bond of the protein or peptide and are called endopeptidases. The optimal pH for proteolytic activity is another basis for classifying these enzymes into acid-, neutral-, and alkaline-proteases.

Based on catalytic residues, proteases are classified into seven groups: serine, cysteine, threonine, aspartic, glutamic, metalloproteases, and asparagine peptide lyases. Alongside the asparagine peptide lyases, which work via elimination reaction mechanisms, all other proteases work via hydrolysis and differ only in the type of nucleophile used. The mechanism of hydrolysis involves attacking the cleavable carbonyl group (C=O) with an activated nucleophile following polarization of the amide bond (Li et al., 2013). Serine and threonine proteases use the hydroxyl side chains on these amino acids at the active site as the nucleophile. Water serves as the nucleophile in aspartic proteases and glutamate proteases; in cysteine proteases and metalloproteases, thiol groups and metal ions are used as the nucleophile, respectively (López-Otín and Bond, 2008; Rawlings et al., 2011).

9.3.1.2 Sources of Proteases for Food Applications

Proteolytic enzymes are ubiquitous in nature, but for the purpose of food and industrial applications, they are sourced from microorganisms, plants, and mammalian gastrointestinal (GI) tracts (Gupta et al., 2002b; Sumantha et al., 2006). A number of plant proteases such as bromelain (pineapple), papain (pawpaw), actinidin (kiwi), ficin (fig), and vegetable rennet (cardoon) have been used in the production of peptides from various proteins (Bah et al., 2016; Mazorra-Manzano et al., 2017), but plant proteases suffer from seasonal variability and long maturation times, and are therefore not ideal for industrial-scale production of bioactive peptides (Agyei and Danquah, 2011). Animal proteases of GI origin are also attractive for the production of bioactive peptides. There is a large number of enzymes in this category, with chymosin, trypsin, chymotrypsin, pepsin, carboxypeptidase A, elastases, and erepsin being examples. Together, these enzymes provide a plethora of activities that can be exploited for the generation of unique peptide sequences. The use of GI proteases provides an avenue to simulate the potential release of bioactive peptides during the normal course of protein digestion in the human digestive system. However, in addition to generating undesirably bitter protein hydrolysates, GI enzymes are fraught with animal right concerns as their acquisition often involves the killing of animals (Rao et al., 1998).

Microbial proteases are the most widely used for industrial application including bioactive peptide production. Microbial proteases offer several advantages over proteases from other sources: (1) the nutritional requirements and cost for culturing microorganisms is relatively cheaper, and microorganisms have short maturation time; (2) the cost and labor involved in the downstream processing of microbial proteases are relatively low, as most

microbial proteases (particularly from lactic acid bacteria) are expressed and anchored on the cell-membrane, making harvesting and purification easy; (3) microbial proteins are thought to have better storage stability with little loss of activity under storage (Gupta et al., 2002a); and (4) there is a wide diversity in the microbial world. Also, reports on microorganisms living in extreme environments have shown that they are express proteases with unique stability and catalytic properties. This suggests an almost infinite number of proteases can be exploited for food applications (Agyei and Danquah, 2011; Rao et al., 1998). For example, in lactic acid bacteria alone, there are cell-membrane-bound proteinases, and a plethora of intracellular peptidases (i.e., endopeptidases, aminopeptidases, tripeptidases, and dipeptidases) (Gobbetti et al., 1996; Khalid and Marth, 1990). All these proteases offer different but unique enzymatic activities. Industrial-scale microbial proteases for the production of bioactive peptides are often produced by *Bacillus* spp., and lactic acid bacteria (Ferrero, 2001).

9.3.2 Bioactive Peptides: Protease-Derived Functional Food Ingredients

Bioactive peptides have been described as peptides sequences that, upon introduction into the body, impart health functions beyond nutrition (Gnasegaran et al., 2017; Korhonen and Pihlanto, 2006; Park and Nam, 2015). The peptides are able to trigger certain physiological responses in living body systems thus prompting a wide interest for their use in the development of functional foods and peptide therapeutics (Agyei et al., 2016, 2017; Korhonen and Pihlanto, 2006; Powers and Hancock, 2003). A collection of some biological properties of bioactive peptides is shown in Fig. 9.1. Some bioactive peptide sequences, termed as multifunctional peptides, have been shown to display more than one biological activity (Meisel, 2004). Examples of multifunctional peptides include lactoferricin (which has anticancer, antitumor, antiparasitical, and antimicrobial properties) (Eliassen et al., 2002; Korhonen and Pihlanto, 2006), lunasin (which shows anti-inflammatory, antioxidant, and antihypertensive properties) (Hernandez-Ledesma et al., 2009; Seber et al., 2012), and other peptides from other protein sources (marked with footnote "a" in Table 9.1). Multifunctional peptides are very attractive for targeting disease conditions that present multiple symptoms (Li and Aluko, 2010; Sistla, 2013).

Consumer interest in food formulations that perform health functions has grown remarkably in recent years, making the development of functional foods from bioactive peptides an attractive investment by the food industry. Research is growing in the exploration of proteases and proteins that generate novel peptide sequences which have high biological potency and improved properties (Udenigwe and Aluko, 2012). There have also been advances in options for bioprocessing and downstream purification of peptides (Agyei et al., 2016). The recent growth in the development of bioinformatics and "-omic" techniques for predicting the potential release of specific peptides from proteins is also promising (Minkiewicz et al., 2008; Udenigwe, 2014). These advances will facilitate the development of food formulations that contain bioactive peptides as active ingredients.

9.3.3 The Use of Lipases for Omega 3 Oil Production From Lipids

Omega-3 fatty acids are polyunsaturated fatty acids (PUFAs) with multiple double bonds (Akanbi et al., 2013). Generally, these fatty acids have 18 to 22 carbon chains with two or more double bonds. Notable omega-3 fatty acids are alpha-linolenic acid (ALA, C18:3n-3),

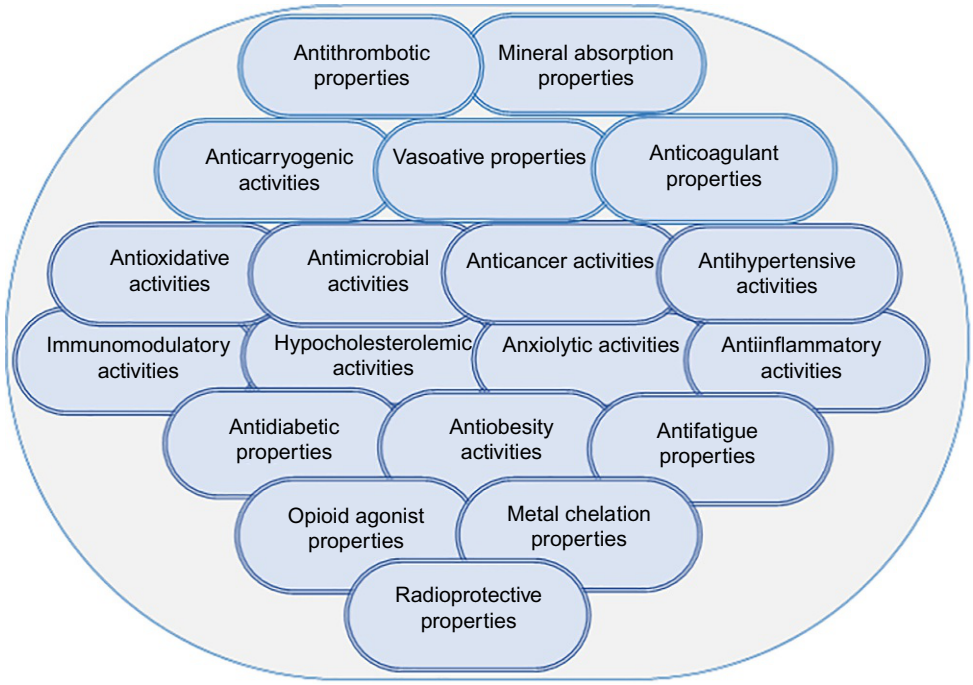

FIG. 9.1 Array of biological activities triggered by bioactive peptides.

TABLE 9.1 Array of Bioactive Peptides and the Protein/Enzyme Combination Used for Their Production

Protein Source	Enzyme Used	Peptide Sequences	Bioactivity	Reference
Chicken muscle	Thermolysin	Leu-Pro-Lys	ACE inhibition properties	Murray and Fitzgerald (2007)
Maize protein (α-zein)	Thermolysin	Leu-Arg-Pro	ACE inhibition properties	Murray and Fitzgerald (2007)
Maize protein (α-zein)	Thermolysin	[a]Val-His-Leu-Pro-Pro-Pro	PEP inhibition activities and antiamnesic properties	Miyoshi et al. (1995)
Milk protein (α-casein)	*Lactobacillus helveticus* CP790 proteinases	Tyr-Lys-Val-Pro-Glu-Leu	ACE inhibition properties	Murray and Fitzgerald (2007)
Egg protein (lysozyme)	Clostripain	Ile-Val-Ser-Asp-Gly-Asn-Gly-Met-Asp-Ala-Trp-Val-Ala-Trp-Arg	Antimicrobial activities	López Expósito and Recio (2006)

TABLE 9.1 Array of Bioactive Peptides and the Protein/Enzyme Combination Used for Their Production—cont'd

Protein Source	Enzyme Used	Peptide Sequences	Bioactivity	Reference
Milk protein (α-casein)	Chymotrypsin	[a]Gln-Lys-Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe; [a]Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-Leu	ACE inhibition; PEP inhibition; Antioxidant; Antimicrobial activities	Sistla (2013)
Flaxseed protein	Papain	Flaxseed protein hydrolysates, (sequence not determined)	ACE inhibition properties	Udenigwe et al. (2009)
Flaxseed protein	Ficin	Flaxseed protein hydrolysates, (sequence not determined)	Renin inhibition properties	Udenigwe et al. (2009)
Proteins from macroalga (*Palmaria palmata*)	Papain	Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala	Renin inhibition properties	Fitzgerald et al. (2012)
Bovine serum albumin	Papain	Ser-Leu-Arg	Renin inhibition properties	Lafarga et al. (2016)
Bovine serum albumin	Papain	Tyr-Tyr, Glu-Arg, Phe-Arg	ACE inhibition properties	Lafarga et al. (2016)
Milk protein (α-casein)	Pepsin	Tyr-Phe-Tyr-Pro-Glu-Leu	Antioxidant properties	Pihlanto (2006)
Milk protein (β-lactoglobulin)	Corolase PP	Tyr-Val-Glu-Glu-Leu	Antioxidant properties	Pihlanto (2006)
Milk protein (κ-casein)	Trypsin	Phe-Phe-Ser-Asp-Lys	Immunomodulatory properties	Gill et al. (2000)
Egg protein (ovolin)	Trypsin	Val-Tyr-Leu-Pro-Arg	Anxiolytic properties	Oda et al. (2012)
Plant protein (Rubisco)	Pepsin and pancreatin	[a]Rubiscolin-6 (Tyr-Pro-Leu-Asp-Leu-Phe); [a]Rubimetide (Met-Arg-Trp)	Opioid, blood pressure-lowering and anxiolytic properties	Zhao et al. (2008)

[a] *Multifunctional peptides.*
ACE, angiotensin converting enzyme; *PEP*, prolyl endopeptidase.

stearidonic acid (STA, C18:4n-3), eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3). DHA and EPA are the most widely studied because of their well-established health benefits (Mazza et al., 2007). The chemical structures and nomenclatures of DHA and EPA are presented in Fig. 9.2.

Oily fish is the major source of omega-3 fatty acids and generally contains a maximum of 30% DHA and EPA (Akanbi and Barrow, 2016, 2017; Akanbi et al., 2014; Wang et al., 2010).

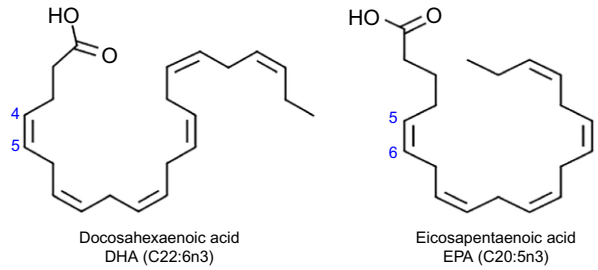

FIG. 9.2 Chemical structures of DHA (Δ4,7,10,13,16,19-docosahexaenoic acid) and EPA (Δ5,8,11,14,17-eicosapentaenoic acid). Numbers 4-5 (DHA) and 5-6 (EPA) mean the carbon number where the first double bond is located (counting from the carboxylic end).

Interestingly, the growing markets for nutritional supplements, food, and pharmaceutical have a high demand for high-quality DHA and EPA concentrates (Akanbi et al., 2013). Therefore, methods to produce concentrates of these omega-3 fats are industrially important. Industrial production of DHA and EPA concentrates involve fractional distillation and urea complexation techniques. However, these techniques are environmentally unfriendly and can cause partial oxidation and polymerization of these fatty acids (Shahidi and Wanasundara, 1998). Greener and milder techniques involving lipases are being applied as viable alternatives (Akanbi et al., 2013, 2014; Akanbi and Barrow, 2017). Therefore, in this section, the use of lipases to concentrate omega-3 fatty acids will be discussed.

9.3.3.1 Structure and Catalytic Behavior of Lipase Classes

Lipases are a group of enzymes with the ability to hydrolyze triacylglycerols (TAG) at the lipid water interface. They act at the interface and catalyze the hydrolysis of TAG to free fatty acids and glycerol (Akanbi et al., 2013; Haba et al., 2000; Hiol et al., 2000; Olusesan et al., 2011b). Because of their compatibility with a broad range of solvents, lipases can also catalyze a range of reactions (Akanbi and Barrow, 2017; Houde et al., 2004). These unique features separate them from the closely related hydrolytic enzymes, esterases (Fojan et al., 2000; Olusesan et al., 2011a).

The ability of lipases to catalyze the hydrolysis of fats or synthesize esters depends largely on their structures. All lipases, regardless of the source, have an α/β hydrolase fold as well as the helical portion known as the lid, which covers the enzyme's active site (Nardini and Dijkstra, 1999). As soon as contact is established between lipase and lipids under suitable conditions, the active site opens and hydrolysis begins. The amino acid residues at the active sites of most lipases are serine (Ser), aspartic (Asp), or glutamic (Glu) acid, and histidine (His) and these are always arranged in a catalytic triad-like configuration (Ollis et al., 1992; Van Pouderoyen et al., 2001). The catalytic triad of a *Bacillus subtilis* lipase and its secondary structure detailing its α/β hydrolase fold are shown in Fig. 9.3. Based on their active and binding site configurations, lipases can be divided into three groups as previously reported: (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (example, lipases from *Rhizomucor* and *Rhizopus*); (2) lipases with a funnel-like binding site (example, lipases from *Candida antarctica, Pseudomonas* and mammalian pancreas), and (3) lipases with a tunnel-like binding site (example, lipase from *Candida rugosa*) (Pleiss et al., 1998).

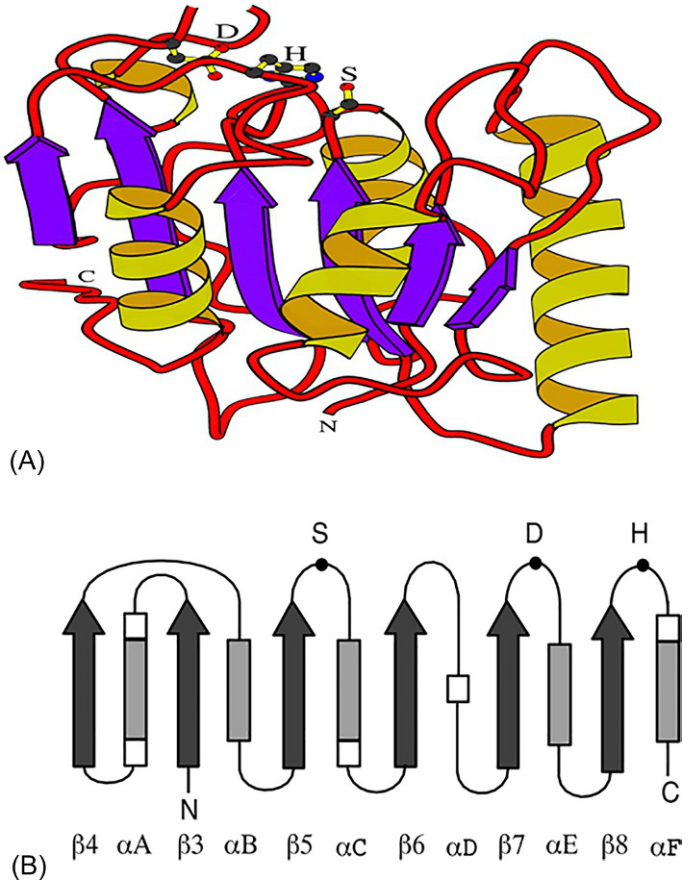

FIG. 9.3 Structure of *Bacillus subtilis* lipase showing its catalytic triads Ser77 (S), His156 (H) and Asp133 (D) and **(b)** secondary structure topology of its α/β hydrolase fold. *Reprinted from Journal of Molecular Biology, Volume 309, Issue 1, Van Pouderoyen, G., Eggert, T., Jaeger, K.-E., & Dijkstra, B. W., The crystal structure of Bacillus subtilis lipase: a minimal α/β hydrolase fold enzyme, 215-226, Copyright (2001), with permission from Elsevier.*

9.3.3.2 Lipase-Based Concentration and Stabilization of Omega-3 Fatty Acids

The most efficient method of omega 3 concentration by lipases is hydrolysis. Lipase-catalyzed hydrolysis of oil ensures partial removal of saturated and monounsaturated fatty acids while concentrating the less hydrolysable omega-3 fatty acids. To achieve this, the fatty acid and positional selectivity of lipases must be known. Lipases hydrolyze specific fatty acids at specific positions on the glycerol backbone of oil (Akanbi et al., 2013; Lanser et al., 2002). These positions are numbered using the stereospecific numbering (*sn*) system giving *sn*-1, *sn*-2, and *sn*-3. The majority of lipases can hydrolyze fatty acids on the outer parts of the glycerol backbone (i.e., *sn*-1 and *sn*-3 positions) and are therefore *sn*-1,3 regio-specific. Very few lipases can hydrolyze fatty acids in the middle (*sn*-2) position (Akanbi and Barrow, 2017; Baharum et al., 2003).

To concentrate omega 3 DHA and EPA from fish or other marine oils, the positional distribution of these fatty acids on glycerol backbone of the oil and the regioselectivity of lipases must be taken into consideration. Most fish oils have EPA and DHA predominantly located at the *sn*-2 position, and as such, an *sn*-1,3 lipase will be suitable for concentrating these fatty acids. Additionally, most lipases discriminate against polyunsaturated fatty acids based on their chain length and their multiple double bonds that are difficult to break. *Thermomyces lanuginosus* lipase (TL 100 L) has been used to concentrate EPA and DHA from anchovy oil (Akanbi et al., 2013). The relative amounts of these fatty acids increased in the acylglycerol portion of the oil as saturated and monounsaturated fatty acids were progressively removed. Carbon (^{13}C) nuclear magnetic resonance (NMR) analysis showed that both EPA and DHA were concentrated in all the three glycerol positions (i.e., *sn*-1,3 and *sn*-2) (Akanbi et al., 2013). Also, when this enzyme (TL 100 L) was treated with a protic ionic liquid, it concentrated both EPA and DHA from 30% to 52%, suggesting that solvent engineering could enhance the hydrolytic activity of lipases (Akanbi et al., 2012). With porcine pancreatic lipase, EPA plus DHA from seal and anchovy oils increased from 15% and 30% to 35% and 60%, respectively after partial hydrolysis (Akanbi et al., 2012). It was recently found that a combination of *Thermomyces lanuginosus* lipase (TL 100 L) and *Candida antarctica* lipase A (CAL-A) could be used to prepare highly pure (~90%) DHA concentrate from alga oil (Akanbi and Barrow, 2017). These results and others (Gamez-Meza et al., 2003; Kahveci and Xu, 2011; Wanasundara and Shahidi, 1998; Xia et al., 2017) have shown that lipases can be used to produce high quality omega 3 concentrates useful for food fortification and pharmaceutical applications.

Because omega-3 fatty acids are oxidatively unstable, research efforts have been intensified to solve this problem. One method of stabilizing omega-3 fatty acids is microencapsulation. Microencapsulation involves forming an outer shell that helps protect oil against oxidation. Multicore complex coacervation technique has been found to significantly stabilize fish oil against oxidation than spray drying because oil resides closer to the center of the particle and a thick outer shell is present providing better protection against oxygen penetration (Barrow et al., 2007; Wang et al., 2014; Wang et al., 2015). Lipases have also been used to synthesize antioxidants for use in oil stabilization (Medina et al., 2009; Pande and Akoh, 2016; Pereira-Caro et al., 2009; Tan and Shahidi, 2011; Wang and Shahidi, 2013; Warnakulasuriya and Rupasinghe, 2016).

9.3.3.3 Health and Food Application of Omega-3 Fatty Acids

Health benefits of omega-3 fatty acids, particularly EPA and DHA, have been widely reported. They have been found to control and regulate various cellular activities including the expression of an array of important genes in the body (De Caterina and Massaro, 2005). Because humans are unable to synthesize omega-3 fatty acids, they must be obtained through omega-3 rich diets or supplements (Mahaffey, 2004).

It has been reported that the consumption of omega-3 fatty acids, especially EPA and DHA, has helped reduce the prevalence of cardiovascular diseases such as arrhythmias (Albert et al., 2002; Din et al., 2004), atherothrombosis (Marchioli et al., 2002; Nambi and Ballantyne, 2006), inflammation (Mori and Beilin, 2004), and hypertension (Von Schacky and Harris, 2007). Research and clinical evidence shows that omega-3 fatty acids, especially EPA and DHA, help in the prevention of common cancers (Takahashi et al., 1993, 1997). Dietary omega-3 fatty acids were reported to have prevented non-melanoma skin cancer by significantly lowering the pro-inflammatory and immunosuppressive prostaglandin E synthase type 2 (PGE_2) levels in

human skin irradiated with ultraviolet B (Black and Rhodes, 2006). DHA is very important for the development of brains and eyes of infants (Amminger et al., 2007; Innis, 2007). It has also been shown that omega-3 fatty acids may help improve mood in people who are depressed (Conklin et al., 2007; Parker et al., 2006). Deficiency in EPA and DHA has been found to be one of the major causes of human mood disorders (Parker et al., 2006). A US study found that consumption of fatty fish was linked with a reduced risk of dementia and Alzheimer's disease (AD) (Huang et al., 2005). DHA has helped in preventing cognitive aging and dementia in humans (Whalley et al., 2008), and DHA and EPA have been found to lower plasma cholesterol and triglyceride levels thereby helping prevent obesity (Harris and Bulchandani, 2006). They have also been found to help in the treatment and prevention of arthritis (Covington, 2004; Miles and Calder, 2012).

Omega-3 fatty acid products account for a rapidly growing $25 billion industry (Dellinger et al., 2016) and is expected to continue to grow. Concentrates of omega-3 fatty acids are being sold as nutritional supplements around the world. Most infant formulas now contain omega-3 DHA, and consumer acceptance of these products is high. Omega-3 fatty acids are also being incorporated into foods such as bread, cereal products, spreads, milk, mayonnaise and salad dressings, and crackers and bars (Shahidi, 2004). Because these fatty acids are highly susceptible to oxidation, their application will be more successful in products that are consumed within a short period of time (Shahidi, 2004).

9.3.4 The Use of β-Galactosidases for the Production of Galacto-Oligosaccharide Prebiotics

9.3.4.1 Structure and Properties of β-Galactosidases

Among the hydrolytic class of enzymes, glycoside hydrolases are responsible for the hydrolysis of glycosidic bonds in carbohydrates, or between a carbohydrate and non-carbohydrate moiety. β-Galactosidases (β-D-galactoside galactohydrolase; EC: 3.2.1.23), a gene product of *lacZ* is one of the most well-studied carbohydrate-acting enzymes (Juers et al., 2012a). It is a member of family 1 of the glycoside hydrolases according to the database of CArbohydrate-Active EnZymes, CAZy (Lombard et al., 2014). It is an exoglycosidase that cleaves the β-bond of galactosides, thereby releasing galactose and another organic moiety. An ability to cleave α-L-arabinosides, β-D-fucosides and β-D-glucosides has also been reported for some β-galactosidase enzymes (BRENDA, 2017).

The sequence of β-galactosidase was first unraveled in *E. coli* (Fowler and Zabin, 1977). Functionally active β-galactosidase enzymes from *E. coli* is a 2,2,2-point symmetric homotetramer with each of the four subunits comprising of about 1023 amino acids and an overall molecular mass of 465 kDa (Bartesaghi et al., 2014; Jacobson et al., 1994). Each of the subunit contains five domains, and domain 3, and its α/β barrel structure, holds the active site at the C-terminal of the barrel (Matthews, 2005). The enzyme requires monovalents (Na^+ or K^+) or divalent (Mg^{2+}) ions for maximum activity, and the binding sites for one of the pairs of ions are located in the active site (Jacobson et al., 1994).

For the purposes of food application, β-galactosidase is sourced from yeast species such as *Kluyveromyces lactis* rather than from *E. coli*. Although β-galactosidase from *K. lactis* shares some structural similarities with that of *E. coli*, a comparison of active sites shows a difference for the determinants of reaction mechanism in these two species (Pereira-Rodríguez et al., 2012). For example, both dimeric and tetrameric forms of β-galactosidase are active in

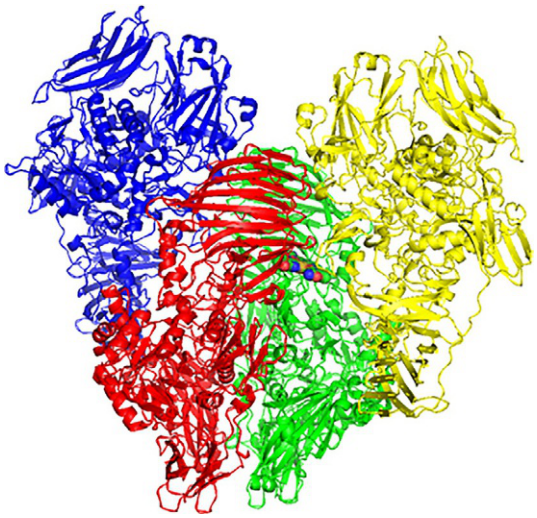

FIG. 9.4 Structure of β-galactosidase from *K. lactis* showing tetramer. Chain A is shown in green, B in red, C in yellow, and D in blue. Active site is shown by the ball and stick image. *Source: PDB 3OBA (Pereira-Rodríguez, Á., Fernández-Leiro, R., González-Siso, M.I., Cerdán, M.E., Becerra, M., Sanz-Aparicio, J., 2012. Structural basis of specificity in tetrameric Kluyveromyces lactis β-galactosidase. J. Struct. Biol. 177, 392–401).*

K. lactis, and each monomer has ~124 kDa (Becerra et al., 1998). Also, in *K. lactis*, residues from domains 1, 3, and 5 make up the active site. In addition to a Mn^{2+} in the active site which is thought to help stabilize interactions between different subunits, there are binding sites for one Mg^{2+} and two Na^{+} in the active site (Pereira-Rodríguez et al., 2012). The structure of β-galactosidase form *K. lactis* is shown in Fig. 9.4.

9.3.4.2 Mechanisms for the Production of Oligosaccharide Prebiotics

β-Galactosidase is able to perform two catalytic activities: the hydrolysis of lactose and the conversion of lactose to allolactose (Matthews, 2005). The mechanism of lactose hydrolysis in β-galactosidase from *K. lactis* is mediated by the Mg ions and two glutamic acid residues: Glu-482 and Glu-551. Glu-482 donates a proton to a glycosidic oxygen of lactose which results in a covalent bond between the enzyme and the sugar. Attack of the protonated lactose via a covalent bond at the galactose moiety by the nucleophilic Glu-551 results in the severing of the glucose residue. Finally, a water molecule releases the galactosyl residue from the enzyme and re-protonates the two glutamic acid residues (Bell et al., 2013; Matthews, 2005). Hydrolysis of lactose is the basis for the production of lactose-free milk for lactose intolerance, a condition that afflicts about 70% of the world's adult population. Lactose hydrolysis is also important for controlling undesired crystallization of lactose in dairy products such as ice cream and sweetened condensed milk. It also helps in the degradation of lactose in whey wastes into glucose and galactose, which have better sensory (sweetening) properties and solubility than lactose (Bell et al., 2013; Pereira-Rodríguez et al., 2012).

The conversion of lactose to allolactose (disaccharide of D-galactose and D-glucose linked through a β-1-6 glycosidic linkage) occurs through transgalactosylation mechanisms which

share similarity with the aforementioned hydrolytic scheme, except that in this case, glucose—and not water—is used as the acceptor in the final step (Matthews, 2005). Following breakage of the β-1-4 linkage of lactose, the hydroxyl group on C6 of glucose reacts with C1 of galactose to form allolactose indicating a direct transfer of galactose to the 6 position of free glucose (Huber et al., 1976; Juers et al., 2012b).

There is always a competition between the hydrolase and transgalactosylase activities of β-galactosidase as the direction of either of these pathways is dependent on several factors. For example, high lactose concentrations, use of β-lactose, high pH (>7.8), use of immobilized enzyme forms, and presence of Mg^{2+} ions, reduction of product inhibition by glucose have all been shown to increase transgalactosylase activities of β-galactosidase, leading to an increase in production of allolactose and oligosaccharides (Huber et al., 1976; Park and Oh, 2010).

9.3.4.3 *Applications of β-Galactosidase-Derived Oligosaccharide Prebiotics*

The transgalactosylation reaction of β-galactosidase is the basis for the production of galacto-oligosaccharides (Fig. 9.5), an important food ingredient. Galacto-oligosaccharides (GOS), also called oligogalactosyllactose or transgalactooligosaccharides, are a group of non-digestible fibers that are able to perform prebiotic function by stimulating the growth and activities of probiotic bacteria in the gut (Pereira-Rodríguez et al., 2012). Probiotic bacteria in the human gut have been scientifically recognized for their ability to promote human health and well-being (de Vrese and Schrezenmeir, 2008; Gorbach, 2000). The healthful role of gut bacteria such as bacteriodes, lactobacilli, and bifidobacteria has been widely reported. The role of probiotic bacteria in controlling certain central nervous system disorders has been linked to biochemical signaling described in the so-called microbiome brain-gut axis (Wang et al., 2016). In fact, the term psychobiotics has been coined to describe a new class of probiotics which, upon ingestion in adequate amounts, can provide antidepressant and anxiolytic functions that relieve some symptoms of psychiatric illnesses including depression and chronic fatigue syndrome (Dinan et al., 2013).

As prebiotics, GOS act as "food" for gut bacteria and gets fermented in the large intestine to produce other compounds, such as short-chain fatty acids, which alter the composition and/or activity of gut microflora (Roberfroid, 2000). These short-chain fatty acids (e.g., butyric acid, propionic acid, acetic acids) promote the health of colon mucosa cells and help control colon inflammation (Eswaran et al., 2013). Consequently, GOS are an attractive high-value prebiotic and functional food ingredient whose health benefits have been demonstrated in human clinical studies (Niittynen et al., 2007). There is also a growing industry dedicated to

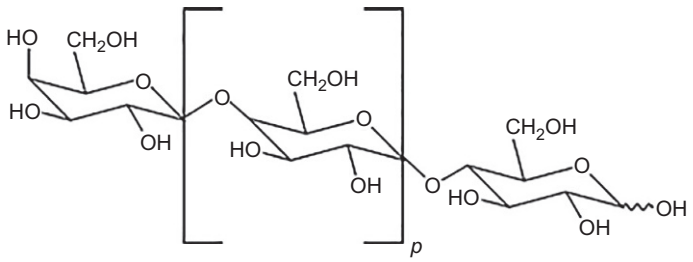

FIG. 9.5 Structure of a typical galacto-oligosaccharide; *p = 1 - 4. Adapted from Meyer, T.S.M., Miguel, Â.S.M., Fernández, D.E.R., Ortiz, G.M.D., 2015. Biotechnological production of oligosaccharides—applications in the food industry. In: Eissa, A.H.A. (Ed.), Food Production and Industry. InTech, Rijeka.*

the fortification of foods and supplements with probiotics designed to exert health effects in humans (de Vrese and Schrezenmeir, 2008). GOS are used as an ingredient in a wide variety of foods (infant formula, satietogenic foods, as low calorific sweeteners, powdered milk, etc.), as well as in cosmetic and pharmaceutical products (Park and Oh, 2010). The global market for GOS is expected to reach $1.7 billion by 2025 (Grand View Research, 2016b).

9.4 CONCLUSION AND FUTURE OUTLOOK

Research has shown the feasibility of using exogenous enzymes to produce functional foods/ingredients. Finding (novel) enzymes with unique catalytic properties is essential in order to develop functional foods. Also, techniques used for bioprocessing, purification, and fractionation should be advanced to improve the efficiency of enzyme production.

Various attempts have been conducted to isolate, purify, or concentrate the targeted functional ingredients or fractions coming from enzymatic reactions. Unfortunately, it is relatively more costly than the production of chemically synthesized ingredients. So far, the bioactivities of the isolated-reaction products obtained from enzymatic reactions, such as protease, lipase, and galactosidases, have been demonstrated in vitro. However, this finding needs further validation in vivo to understand the stability and functionality of these functional foods and ingredients during human digestion.

In the last decades, consumers have demanded more and more "natural" foods. To meet this demand, encapsulation technology has been extensively developed to protect functional ingredients from direct contact with other food components (such as minerals, protein, lipids, fiber, etc.) when embedded in food matrices to improve their stability during food preservation and processing, as well as to control their release during digestion. However, further studies are required to validate the in vivo bioactivity of these encapsulated ingredients, and more efforts should be spent to develop food compounds suitable as a substrate for gastrointestinal enzymes that can be converted to functional compounds during digestion and can be directly absorbed by the human body.

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CHAPTER

10

Enzymes as Additives in Starch Processing: A Short Overview

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10.1 INTRODUCTION

Enzymes are biocatalysts used to drive chemical reactions outside their natural localization. The use of biocatalysts as food additives and in processing raw materials into valuable products has been practiced for a long time. The 20th century success of isolating of enzymes from living cells paved the way for commercial production of these enzymes thus their wider application in the food industry (Fernandes and Carvalho, 2016; Fraatz et al., 2014; Graphame et al., 2015; Husain, 2017; Narra-Rodrigues et al., 2017). Processing food through the use of biological agents is a well-established historical approach. The earliest applications dates back to 6000 BCE—or earlier—with the brewing of beer, bread baking, and cheese making. Furthermore, starch, a polysaccharide, is present as a storage compound in the leaves, tubers, seeds, and roots of many plants. Several of these starch-containing plants have been domesticated and are important agricultural crops (e.g., corn, wheat, rice, potato, yam, cocoyam, and cassava). In addition to utilizing the aforementioned food crops as food for direct human consumption, the roots, tubers, and seeds are processed to harvest the starch. The starch is usually modified chemically or enzymatically to a wide variety of derivatives. Against this background, this chapter presents a review on the enzymes for food processing, starch, and starch-active enzymes, and enzymes as additives and their effects on starch processing. The process requires the crude enzyme and exogenous enzymatic production of syrups using flour of a carbohydrate source, as well as an extracted starch.

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00010-4

10.2 ENZYMES FOR FOOD PROCESSING

The enzymes used in the food industry are diverse due to their roles and abilities in the production of food and beverage products (Graphame et al., 2015; Sanromán and Deive, 2016). Based on the type of reactions they catalyze, enzymes are classified into 6 main categories: (1) oxidoreductases (dehydrogenases, reductases, or oxidases), (2) transferases, (3) hydrolases, (4) lyases, (5) isomerases (racemases, epimerases, cis-tran-sisomerases, isomerases, tautomerases, mutases, cycloisomerases), and (6) ligases (synthases) (Ako and Nip, 2015; Fernandes, 2010; Fraatz et al., 2014; Graphame et al., 2015; James et al., 1996). The activity of an enzyme depends on several factors such as temperature, pH, amount of substrate, and type of substrate. Depending on the processing conditions (pH and temperature), enzymes could have a higher efficiency even at low concentrations, and could considerably speed up production processes compared to other catalysts (Fraatz et al., 2014; Simpson et al., 2012; Van Oort, 2010). Even at low concentrations of around 0.1% or less of the product (Li et al., 2017; Ray et al., 2016), under mild conditions such as at low temperatures, their efficiency is high. In addition, enzymes work several times faster than other catalysts (James et al., 1996). The ability to immobilize them onto stationary support materials and reuse them can help to reduce costs in food processing (Simpson et al., 2012; Van Oort, 2010; Zen et al., 2017). Recent developments in industrial biotechnology, such as recombinant DNA, have paved the way for the introduction of genes into microorganisms from traditional sources that encode them into special vectors to produce enzymes with a higher degree of purity and yields at reduced costs (Zen et al., 2017). Furthermore, recent developments in fermentation technologies provide novel tailor-made enzymes to suit specific food processing applications. Therefore, enzymes produced using these technologies have superiority to traditionally produced enzymes in functioning under harsh processing conditions such as extreme pH and temperature (Van Oort, 2010). Because of these and some other advantages, enzymes are used in the food industry for numerous applications including production of beverages, baking, milling, as well as the manufacturing of dairy, egg, fish, meat, cereal, and confectionery products (Ermis, 2017; Fraatz et al., 2014; Graphame et al., 2015; Santelia and Lunn, 2017; Simpson et al., 2012).

According to previous researchers, approximately 260 different enzymes are available in the EU with around 91% obtained through fermentation (58% from fungi, 5% from yeasts, and 27% from bacteria) and 9% through extraction (3% from plant and 6% from animal) (Fraatz et al., 2014). Ako and Nip have reported recommended names, systematic names, and enzyme codes (EC) for some common food enzymes (Ako and Nip, 2015). According to EU enzyme database, there are about 15 different enzymes and their mixtures extracted from animal sources widely used in the food industry. These include catalase, thrombin, trypsin, chymotrypsin, elastase, carboxipeptidase, lactoperoxidase, lysozyme, pancreatin, phospholipase, chymosin (rennet), pepsin, and triacylglycerol lipase (EC, 2016; Madhu and Chakraborty, 2017; Ray et al., 2016; Simpson et al., 2012; Van Oort, 2010). Enzymes derived from animal sources like catalase, chymotrypsin, trypsin, peptidase, protease, pepsin, lipases, peroxidase, and chymosin could also be alternatively produced through fermentation (Ermis, 2017).

10.3 STARCH AND STARCH-ACTIVE ENZYMES

Starch is composed of amylose, a virtually linear glucose polymer in which the glucose residues are linked via α-1,4 glycosidic linkages, and amylopectin, in which most of the glucose residues are linked via α-1,4 glycosidic linkages with up to 5% α-1,6 linked side chains. These glycosidic linkages are stable at a higher and neutral pH but hydrolyze chemically at a lower pH. At the end of the polymeric chain, a latent aldehyde group, known as the reducing end, is present (Henrissat et al., 2001; Terrapon et al., 2017; Van Der Maarel et al., 2002). Amylose is a relatively small molecule ranging in size from several hundred to a few thousand glucose residues, containing one reducing and one non-reducing end. Amylopectin is a much larger molecule containing up to 100,000 glucose residues. It has one reducing and many nonreducing ends. Amylose and amylopectin are packed together in starch granules, the size and shape of which vary greatly among the different botanical sources (Romani et al., 2017; Tester et al., 2006). In principle, granules can be considered as relatively inert substrates that are only slowly degraded by enzymes. The amount of amylose in starch varies from almost nothing (waxy variants having almost 100% amylopectin), to 70% (high amylose corn variants) (Henrissat et al., 2001). Wild-type starches on average have 20%–25% amylose and 75% to 80% amylopectin. Enzymes, on the other hand, are organic catalysts which are proteinous in nature with a high degree of specificity and efficiency for the transformation of organic compounds through defined reaction sequences. These properties, together with the fact they are controlled by pH and temperature and readily inactivated when the reaction is complete, make them useful tools in the study of foods. While enzymes may cause deterioration of foods, they can also be used in food processing to produce particular products or to modify the characteristics of particular products (Barchiesi et al., 2017; Terrapon et al., 2017) Enzymes tend to increase the rate of reactions by decreasing the activation energy but do not alter the equilibrium constant (IFIS, 2005). A large variety of enzymes active toward amylose or amylopectin have evolved in nature. Basically, starch-active enzymes are divided into two major groups depending on their mode of action: (1) *exo*- and *endo*-acting hydrolases that hydrolyze the α-1,4- and/or α-1,6-glycosidic linkage using water, and (2) *glucanotransferases* that break an α-1,4- which show benefits compared to the commonly applied acid hydrolysis. In the 1970s, glucose isomerase was introduced for the production of high fructose syrups. Glucose *iso-merase* converts glucose into fructose, thus creating a product with a higher sweetness. Later, thermostable *α-amylase* and *pullulanase* were introduced for a faster and better hydrolysis of starch.

10.4 ENZYMES AS ADDITIVES ON STARCH AND THEIR EFFECTS

10.4.1 Debranching Enzymes on Starch

When debranching enzymes are applied on starch, α-1,6 linkages are broken down by enzyme action (Sarka and Dvoracek, 2017). Starch is the main storage of carbohydrate in plant organisms. Normal starch in granular form is generally composed of two types of molecules: amylose and amylopectin. Amylose is a linear (1,4)-α-D-glucan, although there is

evidence of a few 1,6 branches in some amyloses (Sarka and Dvoracek, 2017). Amylopectin is a branched structure containing both (1,4)-alpha-D linkages between D-glucose residues and (1,6)-alpha-D branch points. Waxy starches consist almost exclusively of amylopectin, a high molecular weight molecule (Murray and Phisarnchananan, 2014). The branches in amylopectin are arranged in a clustered structure that allows adjacent chains to form double helices (Bresolin et al., 2006; Dhital et al., 2017; Zeeman et al., 2010). The packing of these helices results in crystalline regions of the starch. The crystalline organization of the starch granule is linked to the amylopectin branch chain distribution profile, which is typical for the source of starch (Mouliney et al., 2011). Starch can be debranched at α-1,6 linkages by debranching enzymes (e.g., isoamylase and pullulanase) under specific conditions. These debranching enzymes serve as additives in starch processing. For waxy starches, only short linear chains are released when starch is debranched, and therefore these chains have a relatively narrower molecular weight distribution. Debranched starches are used in preparations of retrograded amylose-resistant starch and nanoparticles (Sarka and Dvoracek, 2017). Starch-based nanoparticles are used as fillers and reinforcing agents in polymer composites, carriers for drug delivery, barrier coating materials, and stabilizers in oil-in-water emulsions. Annealing and heat moisture treatment are often used to modify the physicochemical properties and digestibility of starch granules (Sarka and Dvoracek, 2017), but enzymes are most effective when added to modify physicochemical properties and digestibility of starch granules (Liu et al., 2015; Liu et al., 2016; Osuji and Okafor, 2013). Waxy starch is mainly used in food technologies (e.g., confectionary or bakery) where it minimizes retrogradation (Sarka and Dvoracek, 2017). Native starch is often extensively modified to achieve the rheological properties necessary for specific industrial applications (Ashogbon and Akintayo, 2014; Li et al., 2017), the 1,4-glucan-branching enzyme (GBE, EC 2.4.1.18) has been shown to catalyze the intramolecular or intermolecular transglycosylation of starch molecules through the cleavage of 1,4-glycosidic bonds and subsequent transfer of the cleaved oligosaccharide to create −1.6 branches (Kajiura et al., 2011; Mukerjea et al., 2013; Li et al., 2017). Thus, the glucan-branching enzyme decreases the amylose content of starch and produces shorter branches within a more highly branched structure (Li et al., 2016). A higher proportion of short-chain amylopectin results in a greater decrease in the viscosity of starch, and a lower amylose content means a better structured network (Ai and Jane, 2015; Li et al., 2017; Yuan et al., 2008). Enzymatic modification of cassava starch with a glucan-branching enzyme always results in significant changes in its molecular structure and rheological properties. Selective and particular attack of glucan-branching enzymes on starch granules creates broken and larger openings. The action of glucan-branching enzymes on starch granules produces amylopectin with higher proportions of short chains and stimulates the amylose recrystallization process. Enzymatic modification with the glucan-branching enzyme increases the shear resistance, fluidity, and elastic characteristic of cassava starch paste (Bai et al., 2017; Ding et al., 2017; Li et al., 2017). The viscosities of modified starch pastes do not respond to temperature changes during processing and storage when compared to those of native pastes (Li et al., 2017). Because starch is an important food additive due to its thickening and gelling properties, it helps provide proper texture and controls moisture mobility, thereby improving the quality and stability of processed foods (Ahmt et al., 2004; Dang et al., 2009). The rheological properties of starch products are the major functional properties that govern their use in food processing. Rheological properties are closely related to the quality of starch-based foods (e.g., hardness,

stickiness, and chewiness), as well as being crucial in processing-related parameters such as transportation, agitation, mixing, and energy consumption (Jiang et al., 2003; Jiang et al., 2015; Liu et al., 2009). The key rheological characteristics of starch pastes include their flow behavior, their viscoelastic properties, etc. Starches with different molecular conformations and structures exhibit different flow behavioral patterns and deformation characteristics in response to applied stress (Ai and Jane, 2015). The rheological properties of starch are very sensitive to several factors, such as temperature, starch concentration, pH, and the presence and concentration of other components (gums, proteins, salts, and acids). In particular, the amylose/amylopectin ratio, molecular weight, and the chain length distribution of starch have tremendous effects on its rheological behavior (Choi and Yoo, 2008; Moran et al., 2011). By means of enzymatic modification using the glucan-branching enzyme as an additive, the production of cassava, yam, potato, and cocoyam starch-based products with desirable rheological properties is feasible.

Rapidly digestible starches can be modified to be resistant to enzymes (Jochym and Nebesny, 2017; Kamila and Ewa, 2017). Starch is generally consumed in gelatinized form, which is readily and quickly digested (Raigond et al., 2015). This type of starch is known as rapidly digestible starch (RDS). If present in high proportions in food, it will rapidly release glucose to the blood, elevating both its levels and insulin response, which is detrimental to health. RDS is associated with obesity, Type 2 diabetes, and cardiovascular disease (CVD) risk. This problem could be overcome by changes in dietary habits, such as increasing resistant starch consumption. The American Association of Cereal Chemists and the Food Nutrition Board of the Institute of Medicine of the National Academies have defined resistant starch (RS) as a type of dietary fiber (DF). The process of developing new fiber formulations has resulted in increased interest in the chemical modification of starch, which could be a source of Type 4 resistant starch (RS4). RS4 is obtained by applying standard methods of chemical modification of starch such as cross linking and substitution (Sang and Seib, 2006; Xie et al., 2006). Similarly, products of starch dextrinization, conducted under specific conditions, must result in starch that is resistant to enzymatic digestion (Jochym and Nebesny, 2017). If enzymes are added to process such starches during digestion, it becomes resistant to all enzymatic actions (Jochym and Nebesny, 2017). When starch is chemically modified, it becomes resistant to enzymatic digestion because of the formation of steric hindrance at the site of enzymatic action. On the other hand, in the course of starch dextrinization, 1,2- and 1,3-glucosidic bonds are generated at the expense of 1,4- and 1,6-glycosidic bonds, which are characteristic of starch. This is the result of complex processes occurring under the influence of temperature and acid catalysts on the starch, including depolymerization, transglucosidation, and repolymerization. The formation of new bonds makes dextrins less susceptible to the activity of digestive enzymes by reducing the number of targets for potential attack (Jochym and Nebesny, 2017).

10.4.2 Enzyme as Additives in Bread Dough

Application of combined enzymes on bread dough affects its fundamental rheological parameters with a high content of resistant starch (RS). Dietary fiber provides health benefits such as the decrease of intestinal transit time, increase of stools bulk. Being fermentable by colonic microflora, fiber also reduces total and/or LDL cholesterol levels of the blood and reduces the post-prandial blood glucose levels (FAO/WHO, 2009). This makes dietary fiber

an interesting ingredient for the development of functional foods in response to the epidemic of noncommunicable diseases like cardiovascular diseases, cancer, and diabetes (WHO, 2011). Resistant starch (RS), which is not digested, allows fermentation in the colon which can be considered a kind of dietary fiber. Four types of RS have been described: (1) RS_1, that is physically inaccessible to digestion such as the starch found in grains or seeds; (2) RS_2, one whose granules are structured in a way that does not allow enzymes to hydrolyze it; 93) RS_3 which is the retrograded starch formed when foods are cooked and cooled; and (4) RS_4, which is chemically-modified starch (Dura and Rosell, 2017; Fuentes-Zaragoza et al., 2010). High amylose maize starch, defined as RS_2, is a fine white powder, obtained from a specific hybrid of corn naturally rich in amylose. Adding it to bread dough dilutes the gluten yielding dough with poor rheological properties and baking performance (Dura and Rosell, 2017; Ruiz-Rodríguez et al., 2015; Sanchez et al., 2014; Verdú et al., 2017). Production of bread with poor textural properties limits the application of RS_2 in bread making (Almeida et al., 2013; Altuna et al., 2016; Dura and Rosell, 2017; Liu et al., 2015). To de-limit the application of RS_2 in making bread, additives such as enzymes are used to minimize these effects. Enzymes transglutaminase (TG), glucose oxidase (Gox), and fungal xylanase (HE) have a wide application in the bakery industry. TG is a strong protein cross-linking enzyme, improving dough strength and bread volume (Dura and Rosell, 2017) Glucose oxidase catalyzes the oxidation of glucose to gluconic acid with simultaneous formation of hydrogen peroxide (Altuna et al., 2016; Bankar et al., 2009). Hydrogen peroxide is capable of oxidizing free sulfhydryl groups forming disulfide bonds within the gluten network, resulting in its strength enhancement (Dura and Rosell, 2017; Miao et al., 2014). Fungal xylanase breaks down the hemicellulose in wheat flour helping the redistribution of water and leaving the dough softer and easier to knead (Altuna et al., 2016). When studying bread dough, rheological measurements (fundamental or empirical and of large or small deformation) constitute an important approach which can be correlated to bread quality as reported by many researchers (Dobraszczyk and Salmanowicz, 2008). Empirical measurements are the most used in the bread industry, but the accuracy depends on the type of equipment employed in the collection of data. Otherwise, fundamental measurements provide physical parameters like force, deformation, torque, energy, and the results are independent of the test equipment and can theoretically be used to model the flow conditions encountered by the dough during mixing, proofing and baking (Altuna et al., 2016). Small deformation tests provide fundamental parameters, but they are not directly related to the baking process in which the dough is submitted to large deformation. During kneading, dough is stretched and stressed, and a small amount of air is occluded in the dough, forming small spherical gas cells whose size increases during the fermentation stage in which part of the carbon dioxide produced by the yeast migrates into them. For that reason, researchers have proposed approaches that allow obtaining fundamental parameters in large deformation tests (Dunnewind et al., 2004). Dobraszczyk (2003) suggested that existing studies show better relationships between rheological properties with large deformation extensional and relaxation properties and baking performance. In one study, enzymes transglutaminase, glucose oxidase, and fungal xylanase were added to bread dough with RS in different concentrations and their baking performance was found to be comparable to regular dough without RS (Altuna et al., 2016; Dura and Rosell, 2017). Addition of resistant starch to dough without enzymes added reduces its expansion during fermentation, while dough formulated with RS and enzymes could circumvent this undesirable effect (Altuna et al., 2016).

Application of enzymes in dough improves the rheological behavior of it with or without resistant starch involved.

10.5 ENZYMATIC PRODUCTION OF SYRUPS

Most enzymatic starch conversion processes start with heating water-starch slurry to disrupt the granular structure and bring the two glucose polymers (amylose and amylopectin) into solution. The starch granule itself is more or less insoluble in water. However, upon heating, the granules take up water, resulting in a swelling and increase in viscosity. Continuation of heating results in the disruption of the granules and a release of the amylose and amylopectin. During cooling, the free amylose and amylopectin side chains start to interact, forming a strong network resulting in increased viscosity. A viscous (sticky) solution is formed (Nweke and Abiamere, 2014; Parker et al., 2010; Poul, 2016; Zainab et al., 2011). Finally, a white, opaque gel is formed. The interaction in this gel is so strong that the gel has become thermo-irreversible to the extent that it cannot be brought into solution again by heating. Starch hydrolysis/degradation occurs in three stages, namely, gelatinization, liquefaction, and saccharification. Gelatinization is the swelling and bursting of starch granules in a hot aqueous solution. The starch molecules set free into this viscous solution are more easily attacked than ungelatinized starch by amylases. The degree of viscosity depends on the extent of water uptake which varies for different starch sources. Liquefaction is the reduction of viscosity of the gelatinized starch by α-amylase. The long chain composed of glucose in starch (amylose and amylopectin) is very rapidly broken open to form smaller chains by alpha amylase. This causes a very rapid reduction in the viscosity of the gelatinized starch. Beta amylase can only slowly degrade the long chains from the non-reducing end. Saccharification is the complete degradation of starch to simpler sugars such as glucose, maltose, maltotriose, and to some extent, dextrins. An iodine test is a qualitative test used to determinw complete hydrolysis of starch. α-Amylase progressively breaks open the chains of amylose and amylopectin to form dextrins containing 7–12 glucose residues. Beta amylase splits off 2 residues (maltose) from the new nonreducing ends produced on these smaller chains. This process inevitably takes longer than the splitting open of the longer chains by α-amylase. Because of the differing lengths of the chains, other sugars such as glucose and maltose are produced as well as maltotriose. In all cases for different carbohydrate sources, saccharification breakdown stops at 2–3 glucose residues away from the α-1,6 bonds in amylopectin because neither amylase nor α-amylase can break down α-1,6 bonds. These limit dextrins are always present in normal syrups and worts except when pullulanases or limit dextrinases are used as an exogenous enzyme during hydrolysis to breakdown 1,6-glucosidic bonds (Nweke and Abiamere, 2014; Parker et al., 2010; Poul, 2016). Some of the enzymes used in starch hydrolysis include α-amylase, β-amylase, amyloglucosidase, pullulanase, and glucose isomerase. The final composition of syrup is solely dependent on the type/nature of enzymes or combination of enzymes and the quality of the raw material used during the syrup production process (Regy and Padmaja 2013; Spinosa et al., 2016). The degree of starch hydrolysis can be monitored or qualified using 0.02 N tincture of iodine (a solution of iodine and potassium iodide in alcohol). This examination is called an iodine test and it is always performed on a cool sample. It is based on the fact that an iodine solution gives a blue-to-black color at room temperature if

starch and large dextrins are present. On the other hand, when iodine is added to the sample and the yellow iodine color remains without being discolored, it shows that starch and large dextrins are no longer present. At this point, the syrup sample is iodine normal, showing that the sample is full of sugars and smaller dextrins (limit dextrins) (Osuji and Okafor, 2013). This means that the sample has been completely hydrolyzed and saccharification is completed. Fig. 10.1 shows a simplified flow chart for the syrup production process.

10.5.1 Crude Enzyme Production of Syrups

Enzymes are integral raw material used in syrup production. They can be categorized as endogenous and exogenous enzymes. The endogenous enzymes, also known as immobilized enzymes/inherent enzymes, are enzymes that are present naturally in the cell/tissue of the food material, while exogenous enzymes are external enzymes that are incorporated into the food material of interest as additives. The endogenous enzymes present in rice and sorghum are developed by malting (Narra et al., 2016; Narra-Rodrigues et al., 2017). For instance,

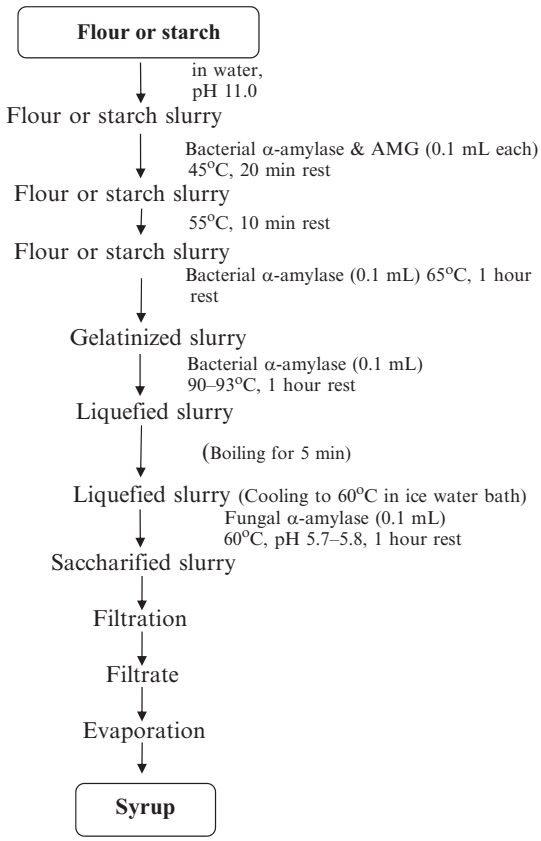

FIG. 10.1 Syrup production process.

crude enzymes have been developed from grains (rice, millet, sorghum) using standard barley malting protocol (Imbadu, 2010; Ubalua, 2016). The malted grains containing activated enzymes can be used in syrup production as crude enzyme additives on starch. Starch, on the other hand, is the major raw material for syrup production. In the United States and in many other parts of the world, extracted starch is the predominant source of syrup production. Although starch extracted from corn and other plant sources have been effectively used in syrup production, there is no information on the use of whole flour or crude starch (nonstarch food components inclusive) of any food material in syrup production. The starch-modification process, with the aid of an enzyme, yields hydrolysates, which may include glucose and combination of other sugars such as maltose and maltotriose. However, the extent of the modification depends on the degree of hydrolysis and the nature of enzymes (Fernandes and Carvalho, 2016; Spinosa et al., 2016). The product (syrup) is purified and clarified using simple unsophisticated techniques that can easily be monitored by untrained hands. In this chapter, results of recent investigation on syrup production from flours of tubers such as cassava, cocoyam, potato, and yam, and from flours of malted and unmalted rice, are presented (Ramasamy et al., 2014; Sanromán and Deive, 2016).

10.5.2 Sugar Syrup Production Using Exogenous Starch-Degrading Enzymes on Tubers

It is important to properly understand the various properties of syrups from tropical tuber (cassava, potato, yam and cocoyam) flours and how some of these properties may vary among different tuber varieties and treatments. The physico-chemical and sugar spectra of the resultant syrup from different tubers are shown in Table 10.1. The pH of the syrup produced from various tubers ranged from 4.65 to 5.59. The pH is one of the main quality characteristics that describe the stability of bioactive compounds in food products. Foods at such pH levels are medium-acid foods and have longer shelf-life than foods above this range, which favor microbial activity. However, there is a relationship between the dextrose equivalent (DE) of the syrups and viscosity. As the DE of syrup increases, the viscosity decreases. The reduction in the viscosity of the syrups corresponds to the reduced amount of total solids present in the syrup which is evident in the higher sugars (Hull, 2010; Regy and Padmaja, 2013; Zainab et al., 2011). The increase in DE will also increase the risk of dextrose crystallization. The variation in the viscosity of the syrups is principally due to varying solid contents and, to a lesser degree, varietal differences and type of treatments. This shows that high-molecular-weight components in syrups, including higher sugars and/or dextrins, contribute significantly to the flow behavior of syrups. Furthermore, the main factors affecting the viscosity of solutions are: (1) the nature of the continuous and the dispersed phases, (2) particle-particle and particle-solvent interaction, (3) concentration, (4) shape, (5) particle size, and (6) temperature (Fernandes and Carvalho, 2016; Narra-Rodrigues et al., 2017). As the percentage of higher sugars increases, the particle-particle interaction and particle-solvent interaction also increases, thus increasing the syrup viscosity. On this note, the viscosity of syrups reduces with the reduction in the level of higher sugars.

The sugar spectra of syrups yield different concentrations of the various sugars probably because of the nature of its carbohydrate sources and the type of enzyme used during hydrolysis. It is very important to note that the quality and type of syrup depends solely

TABLE 10.1 Physico-Chemical and Sugar Spectra of Syrup Produced From Tuber Flours Using Exogenous Enzymes

Samples	Dextrose Equivalent (%)	pH	Viscosity (cp)	Maltose (%)	Glucose (%)	Maltotriose (%)	Sucrose (%)	Raffinose (%)	Stachyose (%)	Higher Sugars (%)
Unsprouted yam[a]	26	4.65	9800	11	18	5	0.38	0.15	1.05	66
Sprouted yam[a]	26	4.65	9800	15	17	7	0.27	0.13	0.95	60
Cassava *Nwocha*[a]	40	5.56	6500	20	14	9	1.99	0.05	1.05	55
TMS 30,572[a]	37	5.54	6900	15	15	7	1.58	0.95	0.95	61
NR(80)84[a]	37	5.59	6800	16	14	7	1.95	0.09	1.09	60
Potato yellow[b]	70	5.1	2200	10	60	8	2.82	0.82	0.82	27
Potato white[b]	80	5.5	1500	10	63	6	3.05	0.93	1.30	16
Cocoyam[a]	50	5.2	5500	48	10	15	1.09	0.79	1.00	28

[a]*Double enzyme application (bacterial α-amylase and fungal α-amylase).*
[b]*Double enzyme application (bacterial α-amylase and amyloglucosidase).*

on the nature and type of enzyme utilized especially during saccharification (Hull, 2010). If amyloglucosidase (glucoamylase) is used as a saccharifying enzyme, syrups with higher DE (70-95DE), higher percentage of glucose with lesser maltose, and lesser maltotriose will be obtained. But if fungal α-amylase is used as a saccharifying enzyme, syrups with moderate DE (42-55DE), a lesser percentage of glucose with higher maltose and maltotriose will be obtained. The lower the DE of some tubers, such as yam and cassava, when compared to that of the other tubers, is as a result of varietal differences. This shows that the nature and type of carbohydrate material contributes to the quality of the syrup sugar, irrespective of whether the hydrolysis is complete or not. This is seen in the compositions of sprouted and un-sprouted yam. With continual variation in the carbohydrate materials being hydrolyzed, syrups produced will definitely have different compositions and DE because of the nature of its starch network. For some tubers, the starch packing may be such that amylase hydrolysis is enhanced by having more accessibility to the substrate while some starch may compose more of amylose starch than amylopectin, thereby resulting in higher DE.

10.5.3 Sugar Syrup Production Using Crude Enzymes on Tubers

The application of crude enzymes (malted grains) to hydrolyze starches and flours is well known in brewing and beverage production, but their application in syrup production is yet to be elucidated. This application of crude enzymes has been practiced successfully using sorghum and rice malt produced in the tropics (Stevnebo et al., 2006). The amylase activities varied with the different treatments and tubers. As mentioned in Table 10.2, the pH of syrups from crude enzyme-hydrolyzed tubers is in the range of 4.30 to 5.50, and they are all in the medium acid region irrespective of the nature of substrate (whether flour or starch sources). Syrups from flours have a higher pH than corresponding syrup from starches. This is because of the alkaline nature of syrups from flours as a result of nitrogenous co-extractives in the form of proteins and amino acids that make up the entire flour component, resulting in higher pH (Gibbah et al., 2009; Imbadu, 2010). As stated earlier, the relationship between DE, viscosity, and higher sugars cannot be overemphasized. The higher percentages of higher sugars from crude enzyme-hydrolyzed flours and starches are an indication of minimal hydrolysis. The minimal hydrolysis results in higher viscosity due to increased concentrations of higher molecular weight compounds in the syrups, having significant particle-particle and solvent-particle interaction, thus the lower DE. Moreover, when crude enzymes are used in hydrolysis of any carbohydrate material, the viscosity of the resultant syrup tends to increase because of the presence of starch as co-extractives in the crude enzyme flours (malted rice and sorghum). The presence of starch in the slurry increases the gelatinization of the slurry/medium, resulting in an increase in the syrup viscosity.

10.5.4 Sugar Syrup Production Using Exogenous Enzymes on Malted and Unmalted Rice

Like tubers, cereals from malted or unmalted cereal flour can also be used for syrup production. When hydrolyzed, the unmalted flours always have higher DE than the malted flours. This is due to malting losses which would have reduced the hydrolyzable sugars in the malted samples. The higher DE in unmalted samples is supported by the higher presence

TABLE 10.2 Typical Physico-Chemical Properties and Sugar Spectra of Syrups Produced From Tubers Using Crude Enzyme Conversion

	Flour								Starch							
Samples	**DE (%)**	**Viscosity (cp)**	**pH**	**Maltose (%)**	**Glucose (%)**	**Maltotriose (%)**	**Sucrose (%)**	**Higher Sugar (%)**	**DE (%)**	**pH**	**Viscosity (cp)**	**Maltose (%)**	**Glucose (%)**	**Maltotriose (%)**	**Sucrose (%)**	**Higher Sugar (%)**
Malted sorghum on cocoyam	22	10,000	5.5	8	8	6	9	69	35	4.50	7500	23	19	6	6	46
Malted rice on cocoyam	20	10,000	5.1	9	7	4	9	71	34	4.90	7800	21	18	6	7	48
Malted sorghum and rice on cocoyam	30	8500	5.5	12	8	10	9	61	40	4.50	6800	23	17	6	6	48
Malted sorghum on yam	28	9000	5.3	9	6	7	8	70	36	4.30	6550	17	15	11	5	52
Malted rice on yam	25	9300	5.2	9	6	5	7	73	32	4.70	8000	15	13	12	7	53
Malted sorghum and rice on yam	33	7800	5.3	11	9	8	8	64	41	4.30	6700	17	15	11	5	52

Malted sorghum on cassava	25	9300	4.7	10	7	6	5	72	35	4.30	7200	16	14	8	9	53
Malted rice on cassava	22	9600	4.9	10	5	4	6	75	33	4.90	7800	15	14	9	6	56
Malted sorghum and rice on cassava	32	8100	4.7	12	8	7	5	68	43	4.30	6600	16	14	8	9	53
Malted sorghum on potato	26	8500	5.3	10	6	5	6	73	38	4.30	7000	20	11	12	8	49
Malted rice on potato	21	9600	5.0	9	5	3	6	77	36	4.30	7500	22	17	10	9	42
Malted sorghum and rice on potato	35	8000	5.3	12	9	8	6	65	46	4.30	6300	22	15	11	8	44

of glucose in unmalted samples (Brunson et al., 2013; Khattab and Kodaki, 2016). Using rice varieties to illustrate the higher DE of 306 when compared to other rice varieties is due to the nature of its starch composition (Table 10.3). The starch packing may have been such that amylase hydrolysis is enhanced by having more accessibility to the substrate (Khattab and Kodaki, 2016). It is also possible that the starch of 306 is composed more of amylose starch than amylopectin. The amylose is known to be more completely hydrolyzed than the amylopectin. This is because amylopectin hydrolysis is limited by the occurrence of the beta-limit dextrin (Osuji and Anih, 2011, Sanromán and Deive, 2016). Syrups with a DE >38% is mostly used in beer brewing because of its high content of fermentable sugars. It helps to increase the productivity and improve the original extract. Dextrose Equivalent (DE) is a measure of the amount of reducing sugars present in a sugar product, relative to dextrose (glucose), expressed as a percentage on a dry basis. Syrups are given a DE number based on the degree of starch breakdown. Low DE syrups contain lots of higher sugars and lots of higher sugars mean high viscosity. High DE syrups, on the other hand, contain fewer high sugars and are therefore less viscous. High DE syrups contain lots of reducing sugars, and reducing sugars mean both sweetness and color reactions.

As mentioned in Table 10.3, if the DE of syrup ranges from 40% to 55%, the most abundant sugar is always maltose. On the other hand, if the DE ranges from 60% to 97%, the most abundant sugar is always glucose. This is because DE describes the degree of starch conversion to glucose. When two glucose molecules are joined together, as in maltose, one of the C_1 positions will no longer be available because it is joined to the next molecule at the C_4 position. This means that whilst there are two glucose molecules joined together, only one reducing site is available, therefore the reducing power of maltose is only about half that of glucose. Similarly, where there are three glucose units joined together, as in maltotriose, the reducing power is only about one-third that of glucose. Basically, as the number of glucose molecules in the glucose chain increases, the reducing power of the sugar is lessened and the DE reduces accordingly. On this note, reduction in DE of malted samples is attributed to the combination of the amount of maltose and maltotriose produced in the malt and wort. This implies that increase in the glucose chain of any sugar molecule decreases the reducing power of the sugar, thus reduces the DE of the sugar. It is important to note that syrups from unmalted samples had a higher DE, higher glucose concentration, lower maltose concentration, and lower maltotriose concentration when compared to their corresponding sugar syrups from malted samples. Malting generally causes a one-fold increase in maltose,but causes reduction in glucose and sucrose. The reduction in glucose is about 50% and the reduction in sucrose is 75%. This implies that maltose is probably generated during the malting action and also by hydrolytic enzymes during hydrolysis. Unlike in the unmalted samples, where glucose and sucrose were being used up in the malting process to sustain the malt metabolism. Sucrose is a natural component of grains and it is not produced during malting or during hydrolysis. In addition, sucrose and glucose are major soluble sugars found in a physiologically matured kernel. The germination process would have depleted the naturally occurring glucose and sucrose (Gajendragadkar and Gogate, 2016; Webster, 2016). The high maltose concentration in the syrups could be due to the combination of bacterial α-amylase and fungal α-amylase enzymes used in the production of these syrups. Bacterial α-amylase enzyme randomly attacks gelatinized starch at 1,4-linkages to produce glucose and maltose but it is unable to hydrolyze the 1,6-linkages. However, fungal α-amylase attacks the 1,4-linkage of liquefied

TABLE 10.3 Syrups Produced From Malted and Unmalted Rice Using Exogenous Enzymes

Sample	Syrup	Viscosity (cp)	pH	Glucose (%)	Maltose (%)	Maltotriose (%)	Sucrose (%)	Raffinose (%)	Stachyose (%)	Higher Sugars (%)
Malted 306	42DE	6500	4.70	4.00	62	9.56	0.75	0.26	1.08	22.35
Unmalted 306	52DE	5300	4.80	6.13	36	0.26	3.13	1.58	1.58	51.32
Malted Brown rice	40DE	6700	4.50	4.50	67	9.66	0.56	0.27	1.07	16.94
Unmalted Brown rice	51DE	5300	4.60	5.87	37	0.29	3.46	1.63	1.69	50.06
Malted Dragon 12	42DE	6600	4.60	4.80	60	10.23	0.73	0.22	1.08	22.94
Unmalted Dragon 12	50DE	5500	4.80	6.05	28	0.24	2.88	1.64	1.68	59.51
Malted FARO 44	39DE	9200	4.60	5.20	65	11.21	0.56	0.20	0.12	17.71
Unmalted FARO 44	50DE	5400	4.80	6.01	35	1.15	2.86	1.54	1.86	51.58

starch to produce predominately maltose, with lesser amounts of glucose. This provides an explanation for the high maltose concentration as compared to other sugars produced in the syrup. The high maltose concentration in the syrup is also due to the ratio of amylose and amylopectin in the starch because high amylose cultivars apparently produce maltose syrups containing more maltose (Abbes et al., 2011; Choi and Yoo, 2008; Husain, 2017; Jia et al., 2017; Mishra et al., 2017).

The reduction in raffinose content occurs during the malting and mashing process leading to degradation of macromolecules. This explains why there is little or no reduction in raffinose content for the unmalted samples. On the other hand, the reduction in sucrose concentration is as a result of transformation of sucrose by cross-linking with raffinose and stachyose. Raffinose is a trisaccharide composed of galactose, glucose, and fructose unit while stachyose is a tetrasaccharide consisting of glucose, fructose, and two galactose units. They are called α-galactosides, and are found in legumes and whole grains.

10.6 CONCLUSION AND FUTURE OUTLOOK

The use of enzymes in food processing has enormous benefits. It has improved food processing and brought it to a higher pedestal with ease. The complete breakdown of the α-1,6-glucosidic linkage of starch structure and hydrolysis of cell wall macromolecules are feasible by means of enzymes as additives in starch processing. Application of cell-wall-degrading enzymes on legumes results in not only hydrolysis but also the transformation and cross-linkages of the hydrolyzed sugars. However, it has been established that the type of syrup produced depends on the quality of the carbohydrate material and nature or type of enzymes used. Malting improves the hydrolysis and modification of starchy endosperm leading to the production of greater syrup yield and a higher carbohydrate profile. Malting generally increases the maltose concentration of sugar syrups produced from different locally available rice varieties to above 50%. Therefore, such syrups, having maltose as the predominant sugar with a concentration of 50%, are regarded as "High Maltose Syrup." Higher DE syrups give rise to fewer higher sugars and fewer viscous syrups. Exogenous (commercial) enzymes have a higher degree of hydrolysis than the crude enzymes from germinated cereals because of the presence of co-extractives that become part of the crude enzyme flour. There is insufficient hydrolysis when crude enzymes are used, although there is enhanced hydrolysis when crude enzymes from different sources (rice and sorghum malt) are combined. Due to developments in biotechnology and numerous advantages of microbial enzymes, the use of genetically modified microorganisms has produced the majority of enzymes with diverse characteristics and applications in food, pharmaceutical, textile, detergent, and cosmetic industries. Furthermore, future work should focus on the isolation, purification, and characterization/identification of the transformed sugars of enzyme-hydrolyzed sugars of legumes, especially soymilk. The identified cross-linked sugars could be used in calibrating standard analytical equipment for proper quantification of hydrolyzed sugars of soymilk. With the increase in the popularity of foods geared toward specific uses, supplemental enzymes can be used to help make the nutrients in these foods more bio-accessible. Syrups produced from flours (starch and other co-extractives) should be further analyzed qualitatively and quantitatively for vitamins, minerals, and amino acid profile in them. Unlike resistant starch, which

becomes a beneficial food source for intestinal bacteria, the products from bacterial fermentation of protein can produce toxins such as urea, phenolics, and branch-chained fatty acids that are very harmful. Selected proteases can be added to protein drinks or ingested with the drinks to ensure that the proteins are completely broken down in the small intestine. Supplemental enzymes can also be used to enhance the effect of herbs and botanicals.

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CHAPTER

11

Lysozyme: A Natural Antimicrobial Enzyme of Interest in Food Applications

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11.1 INTRODUCTION

Lysozyme, also called muramidase, is a type of glycanhydrolase that hydrolyzes the β-1,4-linkages between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid residues in the peptidoglycan of bacterial cell walls (Yang et al., 2011). Lysozyme is mostly found in saliva, tears, blood serum, human and cow milk, and avian egg whites (Wu et al., 2015), and to some extent in certain bacteria and bacteriophages. Furthermore, as indicated in Table 11.1, some plants also possess this enzyme (Liburdi et al., 2014). In terms of its action, it is a well-known fact that lysozyme is bactericidal against Gram-positive bacteria. However, against Gram-negative bacteria, it is essentially ineffective because of the presence of the outer membrane, which contains lipopolysaccharides (Lucera et al., 2012). Nonetheless, several methods are available to enhance the capability of lysozyme to lyse Gram-negative bacteria, including modification of the enzyme by attaching it to other compounds, bringing about the denaturation of the enzyme, using membrane-permeabilizing agents together with the enzyme, or by using the enzyme in combination with a pulsed electric field (Liburdi et al., 2014). This enzymatic protein was discovered by Alexander Fleming in 1921. It has a molecular weight of approximately 14–15 kDa with an isoelectric point of about pH 11. Structurally, it consists of a single polypeptide chain with 129 amino acids and it is found to be cross-linked in four different places by disulfide bridges (Biological Magnetic Resonance Data Bank, 2017; Liburdi et al., 2014; Wu et al., 2015). The disulfide bridges, together with the six helix regions, provide high thermal stability to the enzyme. Certain amino acids such as Asp-52 and Glu-35 that are present in the active site of the enzyme are also quite important for the catalytic action of lysozyme (Biological Magnetic Resonance Data Bank, 2017; Cegielska-Radziejewska et al., 2008).

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https://doi.org/10.1016/B978-0-12-813280-7.00011-6

TABLE 11.1 Lysozyme Content (ppm Fresh-Weight Basis [FW]) Present in Secretions, Body Fluids, and Tissues of Human and Animal Organisms

Source of Lysozyme	Lysozyme (ppm FW)
Hen egg white	2500–3500
Duck egg white	1000–1300
Goose egg white	500–700
Human tears	3000–5000
Human milk	55–75
Cow milk	10–15
Human spleen	50–160
Human thymus	60–80
Human pancreas	20–35
Cauliflower juice	25–28
Papaya juice	8–9
Cabbage juice	7–8

Adapted from Liburdi, K., Benucci, I., Esti, M., 2014. Lysozyme in wine: an overview of current and future applications. Compr. Rev. Food Sci. Food Saf. 13, 1062–1073.

Lysozymes are essentially categorized as the C-type (chicken or conventional type), G-type (goose-type), or the I-type (invertebrate type), based on the differences in the amino acid sequences and protein tertiary structures. Both the chicken egg lysozyme (cLYS) and human lysozyme (hLYS) belong to the C-type category. But in spite of the fact that cLYS can be easily obtained from egg whites, as it accounts for 3.5% of the total egg white proteins, hLYS displays a higher thermal stability and threefold greater antibacterial activity because of the differences in cationic residues and the three-dimensional structures. Furthermore, hLYS is also therapeutically more effective against a wide range of human diseases without essentially producing immunogenicity and side effects. However, the use of hLYS is largely restricted because of its limited source (Callewaert and Michiels, 2010; Cegielska-Radziejewska et al., 2009; Wu et al., 2015).

Thus, lysozyme is an enzyme that is naturally present in substantial amounts in avian eggs and mammalian milk and is generally recognized as safe (GRAS) for direct addition to foods. The lysozyme from the hen egg white is especially widely reported for its application as an antimicrobial in food products and is also commonly used as a preservative in fish and fish products, meat and meat products, milk and dairy products, and fruits and vegetables (Gyawali and Ibrahim, 2014). The enormous importance that lysozyme holds in the field of food applications can be seen from the fact that even the World Health Organization (WHO) allow the use of lysozyme as a preservative in foods. Currently, it is being used in Chinese noodles, cheese, sushi, kimuchi pickles, and wine production (Abeyrathne et al., 2013).

11.2 LYSOZYME—THE EXISTING AND POTENTIAL FUTURE PRODUCTION METHODS

The high demand for natural food preservatives has led to lysozyme becoming increasingly important in the field of food processing. Therefore, there has been a need for developing efficient and simple techniques for lysozyme production. The methods available for lysozyme isolation and purification from chicken egg whites range from membrane separation to using the chromatographic techniques such as gel filtration, affinity, adsorption, and ion-exchange. Then, by direct crystallization, it can also be isolated by using reductants and thermal treatment (Chang et al., 2000). However, a large number of reports show that the adsorption-elution methods are the most effective for lysozyme separation as the outcome is a better yield (Wu et al., 2015).

Further, developments in the field of genetic engineering have also enabled the expression of the human lysozyme (hLYS) in microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*, and even in eukaryotic cells and plants. In recent years, the mammary glands of transgenic animals such as mice, goats, and cattle have generated considerable interest as potential bioreactors for the expression of recombinant proteins because of their unique capability to efficiently bring about appropriate posttranslational modifications of recombinant proteins. In short, it can be presumed that a foreign gene is directly transferred to the mammary gland epithelial cells of adult animals via a vector, and after the recombinant proteins are synthesized in the mammary gland's epithelial cells, they would be immediately secreted into milk. Thus, this will help in making the process of purifying recombinant proteins much easier even by using relatively simple chromatographic methods. So, using the mammary gland bioreactor system of dairy cows provides not only a good new way to produce recombinant human lysozyme (rhLYS), but also a way to transfer the presumed benefits of human milk to cow milk (Yang et al., 2011; Zhang et al., 2014).

On the other hand, transgenic chickens as bioreactors also offer several advantages, such as the lower cost of cultivation, shorter generation time, a more beneficial glycosylation profile of target proteins, and lower immunogenicity of the protein product. With regard to this, Wu et al. (2015) have reported the successful purification and characterization of rhLYS from eggs of transgenic chickens.

From a microbial perspective, the lysozyme obtained from *Bacillus licheniformis* TIB320, which has been isolated from the soil, can also be a promising option as a food or feed additive if efficient protein expression systems could be found. This is because, when *E. coli* BL21(DE3) and *Bacillus subtilis* WB 800 were used as hosts for lysozyme expression, the lysozyme from *Bacillus licheniformis* TIB320 showed good bactericidal effects against both Gram-positive and Gram-negative bacteria such as *Micrococcus lysodeikticus*, *Clostridium sporogenes*, *Pseudomonas aeruginosa*, etc. It also expressed a broader antimicrobial spectrum than the egg white lysozyme, which was found to mainly inhibit Gram-positive organisms (Zhang et al., 2014).

11.3 LYSOZYME—MOLECULAR MODIFICATION AND DENATURATION FOR FUNCTIONAL IMPROVEMENT

Molecules that bring about bacterial membrane disruption are mostly positively charged, amphipathic, and hydrophobic in nature. It is important to note that at the physiological pH,

even lysozyme seems to possess all of these physicochemical criteria. But because the lysozyme activity against Gram-negative bacteria remains limited, there is a need for producing a modified lysozyme that is going to exhibit distinctly new properties and also quite novel antimicrobial activity so that its antimicrobial activity is enhanced to a larger extent (Derde et al., 2014; Lesnierowski and Kijowski, 2007).

So, in order to increase the efficacy of lysozyme as an antimicrobial agent, a number of initiatives to chemically modify the enzyme have been undertaken. First, it was found that the lipophilization of lysozyme with long-chain fatty acids (stearic or palmitic acid) and shorter-chain saturated fatty acids (capric, myristic, or caproic acid) broaden the Gram-negative bactericidal action of lysozyme with little or no loss of enzymatic activity (Lesnierowski and Kijowski, 2007). On the other hand, the glycosylation of lysozyme produced proteins that were more stable in nature. Their conformational stability was also highly improved, and they were also highly resistant to the action of proteases. They also had an overall improvement in the modulated charge effects and water-binding capacity. Thus, the conjugation of lysozyme with dextran increased the antimicrobial activity. It was also found that the emulsifying activity of the conjugate was approximately 30 times that of the native lysozyme. Similarly, when xyloglucan hydrolysates were conjugated with hen egg lysozyme, it was found that the enzymatic activity was totally conserved and the emulsifying properties increased five times higher than that of the native protein. Further, an antibacterial emulsifier prepared by conjugating lysozyme with a fatty acylated saccharide was seen to exhibit considerable resistance to proteolysis and much enhanced emulsifying activity and emulsion stability. It was also observed that the protein component did not exhibit significant conformational changes, and the conjugate itself maintained approximately 70% of the bactericidal activity as compared to that of the native hen egg lysozyme. Furthermore, an egg white lysozyme first modified by glycosylation and subsequently lipophilized with palmitic acid increased the yield of the lipophilized lysozyme, which also showed strong antimicrobial activity against *Escherichia coli*. Thus, the lipophilization of lysozyme combined with the method of glycosylation is a promising way for the possible industrial applications of lysozyme. This is because of the improved yield and the enhanced antimicrobial activity toward Gram-negative bacteria (Abdou et al., 2013).

Now from the aspect of denaturation, it was found that when lysozyme was subjected to heat denaturation, it resulted in the progressive loss of enzymatic activity, but surprisingly it also led to greatly improved antimicrobial activity against Gram-negative bacteria. So what can be made of this is that the partial unfolding of lysozyme can switch the antimicrobial activity so that it would now be able to bring about the lysis of Gram-negative bacteria at the same time without having a detrimental effect on the inherent bactericidal effect against Gram-positive bacteria. Therefore, the possibility of extending the range of lysozyme action to include Gram-negative bacteria, that is, *E. coli*, is offered by both thermal and chemical-thermal modification methods, which often results in the formation of an enzyme preparation with increased content of polymeric forms. Interestingly enough, some of these isoforms are especially more positive and more hydrophobic than the native hen egg white lysozyme (HEWL) itself (Derde et al., 2014; Lesnierowski and Kijowski, 2007).

11.4 LYSOZYME AS AN ALLERGEN—THE CONTROVERSY SURROUNDING IT

The role of lysozyme as an allergen is still controversial. In fact, some studies concluded that lysozyme is only a weak allergen while others support the opposite notion (Brasca et al., 2013). Nonetheless, lysozymes from hen eggs contain a well-known major allergen named Gal d4, which has both conformational and sequential epitopes. Several studies have also supported the view that HEWL can be considered a possible allergen. Furthermore, in some foods the lysozyme content varies between 250 and 400 mg/L as in cheese, and 100–500 mg/L in wine. These quantities are not as small as they seem to be, especially in relation to patients who are already sensitive to lysozyme.

But in Canada and the United States, lysozyme has been declared GRAS. In September 2006, the United States authorized the use of HEWL in or on processed product labels as "organic." Even the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its report stated that "lysozyme is obtained from edible animal tissue commonly used as food and can thus be designated as class I enzyme and regarded as a food. It was therefore considered acceptable for use in food processing when used in accordance with good manufacturing practices" (JECFA, 1993).

However, since 1999, the regulations relating to the use of lysozyme have been subjected to frequent amendments. In October 2011, the European Food Safety Authority (EFSA) published a scientific opinion on lysozyme (EFSA, 2011) concerning its allergenic effects as a processing aid in winemaking. As per this opinion, the EFSA considered that allergic sensitivity to lysozyme was found to be common among egg-allergic individuals, and that the residual amounts of lysozyme even in wines are considered sufficient enough to trigger allergic reactions in susceptible individuals under the conditions of use proposed by the applicant. Hence, it is mandated that wines treated with lysozyme must be subject to specific labeling and this should also be in accordance with the provisions of European Commission Regulation (EU) No. 1266/2010 of December 22, 2010. Additionally, the labeling standards require the word, "Contains," followed by the specific allergen (sulfites, milk, or eggs) detected in the final product at 0.25 mg/L or greater (Liburdi et al., 2014).

11.5 LYSOZYME—THE IDENTIFICATION AND QUANTIFICATION METHODS

In terms of determining the potency of lysozyme, a turbidimetric analysis is recommended (JECFA, 2006). The method is based on lysozyme's lytic activity on the bacterial cells of *Micrococcus lysodeikticus*, which can be quantified by turbidimetric analysis. Apart from it, there are also several other methods that have been developed for the purpose of identification and quantification of the lysozyme molecule, including the gel-diffusion or lysoplate assay, spectrophotometric or turbidometric procedures, high performance liquid chromatography with fluorescence detection (HPLC-FLD), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA), immunocapture

mass spectrometry, and surface-enhanced mass spectrometry (Brasca et al., 2013; Maidment et al., 2009). Further, the HPLC-FLD method that is used to quantify lysozyme in milk and dairy products was published as an ISO Technical Specification in 2009 (Brasca et al., 2013).

11.6 LYSOZYME IMMOBILIZATION—A USEFUL APPROACH IN FOOD PACKAGING SYSTEMS

Although HEWL is useful in food applications, its unwanted allergenic properties necessitate the development of novel approaches that can optimize the activity of lysozyme so as to nullify its presumed drawbacks. On this count, the method of lysozyme immobilization represents an interesting alternative compromise for the use of HEWL in food systems (Liburdi et al., 2014). Furthermore, the application of enzyme immobilization systems to nanostructures and food packaging materials offers new innovative possibilities in this area (Irkin and Esmer, 2015).

Generally, the primary cause of deterioration in many foods is microbial activity, which is also often responsible for the reduction in food quality and safety. Foodborne diseases associated with *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella enteriditis*, and *Listeria monocytogenes* are a major public health concern throughout the world (Rawdkuen et al., 2012a). In regards to packaged foods, they seem to be mostly affected by a variety of intrinsic factors that include pH and the presence of oxygen, or by extrinsic factors that are associated with the storage conditions, including relative humidity, temperature, and time. On top of that, add the growth and survival of common spoilage and pathogenic microorganisms such as *E. coli* O157, *L. monocytogenes*, *Salmonella*, *Bacillus cereus*, *S. aureus*, *Campylobacter*, *Clostridium perfringens*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (Lucera et al., 2012). These challenges continue to persist in spite of the fact that, although traditional food preservation techniques such as freezing, heating, drying, fermentation, and salting are able to extend the shelf life of food products to a large extent, problems arise when recontamination occurs. This may, in turn, render the food unpalatable for the consumers (Malhotra et al., 2015).

So for preventing the growth of undesirable microorganisms in food, the available options are that the antimicrobials can either be directly added into the product formulation, coated on its surface, or incorporated into the packaging material. Among them, the method of direct incorporation of active agents into food results in an immediate but short-term reduction of bacterial populations. Contrary to this, the antimicrobial films can maintain their activity for longer periods of time (Lucera et al., 2012). Thus, packaging based on the immobilization of antimicrobial enzymes provides a promising form of active packaging systems that can be applied in food processing for the prolongation of shelf life of nonsterile, chilled, or minimally processed foods (Hanusova et al., 2013).

To this end, lysozyme has been immobilized to different matrices via varied mechanisms such as entrapment, adsorption, and surface conjugation methods. However, the most effective among them has been the immobilization of HEWL by adsorption, which was also found to have extended the lysozyme's antimicrobial spectrum to Gram-negative bacteria. In this regard, there have been reports that the chitosan-lysozyme films exhibited efficient antimicrobial activity against *E. coli* and *Streptococcus faecalis* (Liburdi et al., 2014). Further, Yuceer and Caner (2014) reported the ability to maintain the internal quality of fresh eggs during

their long-term storage by using the chitosan-lysozyme combinations. In terms of producing bioactive coating materials for the food and pharmaceutical industries, HEWL was reported to have been efficiently adsorbed onto porous calcium carbonate/carboxymethylcellulose ($CaCO_3$/CMC) microspheres, hydrophobic nanospheres, and pH-sensitive polymeric material (Liburdi et al., 2014). Furthermore, Muriel-Galet et al. (2013) indicated the possibility of applying the ethylene vinyl alcohol (EVOH)-lysozyme films (in which the enzyme had been covalently immobilized on the surface of EVOH films) for reducing the growth of Gram-positive bacteria (e.g., *M. lysodeikticus* and *L. monocytogenes*), with the effect in this case being generated even without the need for the lysozyme to migrate from the film.

In another study, Barbiroli et al. (2012) reported that lysozyme incorporated into paper containing carboxymethyl cellulose was most effective in preventing the growth of *Listeria innocua* on thin meat slices. Furthermore, Jebali et al. (2013) reported that the lysozyme-conjugated nanocellulose (LCNC) had good antibacterial and antifungal effects against standard strains of *E. coli*, *S. aureus*, *A. niger*, and *Candida albicans*.

With regard to enzyme immobilization via entrapment, the most common example is the production of antimicrobially active packaging with the capacity of controlled protein release for maintaining food quality and safety. In this context, different polymers such as polyvinyl alcohol (PVOH) and polyethylene terephthalate (PET) films have been used for the entrapment of lysozyme (Liburdi et al., 2014). Further, Malhotra et al. (2015) have reported that when lysozyme, which is an enzyme that inhibits lactic acid bacteria causing wine malolactic fermentation, was incorporated in PVOH films, the degree of cross-linking of the PVOH films helped to maintain the release rates of the antimicrobials so as to provide an effective inhibitory effect. Similarly, Rollini et al. (2016) reported the effectiveness of the lysozyme/lactoferrin-coated PET in decreasing H_2S-producing bacteria such as *Shewanella putrefaciens* and *Pseudomonas fluorescens* on fresh salmon fillets. In a few other related studies (Table 11.2), the injection of lysozyme to the films from whey proteins was also reported to inhibit the growth of *L. monocytogenes* bacteria in smoked salmon. But in the films based on

TABLE 11.2 Effects of Lysozyme-Based Active Components Incorporated in Different Polymers

Active Components	Polymer	Target Microorganisms	References
Lysozyme	Whey protein	*L. monocytogenes*	Krasniewska and Gniewosz (2012)
	Zein	*L. plantarum*, *B. subtilis*	Krasniewska and Gniewosz (2012)
Lysozyme and EDTA	Zein	*B. subtilis*, *E. coli*	Krasniewska and Gniewosz (2012)
Lysozyme and Na_2EDTA	Zein	*L. monocytogenes*, *E. coli*, *S. typhimurium*	Irkin and Esmer (2015)
Lysozyme and EDTA	Starch (wheat-based)	*B. subtilis*, *E. coli*	Khairuddin et al. (2017)
Lysozyme, EDTA and nisin	Alginate	*M. luteus*, *L. innocua*, *S. enteritidis*, *E. coli*, *S. aureus*	Krasniewska and Gniewosz (2012)
Lysozyme and lactoferrin	Cellulose	*L. innocua*	Barbiroli et al. (2012)
Lysozyme and catechin	Gelatin	*E. coli*, *S. aureus*, *L. innocua*, *S. cerevisiae*	Rawdkuen et al. (2012b)

zein, lysozyme was demonstrated to inhibit the growth of *Lactobacillus plantarum* and *Bacillus subtilis*. It was also observed that when lysozyme was introduced into the film from zein in combination with ethylene diamine tetra-acetic acid (EDTA), the combination enhanced the effect of lysozyme against Gram-negative *E. coli* bacteria. Furthermore, the effectiveness of lysozyme action in combination with EDTA and nisin was also investigated in a polysaccharide film from alginate. It was found to possess strong growth-inhibiting effect on a wide spectrum of bacteria ranging from *Micrococcus luteus*, *L. innocua*, *S. enteriditis*, *E. coli*, and *S. aureus* (Krasniewska and Gniewosz, 2012). It has also been reported that zein films incorporated with lysozyme and disodium ethylene diamine tetra-acetic acid (Na_2EDTA) exhibited antimicrobial activity against *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella typhimurium* in refrigerated ground beef patties (Irkin and Esmer, 2015). Further, Khairuddin et al. (2017) reported the effectiveness of the lysozyme-EDTA combination incorporated in a starch (wheat-based) active packaging film in inhibiting the growth of *B. subtilis* and *E. coli*.

In general, it has been observed that the shelf life of food products can be increased by the use of gelatin gels, which are made from fish skin impregnated with lysozyme (Thallinger et al., 2013). Specifically, it was also reported by Rawdkuen et al. (2012b) that fish gelatin-based film incorporated with a catechin-lysozyme combination had a concentration-dependent, growth-inhibitory effect against *S. aureus*, *E. coli*, *L. innocua*, and *S. cerevisiae*.

11.7 FUTURE PERSPECTIVES

In the last couple of years, the application of nanotechnology to food safety has attracted the attention of many researchers due to its considerable potential for the development of antimicrobial delivery systems. This technology could be used for improving the antimicrobial stability either by applying it directly, as a coating, or in the form of packaging in different food systems, so as to inhibit the growth of foodborne pathogens (Gyawali and Ibrahim, 2014).

Although many synthetic polymers (e.g., polyamides, polyacrylamide, polyphenylesters, and polyurethanes) have been successfully used as delivery systems in the biomedical and pharmaceutical areas, these polymers cannot be utilized in the field of food applications that require GRAS ingredients. Therefore, a major challenge in this area is the replacement of non-food grade materials by biobased alternatives.

Food biomaterials, specifically polysaccharides (e.g., carrageenan, alginate, pectin, dextran, and chitosan), proteins (e.g., zein and whey proteins), and lipids (e.g., medium chain triglycerides, tristearin, and corn oil) are somewhat reasonable possibilities to address that challenge because they are biodegradable, food-grade, and nontoxic while also allowing the possibility of developing novel functionalities and applications. But again, the use of these polymers has problems associated with their performance and processing in addition to the costs that are common to most biodegradable food-grade polymers. Thus, the application of nanotechnology to these polymers may open up new possibilities to improve not only some of their physical limitations, but also their cost-price efficiency (Ramos et al., 2014).

In this context, Medeiros et al. (2014) reported that when a nanolaminate coating of alginate and lysozyme produced by the layer-by-layer methodology was evaluated for its ability on "Coalho" cheese preservation during a period of 20 days, it was found that coated cheese

exhibited lower values of pH, mass loss, lipidic peroxidation, and microorganism proliferation as well as higher titratable acidity in comparison to uncoated cheese. Therefore, these results point to the fact that the gas barrier and antibacterial properties of the alginate/lysozyme nanocoating could possibly become one of the options for extending the shelf life of Coalho cheese.

Furthermore, Zimoch-Korzycka and Jarmoluk (2015) demonstrated the effectiveness of hydrosols containing chitosan in combination with lysozyme and nanocolloidal silver together with hydroxypropylmethyl cellulose as a base component in causing the death of Gram-positive bacteria such as *B. cereus* and *Micrococcus flavus* and Gram-negative bacteria such as *E. coli* and *Pseudomonas fluorescens* when applied on the surface of meat samples.

Lastly, in a recent study, Feng et al. (2017) reported the design of a novel antimicrobial electrospunnanofilm, namely polyvinyl alcohol/β-cyclodextrin/cinnamon essential oil/lysozyme (PVA/β-CD/CEO/LYS) film, which showed strong antibacterial activity against *L. monocytogenes* and *S. enteriditis*. In addition, this nanofilm also exhibited an excellent antifungal activity against *A. niger* and *Penicillium*.

11.8 CONCLUSION

In recent years, there has been a general trend that consumers have become concerned about the safety of the synthetic preservatives that are being used in foods. Thereby, concerted efforts have been made to find natural antimicrobials that can inhibit bacterial and fungal growth in foods in order to improve quality and shelf life (Gyawali and Ibrahim, 2014). Owing to its bactericidal activity and thermal stability, lysozyme has been of interest in the field of medicine, cosmetics, and food, but the most important application of lysozyme still remains its use as a food preservative (Zhang et al., 2014).

Thus, lysozyme, a GRAS-status natural antimicrobial enzyme that is mostly obtained from hen egg white and has been commercially used to primarily prevent late blowing in semihard cheeses, caused by *Clostridium tyrobutyricum*, is one of the most important potential candidates for antimicrobial packaging because of its good stability and activity in different films and food systems (Feng et al., 2017; Lucera et al., 2012).

Acknowledgments

The authors express their sincere gratitude to the Principal, Lady Keane College, Shillong, and the DBT-Institutional Biotech Hub, Lady Keane College, Shillong, for the facilities provided during the preparation of the manuscript, and Ramesh Chinta for the generosity in providing some of the valuable resources.

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CHAPTER

12

Ligninolytic Enzymes: An Introduction and Applications in the Food Industry

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12.1 INTRODUCTION

Ligninolytic enzymes are highly versatile and ubiquitous in nature, known for their role in the degradation of various complexes and recalcitrant polymers (Chandra and Chowdhary, 2015; Chowdhary et al., 2016; Wong, 2009). Naturally, there are a number of microbes that are capable of producing ligninolytic enzymes, but among these, white-rot fungi is more effective to produce many enzymes such as manganese peroxidase (MnP) and lignin peroxidase (LiP). The activity of laccase, MnP, and LiP is further enhanced due to the cooperative activity of some other enzymes such as glyoxal oxidase, aryl alcohol oxidase (veratryl alcohol oxidase), pyranose 2-oxidase (glucose 1-oxidase), cellobiose/quinone oxidoreductase, and cellobiose dehydrogenase. Laccases are also known as polyphenol oxidases and enhance the oxidation of several aromatic compounds (phenolic and nonphenolics), especially those having electron donor groups such as anilines and phenolics using molecular oxygen as a terminal electron acceptor (Gianfreda et al., 1999). The food processing industries produce a huge volume of wastewater globally, which can cause various environmental problems. Food industry wastewater is highly rich in derivatives of sugars, which, due to their organic nature, get easily utilized by microbes as C, N, and energy sources. Hence it is preferred as a cheap raw material for the production of secondary metabolites of industrial importance (Piontek et al., 2002). The Laccase, MnP, and LiP play a key role in different industrial processes such as

https://doi.org/10.1016/B978-0-12-813280-7.00012-8

stabilization of wine and fruit juices in food industries, denim washing in textile industries, and the cosmetic and biosensor-producing industries (Ferry and Leech, 2005; Minussi et al., 2002; Pazarlioglu et al., 2005). Enzymes are biocatalysts that are capable of acting under mild pH and temperature. Due to their nature, enzymes are preferred as organic, less-toxic food components and are the natural alternatives of catalysts as food-processing agents by the consumers (Minussi et al., 2002). This chapter focuses on a few ligninolytic enzymes such as laccase, MnP, and LiP, and also describes the current and future prospectives in the food industries.

12.2 STRUCTURE AND FUNCTION OF LIGNINOLYTIC ENZYMES

The term "lignin" originated from the Latin word "lignum," which means wood. It is found in all types of vascular plants, and represents the second most abundant carbon source on earth after cellulose. Lignin has a complex structure having three primary hydroxyl-cinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols, which remain linked to each other by the oxidative-coupling of monolignols (Wong, 2009). Lignification, the synthesis of lignin, takes place either by the cross-linking of monomeric units with a growing polymer or by polymer-polymer coupling via radicals produced by oxidase enzymes. The resulting polymer has an array of structural units produced by ether and C–C linkages due to the coupling of radicals at various sites. Recently "kenaf" and other lignins are reported to have high concentrations of acylated structural units such as 4-propoxy-sinapyl-γ-acetate, whereas grasses have hydroxycinnamic acids esterified at the γ-position of propyl side chains of lignin (Del Rio et al., 2007; Ralph et al., 2004). Besides, LiP, MnP, and laccase, many enzymes such as aryl alcohol dehydrogenase, quinone oxidoreductase, aromatic acid reductase, catalase aromatic aldehyde oxidase, dioxygenase, glyoxal oxidase, vanillate hydroxylase, and veratryl alcohol oxidase are also reported to be involved in the lignin degradation process (Niladevi, 2009). The basic properties of these enzymes mainly depend on the microbial source. Therefore, the characteristics of these enzymes differ widely depending on their source; they are also of less significance. Further, they could act as mediators of lignin degradation by producing the H_2O_2 required to induce the activity of peroxidases or catalyze the breakdown of lignin.

12.2.1 Laccases

Laccases, also known as polyphenol oxidase, are part of the family of blue multicopper oxidases that catalyze single electron oxidation of food-reducing substrate molecules with four electrons of molecular oxygen into water (Piontek et al. 2002). In addition, these enzymes induce the oxidation of a wide range of substrates, including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines, and ascorbate with the concomitant four electron reduction of oxygen to water (Chandra and Chowdhary, 2015; Galhaup et al. 2002). Laccases exhibit a wide range of substrate while oxidizing compounds in the presence of mediators with high redox potential. However, the application of laccases in the food industries requires a large amount of laccase enzyme; for this, many strategies can be applied along with media and process optimization. In addition, the overexpression of laccase in the host organism may provide a change to achieve large production (Couto and Toca-Herrera, 2007). White rot fungi are well known to produce and use this enzyme in the breaking of the plant

lignocellulosic wall to obtain nutrients (Baldrian, 2006). Besides their catalytic characteristics, laccases also play various important roles in the catalytic process, as they are extracellular, inducible and have low specificity but do not require any cofactors.

12.2.1.1 Laccase Structure and Function

Laccases have about 500 amino acid residues that remains clubbed in three consecutive domains with a Greek key β barrel topology. Out of these three domains, the first contains 150 amino acids, the second has 150–300, and the third has 300–500. The structure of laccase stabilizes by two disulfide links present between the first and second domains as well as the first and third (Bertrand et al., 2002; Matera et al., 2008). Moreover, some laccases have three disulfide bridges, i.e., first bridge inside domain I, second and third bridge combines domain I and III, and the last one between domain II and III (Bertrand et al., 2002). In the laccase enzyme, the four copper atoms remain scattered in three copper centers, that is, type 1 Cu (T1), type 2 Cu (T2), and type 3 Cu (T3). These centers comprise two metallic active sites, that is, one in the mononuclear location T1 and the second in the trinuclear location T2/T3 (Kokol et al., 2007; Matera et al., 2008; Plácido and Capareda, 2015). The mononuclear location of copper, that is, 1 Cu (T1) or blue Cu, is a paramagnetic copper having a strong absorption at 600 nm (blue color) due to the generation of a covalent bond between copper and cysteine (Fig. 12.1) (Matera et al., 2008). T1 Cu reflects a different triangular planar coordination similar to other multicopper oxidases. Laccases have two histidine (His) and one cysteine (Cys) as equatorial ligands whereas other MCOs have methionine as an additional axial ligand. Laccases do not have this axial extra bond because, in place of methionine, they have a phenylalanine (Phe) or leucine (Leu) (Bertrand et al., 2002; Hakulinen et al., 2002). T2 Cu reflects the organization of two His and an H_2O molecule whereas T3 Cu reacts with an H_2O and six histidine molecules. In T3 Cu, two Cu molecules share one water molecule and cleave six histidines into two groups of three His (Plácido and Capareda, 2015). The interplay between H_2O and the two Cus takes place when the enzyme is in its oxidized form. The crystalline structures of laccase showed that T2 Cu (EPR active) may be any of the three Cus in the trinuclear site where two Cus reduce and become silent to EPR while the other remains active to EPR. This exchange between T2 Cu and one of T3 could be related to the mechanism of reduction of $2O_2$ molecules (cofactor), which is unclear for blue multicopper oxidase (Garavaglia et al., 2004). In addition to these, laccases are reported to have structural ligands such as monosaccharides (mannose, *N*-acetyl glucosamine), ions such as Ca^{2+}, and organic molecules, that is, glycerol. The presence of these ligands makes a considerable difference between different categories of laccases (Plácido and Capareda, 2015).

12.2.2 Manganese Peroxidase

MnP was isolated by Kuwahara in 1984 from the batch cultures of *Phanerochaete chrysosporium*, which are glycoproteins having a molecular weight ranging from 38 to 62.5 kDa, ~350 amino acid residues, and 43% identity with LiP sequences (Martin, 2002). The molecular structure of MnP has two main domains with the heminic group in the middle, 10 major helixes, a minor helix, and five disulfide linkages. Out of these disulfide linkages, one is present at the manganese (Mn) bonding site, which is characteristic of MnP and distinguish between

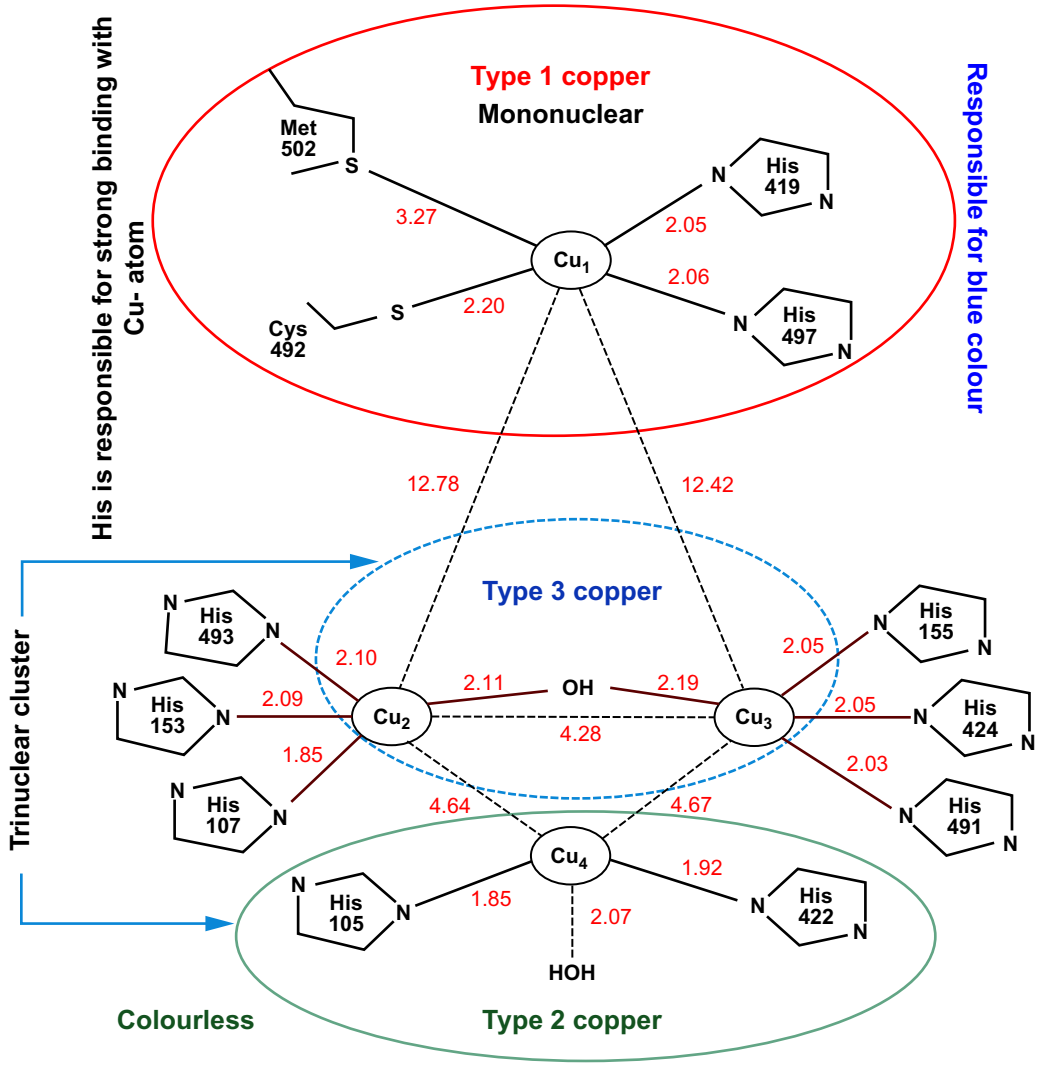

FIG. 12.1 The laccase active site of the copper atoms, including interatomic distances among all relevant ligands. *Modified from Enguita, F.J., Martins, L.O., Henriques, A.O., Carrondo, M.A., 2003. Crystal structure of a bacterial endospore coat component: a laccase with enhanced thermostability properties. J. Biol. Chem. 278, 19416–19425.*

other peroxidases (Sundaramoorthy et al., 1994). The catalytic cycle of the enzyme initiates with the transfer of two electrons from the heminic group to H_2O_2, resulting in the generation of compound I and H_2O. Compound I induces the oxidation of one substrate molecule with the generation of a free radical whereas compound II oxidizes Mn^{2+} into Mn^{3+}, which causes oxidized aromatic compounds. Compound II requires the presence of Mn^{2+} for its catalytic reaction whereas the compound I can oxidize Mn^{2+} or the other substrate. Mn^{3+} is a small size compound with high redox potential that diffuses easily in the lignified cell wall. After

stabilization, Mn^{3+} reacts nonspecifically with organic molecules and removes one electron and one proton from the substrate (Martin, 2002). MnP oxidizes Mn^{2+} to Mn^{3+}, which is stabilized by organic acid chelators such as oxalate, malonate, glyoxylate, etc. It acts as a low molecular mass, diffusible, redox mediator that attacks organic molecules and oxidizes several compounds (nonspecifically) via hydrogen and one electron reduction (Niladevi, 2009). The release of Mn (III) from the active site of the enzyme is facilitated by these organic acids. The one electron oxidation of Mn (II) to Mn (III) in a multistep reaction cycle is as follows:

$$\mathrm{MnP} + \mathrm{H_2O_2} \rightarrow \mathrm{MnP\,compound\,I} + \mathrm{H_2O} \tag{12.1}$$

$$\mathrm{MnP\,compound\,I} + \mathrm{Mn(II)} \rightarrow \mathrm{MnP\,compound\,II} + \mathrm{Mn(III)} \tag{12.2}$$

$$\mathrm{MnP\,compound\,II} + \mathrm{Mn(II)} \rightarrow \mathrm{MnP} + \mathrm{Mn(III)} + \mathrm{H_2O} \tag{12.3}$$

12.2.3 Lignin Peroxidase

Lignin peroxidase (LiP) is one of the most commonly known ligninolytic enzymes participating in lignin degradation and in 1983 from the WRF, *P. chrysosporium* (Dias et al., 2007; Niladevi, 2009). Lignin peroxidase induces the H_2O_2-dependent oxidative depolymerization of lignin polymer (Wong, 2009). LiP has high redox potential (700–1400 mV), low optimum pH of 3–4.5, and the ability to catalyze the degradation of several aromatic groups such as veratryl alcohol (3, 4-dimethoxybenzyl) and methoxybenzenes. LiP is comparatively nonspecific to its substrates and has been known to oxidize phenolic aromatic compounds and also a number of nonphenolic lignin model compounds as well as variety of organic compounds with a redox potential up to 1.4 V (in comparison to normal hydrogen electrode) in association with H_2O_2. LiP is nonspecific to its substrates and known to oxidize phenolic compounds and also a number of nonphenolic compounds as well as variety of organic compounds. Lignin peroxidises are monomeric glycosylated enzymes of 40 kDa with 343 amino acid residues, 370 H_2O, a heme group, four carbohydrates, and two calcium ions (Choinowski et al., 1999). The secondary structure of LiP is mainly helicoidal, which comprises eight major helixes, eight minor helixes, and two antiparallel beta sheets. LiPs have two domains with heminic groups at both sides. This group is inlaid in the protein, but has access to solvents via two small channels.

12.2.3.1 Lignin Peroxidase Structure and Function

Lignin peroxidase exists in a globular shape with a size of about $50 \times 40 \times 40$ Å (Falade et al., 2017). LiP has two domains, that is, proximal and distal domains that remain segregated by heme, which remains completely fit in the proteins. The LiP folding motif consists of eight major α-helices, eight minor helices, and three short antiparallel β sheets (Angel, 2002; Choinowski et al., 1999). The catalytic cycle of LiP is comparable to heme-peroxidases. However, some structural differences between LiP and other heme-peroxidases do exist. The molecular weight of LiP ranges from 38 to 43 kDa, an isoelectric point range of 3.3–4.7, and an optimum pH of pH 3.0 with veratryl alcohol as the substrate (Falade et al., 2017; Furukawa et al., 2014). Structural differences such as LiP have four disulfide bonds while all these

remain in cytochrome *c* peroxidase (CcP). LiP is larger in size, containing 343 amino acid residues whereas, CcP contains 294 residues. Such details have been reported from crystallographic studies of CcP and LiP (Edwards, et al., 1993). Moreover, CcP is also thought to be abundantly enriched with oxidizable amino acids (seven tryptophans, 14 tyrosine residues, five methionines, and one cysteine) whereas LiP has only three tryptophans and eight methionines. Tyrosine and free cysteine are absent in LiP. Further, a remarkable difference between LiP and CcP includes the presence of phenylalanine at the contact site between the distal and proximal heme surfaces in LiP, whereas in the case of CcP, phenylalanine gets replaced with tryptophan. Likewise, in LiP, the Asp-183 remains hydrogen-bonded with the heme propionate whereas, in CcP, Asn-184 performed this function. A low pH of lignin peroxidases is expected that disrupts the aspartic acid-propionate hydrogen bond, resulting in the destabilization of the heme pocket. The bond between the heme iron and the Nε2 atom of the proximal histidine residue in LiP is longer than that in CcP (Choinowski et al., 1999). The weaker Fe–N bond in LiP makes the heme more electron-deficient, thereby destabilizing its high oxidation states. This has been suggested as a logical explanation for the higher redox potential of lignin peroxidase when compared to cytochrome *c* peroxidase.

12.2.3.2 Lignin Peroxidase Catalytic Reactions

Lignin peroxidase is well known to oxidize various nonphenolic lignin model compounds that include β-*O*-4 linkage-type arylglycerol-aryl ethers. The other crucial oxidative properties of LiP involve the formation of radical cations through one electron oxidation, leading to side-chain cleavage, demethylation, intramolecular addition, and rearrangements (Falade et al., 2017; Wong, 2009). The other catalytic oxidative processes associated with LiP are hydroxylation of benzylic methylene groups and oxidation of benzyl alcohols to their respective aldehydes or ketones, and phenol oxidation (Furukawa et al., 2014; Paliwal et al., 2012).

12.3 VARIOUS FOOD INDUSTRIES AND THEIR PRODUCTS

Ligninolytic enzymes have numerous applications for multiple purposes in different sectors of the food industry, right from the amendment of the food sensory parameters and textures to the enhancement of shelf life, including the determination of certain compounds in the beverage industries. In fact, laccase, lignin peroxidase, manganese peroxidase substrates, most phenols, thiol-containing proteins, and unsaturated fatty acids are critical ingredients of many foods and beverages. Therefore, their modification can give rise to new functionalities, increase gradation quality, and reduce costs (Minussi et al., 2002; Osma et al., 2010).

12.4 ENZYMES AND ITS APPLICATIONS

Applications of ligninolytic enzymes (Laccase, Manganese peroxidase, Lignin peroxidase) to some processes that improve or modify the color of food or beverages for the elimination of unwanted phenolics accounts for the browning, haze formation, and turbidity in clear fruit juice, beer, and wine (Table 12.1 and Fig. 12.2) (Rodríguez Couto and Toca Herrera, 2006). One of the enzymes, laccase, is effectively used in ascorbic acid determination, sugar beet pectin

TABLE 12.1 Ligninolytic Enzymes in the Food Industry and Their Applications

Enzymes	Industry	Application	References
Laccase	Beverages, food industry	Phenolic remotion, baking, beer stabilization, fruit juice oxidation process, cork modification, ascorbic acid determination, sugar beet pectin gelation, oxidizing phenolic compound in apple juice.	Minussi et al. 2002; Maciel et al., 2010; Chandra and Chowdhary, 2015
Lignin peroxidase	Food industry	Source of natural aromatics, production of vanillin.	Lomascolo et al., 1999; Barbosa et al., 2008; Maciel et al., 2010
Manganese peroxidase	Food industry	Production of natural aromatic flavors.	Lomascolo et al., 1999; Zorn et al., 2003; Barbosa et al. 2008; Maciel et al., 2010

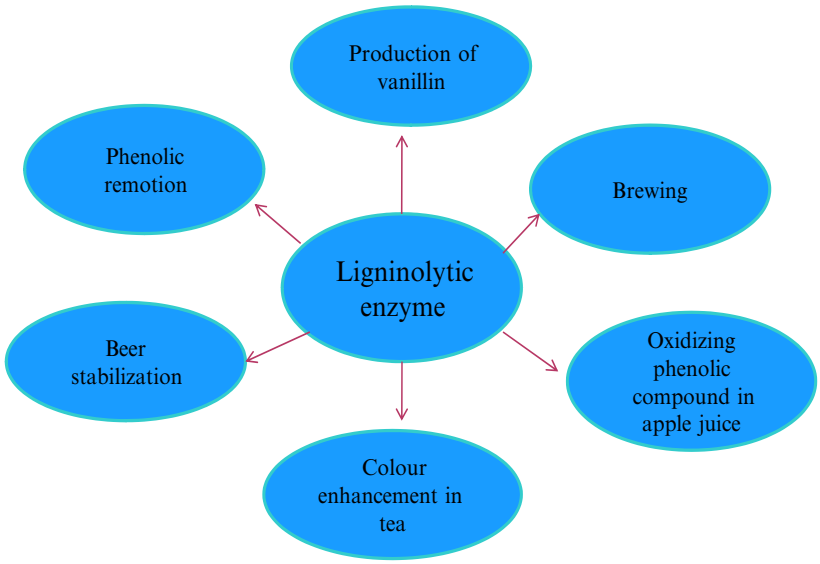

FIG. 12.2 Application of ligninolytic enzymes in food industries.

gelation, baking, and the treatment of olive mill effluent (Minussi et al., 2002, 2007; Rodríguez Couto and Toca Herrera, 2006; Selinheimo et al., 2006). It is also established that LiP and MnP have the capacity to produce natural aromatic flavors (Barbosa et al., 2008; Lomascolo et al., 1999; Zorn et al., 2003).

12.4.1 Use of Laccases in the Food Industry

Several authors have reported the potential multipurpose applications and benefits of laccases in different sectors of the food industry (Madhavi and Lele, 2009; Minussi et al., 2002; Osma et al., 2010; Pezzella et al., 2015). Laccases also have many commercial products in different industries (Table 12.2). It is reaffirmed that its products, such as carbohydrates,

TABLE 12.2 Commercial Laccase-Based Products in Different Industries

Industries	Product Brand Name	Product Application	Manufacturer
Paper and pulp industry	Lignozym process	Pulp bleaching	Lignozym GmbH (Germany) now wacker Chemie
	Lasox	Delignification and bleaching	Bioscreen EK (Germany)
	Novozyme 51003	Paper pulp delignification	Novozymes (Denmark)
	MetZyme	Pulp and biofuel wastewater treatment	Metgen Oy (Finland)
Food Industry	Laccase Y120	Color enhancement in tea, etc.	Ameno Enzyme
	Flavourter	Brewing	Advance Enzyme Technologies Ltd. (India)
	Suberase	Cork modification, Synthesis of phenolic colorants	Novozymes (Denmark)
Textile industry	Denilite	Denim bleaching	Novozymes (Denmark)
	Zylite	Denim bleaching	Zytex Pvt. Ltd. (India)
	NS 51002	Deinking	Novozymes (Denmark)
	NS 51003	Deinking	Novozymes (Denmark)
	Bleach Cut 3-S	Denim bleaching	Season Chemical (China)
	Ecostone LC10	Denim bleaching	AB Enzyme GmbH (Germany)
	Cololace BB	Denim bleaching	Colotex Biotechnology Co. Ltd. (Hong Kong)
	Purizyme Laccase	Bleaching of indigo-dyed garments	Puridet (Asia) Ltd. (Hong Kong)
	Novoprime Base 268	Denim bleaching	Novozymes (Denmark)

Adapted from Osma, J.F., Toca-Herrera J.L., Rodrıguez-Couto S., 2010. Uses of laccases in the food industry. Enzyme Res. 918761.

unsaturated fatty acids, phenols, and thiol-containing proteins, are key components of many foods and beverages. Their increased gradation by laccase can give rise to new functionality, quality enhancement, or even reduced cost (Osma et al., 2010).

12.4.1.1 Food and Beverage Processing

The application of laccases to some processes that induce or transform the color of foods or beverages is enumerated below.

12.4.1.1.1 WINE AND BEER STABILIZATION

One of the most important processes of wine stabilization needs laccases. Polyphenols in wine determine the aroma, color, taste, and texture of wine, and are therefore directly associated with the particular phenolic composition. But the same compounds also impact the maderization process, resulting in turbidity, color intensification, and aroma and flavor modification. Reducing the polyphenol content by laccase treatment has been reported as an efficient method to store wine quality, primarily because the usage of enzymes would alternatively remove target-specific polyphenols without deplorably altering the wine's organoleptic properties. Laccases are well suited with process requirements due to their stability in an acidic medium and reversible inhibition with sulfite (Tanrioven and Eksi, 2005). In addition to the efficiency in polyphenol removal, several workers have underlined the need for additional steps in the wine production process, such as sulfur treatment, clarification, and filtration to restrict the oxidized products produced by laccase (Minussi et al., 2002). Other researchers have also confirmed the application of laccases in prefermentative treatment when coupled with conventional clarifiers such as proteins and poly vinyl pyrrolidone (PVP) (Minussi et al., 2007). Some have highlighted differential selectivity in phenol removal in the improvement of red and white wines. Although the treatment of red wine reduces phenolic compounds, it greatly affects the most important antioxidant properties. Interestingly, the improved treatment of white wines has shown a significant decline in total phenols, more so than in the total antioxidant potential. This results in low production costs and increased shelf life of white wines. Because most wine bottles have cork stoppers, this imparts an unsavory cork off-flavor to wine when it comes into contact with the cork. This is mainly due to phenols naturally present in cork and/or produced by microbial degradation. So the customary usage of laccase treatment of cork stoppers for wine bottles has been envisaged. The most efficient method to decrease the cork taint by phenol oxidation has been patented (Conrad et al., 2000).

12.4.1.1.2 FRUIT JUICE PROCESSING

Juice clarifications by enzymatic protocols have been in practice since the 1930s, according to Minussi et al. (2007). The known challenge of browning, both enzymatic and chemical, is the main reason that causes huge losses in beverages. Many pre- and posttreatment methods are in practice to avoid postturbidity and discoloration of fruit juices. The conventional treatment of stabilization of beverage products by gelatin, bentonite, silica gel, etc., is widely accepted (Osma et al., 2010). Laccase-supported enzymatic treatments are one of the methods that has been initiated for fruit juice stabilization (Piacquadio et al., 1997). However, the reports on laccase-supported apple juice treatments are contradictory. The other report by Gokmen et al. (1998) showed that laccase treatment increases the vulnerability to browning during storage. In this case, delicious golden apple juice was treated with a conventional method (SO_2 added as Meta bisulfite, PVPP, bentonite) and was laccase-free or immobilized on a metal chelate regenerable carrier. Surprisingly, the enzymatically treated apple juice was

less stable than the conventionally treated juice. Further, when laccase was in combination with cross-flow filtration (ultrafiltration) in continuous process without the addition of fining agents, this led to a stable and clear apple juice. Madhavi and Lele (2009) showed that it has the potential to obtain clear and stable juices/concentrates with a light color by means of ultrafiltration and laccase without any large supplementary investment. Thus, any treatment with laccase at a compatible pH followed by "active" filtration or ultrafiltration can improve color and flavor stability as compared to traditional approaches, simply by the addition of ascorbic acid and sulfites.

12.4.1.1.3 BAKING

For a long time, enzymes (amylases, proteases, cellulases, etc.) have been used as bread-improving agents for a variety of reasons. In fact, during the bread-making process, bread and/or dough enhancers are added primarily to improve texture, volume, flavor, and freshness of the bread as well as the enhanced machinability of the dough. The addition of laccase to dough used for producing baked products exerts an oxidizing effect on the dough constituents, thereby helping to enhance the strength of gluten structures in dough and/or baked products; (Si, 1994). The report states that the use of the ligninolytic enzyme (laccase) results in enhanced volume, improved crumb structure, and better softness of the baked product as well as increased strength, stability, and reduced stickiness, thereby improving the machinability of the dough (Selinheimo et al., 2006; Labat et al., 2000). The impact of laccase on dough has been found to be particularly superior when poor quality flour was used.

12.4.1.1.4 SUGAR BEET PECTIN GELATION

The functional food ingredients such as sugar beet pectin can form thermally irreversible gels. These types of gels can be heated while maintaining the gel structure, which is very interesting for the food industry. The present need to find new functional ingredients is a matter of significant attention for the food industry. The specific functional property of sugar beet pectin is that it forms gels by an oxidative cross-linking of ferulic acid (Norsker et al., 2000). Further, it is possible to cross-link the beet pectin through the oxidative coupling of the feruloyl groups using laccase (Littoz and McClements, 2008). By modulating the degree of cross-linking, which can be done by the amount of pectin added and the reaction time, this was found to be very crucial for the hydration characteristics of the powders. An investigation on beet pulp as a substitute source of pectins to apple pomace or citrus peels was also carried out. It showed that peroxidase or laccase can perform the oxidation gelation. Lastly, peroxidase requires the addition of hydrogen peroxide while laccase can utilize the oxygen present in the sample (Kuuva et al., 2003; Norsker et al., 2000). The obtained gel is thus thermoirreversible. It is compelling to note for the food industry that the product can be heated while maintaining a gel structure. This enzymatic gelation was done in three food products with added sugar beet pectin. Making the meat product sliceable was possible due to the cohesive gel formed, which bound the meat pieces together. But, in two of the food products, some unwanted side effects were observed. The enzymes not only catalyzed the cross-linking, but they also oxidized the anthocyanins in the black currant juice and the short-chained fatty acids in milk, which is crucial. This can lead to discoloration of the juice and the development of a strong off-flavor in the milk (Norsker et al., 2000).

12.4.1.2 *Bioremediation of Food Industry Wastewater*

In the ligninolytic enzymes, laccases are well studied with the ability to degrade phenolic compounds (Madhavi and Lele, 2009). The compounds such as aromatic, phenols, and aromatic amines comprise the main class of toxic compounds and are seriously regulated in all the countries globally. The presence of these toxic compounds in drinking and irrigation water causes serious concern for living beings (Pezzella et al., 2015). The immobilization of laccase on organogel supports the removal of naturally occurring and xenobiotic aromatic compounds from aqueous solutions. Laccases immobilized by adsorption on polyethersulfone showed chemical and physical properties that were potentially useful for reducing the phenol concentration in an experimental set up (Lante et al., 2000).

12.4.1.2.1 BEER FACTORY WASTEWATER

Some beer industry wastewaters characterize significant environmental issues due to their great concentration of polyphenols and gray color (dark brown). *Coriolopsis gallica*, a white rot fungus producer of laccase, was able to degrade this high tannin-containing wastewater. The pyrolysis/gas and chromatography/mass spectrometry results revealed a reduction in polyphenol pyrolysis products, primarily phenol and guaiacol, with the retention time (Yague et al. 2000).

12.4.1.2.2 DISTILLERY WASTEWATER

The fermentation of sugarcane molasses called vinasses during ethanol production generates distillery wastewater (Bharagava and Chandra, 2010). It renders an environmental impact due to a high concentration of soluble organic matter and an intense dark brown color (Chowdhary et al., 2016, 2017). The white rot fungus *Trametes* sp. that produces laccase was tested for bioremediation. A maximum effluent decolorization of 73.3% and chemical oxygen demand reduction of 61.7% was achieved after 7 days of fungal treatment to 20% v/v of distillery wastes in the culture medium. In an experimental set up, a 35-fold increase in laccase production by this fungus was observed (Gonzalez et al. 2000).

12.4.1.2.3 OLIVE MILL WASTEWATERS

In the Mediterranean area, olive mill wastewater (OMW) is a main by-product of olive oil production, and it adversely affects the environment. More than 30 million m^3 of OMW is produced in the Mediterranean area, which generate 2.5 L of waste per liter of oil produced (Borja et al., 1992). OMW contains high concentrations of phenolic derivatives (up to 10 g/L) along with high BOD and COD (Strong and Burgess, 2008). OMW has a characteristic color that ranges from dark red to black. The color depends on the age and type of olive processed, a low pH value, a high salt concentration, and a high organic content with high concentrations of aromatic compounds, fatty acids, pectins, sugars, tannins, and phenolic compounds, in particular polyphenols (D'Annibale et al., 2004; Jaouani et al., 2003). Due to the presence of a large number of organic compounds with many polluting, phytotoxicity, and antimicrobial properties, OMW is rendered a waste with highly hazardous effects on living entities and the environment. This makes its dumping one of the main environmental problems in all producing countries (Paredes et al., 1999). A report on laccase was obtained from the white-rot fungus *Lentinula edodes* immobilized on chitosan to treat OMW from an olive oil mill

located in Viterbo (Italy); D'Annibale et al. (1999). It revealed that the treatment of the OMW with immobilized laccase led to limited decoloration as well as to a significant decline in its polyphenol content, and orthodiphenols combined with a decreased toxicity of the effluent. They also showed that an oxirane-immobilized laccase from *L. edodes* efficiently removed the OMW phenolics (D'Annibale et al., 2000). An investigation on the prospects of laccase from *L. edodes* in removing OMW phytotoxicity is significant (Casa et al., 2003). On germinability experiments on durum wheat (*Triticum durum*) in the presence of multiple dilutions of raw or laccase-treated OMW. The treatment with laccase resulted in a 65% and an 86% reduction in total phenols and orthodiphenols, respectively, due to their polymerization, as revealed by size-exclusion chromatography. Interestingly, the germinability of durum wheat seeds was increased by 57% at a 1:8 dilution and by 94% at a 1: 2 dilution, as compared to the same dilutions using untreated OMW.

12.4.2 Use of Manganese Peroxidase (MnP) and LiP

Ligninolytic enzymes (laccases, MnP, and LiP) are broadly distributed to play a key role in various applications, one of which is to reutilize the wastes from food and agricultural industries. Examples of this include corncobs, cassava bagasse, sugar cane bagasse, wheat bran, rice bran, carob pods, and spent brewery grains, among others (Velioglu and Ozturk Urek 2015). Manganese peroxidase and lignin peroxidase have been reported to produce natural aromatic flavors (Barbosa et al. 2008). However, the understanding of molecular mechanisms that regulate enzyme production in lignocellulose bioconversion could be necessary for enhancing the medicinal and edible mushroom production technology. The metabolisms of ligninolytic enzymes have been widely studied using submerged and solid-state fermentation of lignocellulosic substrates (Moldes et al., 2004; Mikiashvili et al., 2005). Basidiomycetes manganese peroxidase (MnP) activity in the submerged fermentation of food industry wastes (Songulashvili et al., 2007) is also reported. The above state of the art on LiP and MnP indicates that less significant study is available on these ligninolytic enzymes. Therefore, the thrust to conduct research on these enzymes in food industrial applications is justified.

12.5 CHALLENGES AND FUTURE PROSPECTS

The ligninolytic enzyme extraction process is very costly and time consuming. A major restriction to the comprehensive use of ligninolytic enzymes is large-scale production of large volumes of highly active enzymes at an affordable cost. Ligninolytic enzymes have great potential applications in many areas of the food industry. Cost-effective sources of laccase production are currently being explored. Even in the promising field of industrial wastewater management, the nutritive potential for the manufacturing of laccases enzymes is being explored.

12.6 CONCLUSION

This chapter illustrates the prospective applications of ligninolytic enzymes in many areas of the food and beverage industry. However, due to large technological advancements and rigorous government regulations, some work has been done to encourage a safe and

ecofriendly treatment process. The above literature also concluded that in the food industry, the ligninolytic enzyme (laccase) has been used more prominently in various applications. In this chapter, many issues have been highlighted, and to solve these, it is necessary to reveal through R&D, ecofriendly ligninolytic enzymes that can degrade/detoxify waste.

Acknowledgments

The authors thank B.B. Ambedkar University, Lucknow for infrastructural support, while Pankaj Chowdhary is grateful to the Rajiv Gandhi National Fellowship (Letter No. RGNF 2015-17SC-UTT-20334) from University Grants Commission (UGC), New Delhi, India for providing the financial support for a doctoral degree.

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CHAPTER

13

Hydrolases of Halophilic Origin With Importance for the Food Industry

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13.1 INTRODUCTION

Enzymes catalyze reactions with high precision, giving efficient production of single stereoisomers while decreasing secondary reactions, which is environmentally friendly. However, most industrial processes take place under specific levels of ionic strength, temperature, and pH. For this reason, some of the enzymes employed in the industry cannot offer their optimal activities under the levels of ionic strength, temperature, and pH used (Enache and Kamekura, 2010). On the other hand, there are reports documenting that haloenzymes are polyextremophilic and remain stable under high pH, temperature, chaotropic agents, and organic solvents, with its stability modulated by salt (Kumar et al., 2012; Munawar and Engel, 2013; Sinha and Khare, 2014). High salt concentration affects catalytic activity in an enzyme-dependent pathway (Gabriel Ortega et al., 2011). The stabilization of halophilic enzymes is modulated by high salt concentration because of a low affinity binding of the salt to specific sites on the folded polypeptide surface, stabilizing the protein active conformation (Mevarech et al., 2000). Halophilic enzymes perform identical functions to their nonhalophilic counterparts, but have a high excess of acidic over basic amino residues (Mevarech et al., 2000).

Halophilic microorganisms that live in salt-rich environments, such as salt lakes, solar salterns, and salt mines (Aljohny, 2015), produce different halophilic enzymes such as proteases, amylases, xylanases, cellulases, lipases, DNases (Setati, 2010), glutaminase

https://doi.org/10.1016/B978-0-12-813280-7.00013-X

(Gupta et al., 2016), L-asparaginase (Shirazian et al., 2016), and glucose dehydrogenase (Esclapez-Espliego et al., 2014). These enzymes have industrial potential for food fermentation, supplements for the food, textile, and pharmaceutical industries, animal feed (Sekar et al., 2016), laundry detergents (to destain blood, ink, and coffee (Sekar et al., 2016)), the leather industry (Setati, 2010), bioremediation (treatment of saline and hypersaline wastewaters (Sekar et al., 2016)), the degradation of biopolymers for treatment of oilfield waste (Moreno et al., 2013), stabilizers for biomolecules, and stress-protective agents (Waditee-Sirisattha et al., 2016). It has even been mentioned that these enzymes have different immunological properties, and may be used for the treatment of acute lymphoblastic leukemia and tumor cells (Shirazian et al., 2016).

Hydrolases catalyze reactions by adding a water molecule, causing the rupture of various chemical bonds. They can hydrolyze substrates such as carbohydrates, lipids, proteins, and nucleic acids. These enzymes are produced in the lysosome, and its membrane keeps enzymes out of the cytosol (Alberts and Bray, 2006). Hydrolases carry out a hydrolysis of ester, ether, peptide, osydic, anhydrous, C–C, and halogen or P–N bonds (Maillet, 2002). Extracellular degrading hydrolases are used by microorganisms to employ organic compounds in different ecosystems (Enache and Kamekura, 2010).

13.2 SOURCES AND SCREENING APPROACH OF FOOD ENZYMES

Enzymes are biological catalysts that can be used in food processing to improve quality, appearance, aroma, freshness, structure, and shelf life or to add functionality and nutritional value in many different food products (Simpson et al., 2012). The enzymes used in the food industry are diverse, but based on the reaction type and what they catalyze, enzymes are classified into six categories (Table 13.1).

Enzymes are used for brewing, ripening, coagulation, baking, cheese or yogurt making, hydrolysis, and cell rupture or molecular structure modification (Simpson et al., 2012). For example, enzymes are used to clarify juices or to improve extraction yields in the cell rupture of oil crops (van Oort, 2010). Within the global industrial enzyme market, food and beverage enzymes represent around 29% of total sales with revenues close to $1.3 billion in 2013; they are expected to reach $2.3 billion by 2018 (Adrio and Demain, 2014; Markets and Markets, 2016). Common enzymes used in the food industry can be produced by extraction from plant and animal tissues or through fermentation using microorganisms (Dodge, 2010). According to Fraatz et al. (2014), approximately 260 different enzymes are available in the European Union with around 91% obtained through fermentation and 9% through extraction (Fig. 13.1).

It is noticeable that fermentation is becoming the main production method because of recent developments in biotechnology and the numerous advantages of microbial enzymes. Within fermentation, the production of enzymes has been mostly performed through submerged-state fermentation, although solid-state fermentation (SSF) has been gaining relevance in recent years (Singhania et al., 2010; Thomas et al., 2013). The activity of an enzyme depends on several factors such as temperature, pH, the amount and type of substrate, and, in some cases, specific properties may be required. For example, high thermal and operational stabilities are required for glucose isomerase in the production of high fructose corn syrup

TABLE 13.1 Mechanism of Action Catalyzed by Some Enzymes Used in the Food Industry

Class	Type of Reaction	Examples
I	Oxidoreductases	Laccase, lipoxygenase, glucose oxidase
II	Transferases	Fructosyl transferase, transglutaminase
III	Hydrolases	Amylase, glucanase, invertase, lactase, lipase, protease, pectinase, phytase, xylanase
IV	Lyases	Acetolactate decarboxylase
V	Isomerases	Racemases, epimerases, cis-tran sisomerases, tautomerases, mutases, arabinose isomerase
VI	Ligases	Synthases

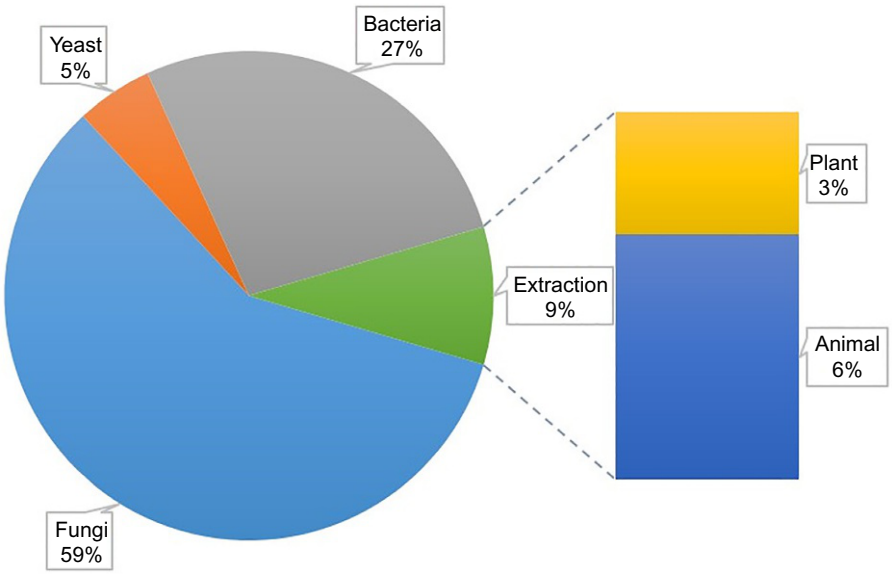

FIG. 13.1 Source pattern distribution of available enzymes in EU.

but cold-active pepsins are need for the riddling process in caviar production (DiCosimo et al., 2013; Zhao et al., 2012). Given the requirement of enzymes capable of working under such specific conditions, as mentioned above, there has been a growing interest in microorganisms from extreme environments as sources for enzymes (Gupta et al., 2014).

To increase the successful application of enzymes in the industrial process, a suitable screening must be applied, considering the required features: activity, enantioselectivity, specificity, stability, and tolerance toward the substrate, product, and solvent used (Singh et al., 2013). However, given the nature of their applications and the implications on public health, enzymes must be assessed for biodegradability and must be labeled as generally

regarded as safe (GRAS), for which they must comply with a set of regulatory constraints (Agarwal and Sahu, 2014; Spök, 2006). Enzyme efficiency is another important parameter: it must be high even at low concentration, which is around 0.1% or less of the product (Paul et al., 2016) under mild conditions. It could be used several times faster than other catalysts, reducing the processing costs in food processing (James et al., 1996; Simpson et al., 2012). It is now possible to obtain enzymes with a higher degree of purity and yields at reduced costs because of recent developments in industrial biotechnology.

13.3 FOOD ENZYMES FROM EXTREME ENVIRONMENTS

An extreme environment can be defined as a set of environmental conditions that can be adverse to the normal development of living things, including microorganisms. These environmental conditions can be a challenge for the survival of living beings that develop there because they may be exposed to acid or alkaline soils, very high or very low temperatures, high relative humidity or low relative humidity, etc. It is often believed that the development of life in extreme environments is almost impossible; however, there are living organisms that are capable of developing in environments such as the one mentioned above. Flora, fauna, and very special microorganisms have overcome the challenge of living in extreme environments, being exposed to different types of climates and different growth conditions. All the above have made these organisms very interesting options for obtaining different biological agents with interest and application in different fields of industry, increasing the added value of natural sources present in extreme environments.

One of the groups of living beings that can be developed in extreme environments is microorganisms, which are able to grow in adverse environments such as semidesert and are capable of producing biological agents of interest. Some microorganisms have been isolated from environments considered extreme, such as the Mexican semidesert. The main microorganisms that have been isolated from this environment have been filamentous fungi (Cruz-Hernández et al., 2005). The isolation of filamentous fungi has been reported from soil and plants that belong to the Mexican semidesert, the main species belonging to *Aspergillus* genus. In previous works, it has been shown that fungi of this genus are capable of producing a variety of enzymes that may have potential applications in different fields of industry (Ramírez-Coronel et al., 2003). For example, the *Aspergillus niger* Aa-20 strain has been reported to produce hydrolases enzymes. The most important enzyme for the degradation of tannins is known as tannase (Aguilar et al., 2001). In addition, other *Aspergillus* species, such as *Aspergillus niger* PSH and *Penicillium commune* EH2, have been reported to be capable of producing enzymes of industrial importance (Cruz-Hernández et al., 2005).

One of the most interesting species that has been isolated from environments such as the Mexican semidesert is the *Aspergillus niger* GH1 strain, which was isolated from a plant characteristic of this area, the creosote bush (*Larrea tridentata*) (Belmares et al., 2009). This strain has the capacity to produce enzymes of importance, and one of them is tannase, which is a hydrolase enzyme that has an industrial application in the food field, for example, in the production of some beverages (Belmares et al., 2003). It has activity at pH 5.0–5.5, at a temperature of 50°C, 60°C, and 70°C with an optimal value of 35°C, and has a protein structure with a relatively high molecular weight (300 kDa) (Pinto et al., 2005). Tannase has been used in the manufacture of instant beverages such as tea, where it has been used as a clarifying agent to

remove undesirable compounds and to make more bioavailable the antioxidant compounds that beverages such as tea have (Mata-Gómez et al., 2009). This enzyme has also been used for the extraction of antioxidant compounds such as gallocatechin, gallic acid, and some other phenolic acids (Pinto et al., 2005). In addition, tannase has been used to clarify fruit juices, beers, and wine, removing phenolic substances that can decrease the quality of the brewed beverage (Ramos et al., 2011).

Fungi of the *Aspergillus* genus (some isolates of environments such as the semidesert), in addition to tannase, produce other enzymes that are important in the food industry. The production of enzymes such as polyphenoloxidase by *Aspergillus niger*, which is used in the food industry as a catalyst for the release of phenolic compounds present in fruit drinks to increase their antioxidant potential (Shi et al., 2005), is being reported. Another enzyme of importance in foods produced by this genus is β-glucosidase, which acts by releasing bioactive compounds from their sugar nuclei to maximize the biological potential that they have and to apply them in the formulation of functional foods (Ascacio-Valdés et al., 2011). The same function has been reported for enzymes such as xylanase (Huang et al., 2007). As mentioned, the *Aspergillus niger* GH1 fungus has been isolated from the semidesert and is capable of producing different enzymes with industrial potential. It has been reported that it can produce tanase, polyphenoloxidase, β-glucosidase, and xylanase, which have importance in foods (Ascacio-Valdés et al., 2014). However, this fungus has been shown to produce a novel enzyme capable of biotransforming phytochemical compounds into biologically active molecules, for example, antioxidants. It has been reported that this enzyme is able to transform compounds present in pomegranate juice, such as punicalagin, to obtain ellagic acid (a potent antioxidant), which made it possible to identify the main intermediates and understand this process of biotransformation (Ascacio-Valdés et al., 2016).

This enzyme was reported as elagitannase. The optimization process has also been reported to produce the best yields of this enzyme using *Aspergillus niger* GH1 in solid-state fermentation. In the next section, we describe some enzymes with industrial potential produced by microorganisms isolated from extreme environments.

13.4 ENZYMES PRODUCED BY HALOPHILIC MICROORGANISMS

Halophiles are "salt-loving" microorganisms that tolerate up to 35% NaCl (Castillo-Carvajal et al., 2014). Based on the concentration of NaCl required for their growth, these microorganisms are categorized into five major classes according to Kushner and Kamekura (1988) (Table 13.2).

Such exceptional microorganisms have been found in locations such as the Dead Sea (Israel), Lake Urmia (Iran), the solar salterns (Tunisia), the Tuzkoy salt mine (Tunisia), and the Great Salt Lake (USA), among others. Enzyme-producing halophilic bacteria have also been reported from the hypersaline Gölü Lake (Turkey). These microorganisms produce enzymes such as amylasesm cellulases, cascinases, gelatinases, lipases, catalases, and oxidases (Birbir et al., 2007). Moreover, Edbeib et al. (2017) isolated a novel dehalogenase-producing bacteria, identified as *Pseudomonas halophila* HX, from this lake. Therefore, this could be a potentially practical ecofriendly strategy for cleaning up coastal contaminated areas, which is relevant because halogenated compounds represent a potential long-term danger to human well-being and health.

TABLE 13.2 Halophile Classification Based on Salt Tolerance (Kushner and Kamekura, 1988)

Class	Name	Salt Tolerance (NaCl)
1	Nonhalophiles	˂0.2 M (approx. 1%)
2	Mild halophiles	0.2–0.5 M (approx. 1%–3%)
3	Moderate halophiles	0.5–2.5 M (approx. 3%–15%)
4	Borderline extreme halophiles	1.5–4.0 M (approx. 9%–23%)
5	Extreme halophiles	2.5–5.2 M (approx. 15%–32%)

On the other hand, the development of renewable energy sources as alternative fossil fuels is gaining attention worldwide and lignocellulosic biomass is a promising source for the commercial production of biofuels and value-added chemicals. However, the structural characteristics of lignocellulose make it extremely resistant to enzymatic degradation (Sun et al., 2016). For this reason, different pretreatments have been proposed. One of them is pretreatment with ionic liquids (ILs), which results in the liberation of amorphous and porous cellulose that is more amenable to degradation by cellulases (Xu et al., 2014). However, the major technical obstacle is the inhibition of cellulases in the presence of ILs commonly used for lignocellulose dissolution because of a low water activity environment. Because hypersalinity also creates a low water activity environment, halophilic cellulases are expected to have IL tolerance as well as thermostable cellulases, which are expected to be resistant to their denaturing effect as they typically have rigid, three-dimensional structures (Xu et al., 2016). This was demonstrated by Mesbah and Wiegel (2017), who found a novel halophilic, alkaliphilic, thermostable and IL-tolerant B-1,4 linkage-specific cellulase from *Alkalilimnicola* sp. NM-DCM1, properties that give potential to this enzyme for application as part of an enzyme cocktail for effective degradation of lignocellulosic biomass.

Other important enzymes are xylanases, which are used in bread making that significantly improve the desirable texture, loaf volume, and shelf life of bread (Dutron et al., 2011). Together with pectinases, carboxymethylcellulases and amylases are used for the clarification of juices (Motta et al., 2013) and they are also used in the brewing industry in order to improve filtration efficiency (Wang et al., 2016). Xylanases can be also used in the production of xylooligosaccharides with prebiotic effects for the development of functional foods. Certain strains of yeast such as *Aureobasidium pullulans* produce extremely high extracellular xylanase, and are also cellulase-free (Li et al., 1993). Yegin (2017) found that *A. pullulans* NRRL Y-2311-1 produced a xylanase with an optimum pH and temperature activity of 4.0 and 30–50°C, respectively. Also, the enzyme was stable in the pH range of 3.0–8.0 and retained almost complete activity at a concentration range of 0%–20% NaCl. Based on the requirement of acidic conditions to exhibit its activity, this xylanase may have potential application in fruit juice production. However, due to its extreme halophilicity, it could have potential application in the processing of seafood and saline foods. Besides its wide pH stability range, this enzyme also showed high tolerance to ethanol, which makes it very useful for wine making or brewing (Yegin, 2017).

13.5 GENERAL STRUCTURE AND PROPERTIES OF HALOPHILIC ENZYMES

Halophilic microorganisms are very interesting at the molecular level for their capacity to survive; they principally need a hypersaline environment. In the last two decades, halophilic microorganisms have been focus of intensive study, the main discovery is that they are able to produce enzymes with potential in different biotech industries. The study of halophilic enzymes is focused, first, on their special structural characteristics because until now some mechanisms and properties have not been fully understood, and second, on possible applications in different industrial areas (Liszka et al., 2012). The key of stability of halophilic enzymes in extreme conditions is provided for two important structural characteristics: (a) an increase of acidic amino acid located in the protein surface, and (b) salt-molecular interactions that allow a "solvation-stabilization model" versus the hypersaline environment (DasSarma and DasSarma, 2015; Sinha and Khare, 2014).

13.6 AMINO ACID COMPOSITION IN HALOPHILIC ENZYMES

Halophilic enzymes tend to have an excess of acidic amino acids and a small number of large nonpolar amino acids on the surface. Particularly, their negative charge is due to low composition of hydrophobic side-chain amino acid residues (phenylalanine, isoleucine and leucine), a small proportion of other small hydrophobic residues (glycine, alanine, serine, and threonine), a deficit of basic amino acids (lysine and arginine), a hydrophobic nucleus and an excess of acidic amino acids (glutamic and aspartic acid) on the surface that increase the loops in the structure of the protein (DasSarma and DasSarma, 2015; Graziano and Merlino, 2014; Kastritis et al., 2007). Around 64% of the total number of amino acids is Asp or Glu, compared with thermophiles that have around 50% of such amino acids. The presence of negative amino acids on the surface favors folded protein conformation, maintaining a weak repulsive protein-protein at high salt concentrations, and allowing an optimum geometry in native conformation surrounded by water and Na^+, K^+ or other ions that stabilize and regulate the catalysis because these ions displace water molecules around the amino group (Asp and Glu, principally) (Graziano and Merlino, 2014; Kramer et al., 2012; Lenton et al., 2016; Sinha and Khare, 2014; Talon et al., 2014). The salt presence (in the case of strict halophilic proteins) can protect them from denaturation, principally in the secondary and tertiary conformation, by providing a molecule more flexible on the surface but more rigid in the protein core with good stability (Sinha and Khare, 2014). Particularly, the preference of short-chain amino acids such as aspartic acid play a role in stability through the participation of hydrophobic packing, increasing the number of interactions (Lenton et al., 2016; Madern et al., 2008; Tadeo et al., 2009; Zaccai, 2013). Asp and Glu are able to create hydrogen bonds for carboxylate chains with the water molecules having a major affinity for Asp (Rhys et al., 2012). A study of acidic amino acids on protein surfaces led to understanding their functions under folded conformation, molecular interactions, aggregation prevention, and other structural aspects of halophilic proteins (Lenton et al., 2016).

13.7 SALT-PROTEIN DYNAMICS: SOLVATION PROCESSES

To maintain protein solubility, different mechanisms have been developed by halophilic microorganisms. In the presence of a high concentration of salts, protein is enveloped by a solvation shell, allowing an equilibrium due to the exclusion of small solutes by the solvation shell, avoiding protein precipitation (Talon et al., 2014). The haloadaptation is given for a weakening of hydrophobic interactions, particularly in the conserved contact region but compensated with interaction with NaCl in the medium. To generate protein interactions, the halophilic protein mechanisms are directed toward the interaction of hydrated ions with a negative surface, where acidic amino acids are responsible for the formation of shells of water protecting the protein from low aqueous activity (Graziano and Merlino, 2014). It has been observed that a great organization of several water molecules allows shell formation, generating stability by charge repulsion (Esclapez et al., 2007). To reduce the repulsive electrostatic interactions, the negative charges on the surface are organized geometrically and interact favorably with water and Na^+ molecules, forming a solvation shell that reduces the negative effect of NaCl as a solute in the medium (Graziano and Merlino, 2014).

The carboxyl groups present in acidic amino acids form strong hydrogen bonds that form a solvation shell cooperating with hydrated salt ions. The nature of cations and anions has an influence on interactions and salt bridge formation. For example, in the case of 2Fe–2S ferredoxin from *Haloarcula marismortui*, the K^+ and in *Hm*MalDH, Cl^- ions were important to these salt bridge formations and molecular interactions (Mevarech et al., 2000; Zaccai, 2013). In the same model, such as *Hm*MalDH, (Ginzburg et al., 1970) it has been suggested that the presence of KCl promotes flexibility compared with NaCl, which has more rigidity for enzymatic activity (Zaccai, 2013). During the salting-out processes, halophilic enzymes have a stability given by the hydrophobic effect with the solvent, excluding salt and binding with water (Madern et al., 2008; Reed et al., 2013). The effect of electrostatic interactions, such as salt bridges, stabilizes the protein that interacts with the negatively charged side chains, as well as allowing the interaction of the negative surface with the solvent, while the internal charged residues allow the hydrogen bonding improve thermostability and contribute to alkaline adaptation, a characteristic of some halophilic enzymes (Liszka et al., 2012). Knowledge of structural and mechanisms of halophilic enzymes is conducted to understand their capacity to act principally in low aqueous activity environments, where salt has a "protective effect" against different harsh conditions such as temperatures and solvents. This makes them potentially good candidates for synthesis reactions in biocatalysis processes (Sinha and Khare, 2014).

13.8 HALOPHILIC HYDROLASES

Halophilic enzymes have been isolated from halophilic and halo-tolerant microorganisms. These enzymes, dubbed haloenzymes, possess a conformation and are functionality stable at different salt concentrations as well as other extreme conditions such as temperature. In general, the halophilic proteins have differential features such as high amino acid content on the surface, low lysine content, low hydrophobicity, and increased salt bridge. Characteristics and generalities of some halophilic enzymes such as amylases, proteases, pectinases, lipases, DNAses, and other hydrolases are mentioned in the following (Karan et al., 2012).

13.8.1 Amylases

Amylases catalyze the hydrolysis of starch and can be divided into exoamylase and endoamylase. The α-amylase is one endoamylase that hydrolyzes α-1,4 glycosidic linkages to produce glucose, maltose, and dextrin; it is produced by animals, plants, and microorganisms (Zhang et al., 2017). Amylases have been isolated from halophilic microorganisms found in hypersaline environments with different salt concentrations, such as salt lakes, subterranean rock salt, saline soil, sea sand, and sea. The salt concentrations ranged from 44 to 256 g/L (Enache et al., 2015). Under these salt concentrations, some α-amylases contain a negative charge on their surface that leads to greater stability. Some amylase enzymes with potential uses in several fields of biotechnology, such as detergent formulation and starch hydrolysis at lower temperatures (Chakraborty et al., 2011, Shafiei et al., 2010) and the treatment of saline wastewater, have been reported (Mohammadipanah et al., 2015).

Stability to the organic solvent by different α-amylases has been reported, such as tolerance of α-amylases from *Thalassobacillus* sp. LY18 to DMSO, DMF, methanol hexane, acetone (Li and Yu, 2012); tolerance of amylase from *Amphibacillus* sp. NM-Ra2 to ethanol, methanol, chloroform, and benzene (Mesbah and Wiegel, 2014); the tolerance of α-amylases from *Nesterenkonia* sp. strain F to benzene, chloroform, toluene, and cyclohexane (Shafiei et al., 2011); and the tolerance of α-amylases from *B. agaradhaerens* Mi-10-6 2 to decane, heptane, hexane, and methanol (Pandey and Singh, 2012). Some amylase types produced by *Halomonas meridian*, *Haloarcula hispanica*, and *Natronococcus amylolyticus* are used for the hydrolysis of corn starch to produce high fructose corn syrup (Patel and Saraf, 2015).

Some amylases have been overexpressed to produce recombinant proteins such as α-amylase (EAMY) in *E. coli* JM109 to improve production in other nonhalophilic bacteria with high activity. This enzyme can be used in processed food and fermented food (Wei et al., 2013). Another amylase cloned and expressed in *E. coli* was α-amylase AmyZ2 from the marine bacterium *Zunongwangia profundal*, with potential application in harsh industrial processes (Wu et al., 2014).

13.8.2 Proteases

Proteases are enzymes that break the peptide bonds of proteins; they are divided into acid, neutral, and alkaline proteases. These enzymes can be obtained from plants, animals, and microorganisms in several conditions, such as high salt concentrations. The halophilic proteases possess stable activity at high temperature and ionic strength in presence of organic solvents (Raval et al., 2015). Some protease enzymes have potential uses in detergents, the pharmaceutical industry, bioremediation processes, and food industries (Mohammadipanah et al., 2015).

Stability to the organic solvent by different proteases has been studied, such as the tolerance of protease from the *Halobacillus blutaparonensis* strain M9 to ether, isooctane, and cyclohexane (Santos et al., 2013); and the tolerance of the *Geomicrobium* sp. EMB2 to ethanol, benzene, cyclohexane, and heptane (Karan et al., 2011). In food industries, proteases play a role in fermentation processes to produce compounds with specific characteristic of flavor and aroma. Proteases have also been used in fish sauce preparation (Akolkar et al., 2010), in the pretreatment of leather in the tanning industry, and in the formulation of therapeutic dietary products (Raval et al., 2015).

13.8.3 Pectinases

Halophiles are an excellent source of enzymes such as pectinases with hydrolytic activity, which are salt- and temperature-tolerant (Rohban et al., 2009). These enzymes are also polyextremophilic, thermostable, and alkaliphilic, properties that are important for distinct industrial processes (Yin et al., 2015). Pectinases catalyze the decomposition of pectin, a key plant carbohydrate. However, this is not a single enzyme, but instead a heterogeneous group of enzymes. They are employed in various applications and are also among the most sold enzymes, contributing 40% to the participation of all food enzymes (Adapa et al., 2014). These enzymes have been an important part of food processing, where they have been employed for pectin degradation. They also facilitate distinct stages of processing such as clarification, liquefaction, and extraction of juice as well as being used in the vinification and extraction of natural oils (Adapa et al., 2014). Pectinases, together with xylanases, carboxymethylcellulases, and amylases, are used in the food industry for the clarification of juices (Yegin, 2017), these enzymes play an important role in many physiological processes such as fruit ripening (Kutateladze et al., 2009).

Babavalian et al. (2013) found that gram-positive bacilli, gram-negative bacteria, and gram-positive cocci are good producers of pectinases. Kutateladze et al. (2009) reported that pectinase producers are represented by *Aspergillus, Trichoderma, Sporotrichum,* and *Penicillium* genera. It has also been mentioned that pectinase is produced by different yeasts such as *Debaryomyces nepalensis* NCYC 3413 (Gummadi et al., 2007), *Kluyveromyces marxianus*, *Saccharomyces cerevisiae,* and *Geotrichum klebahnii* (Cavello et al., 2017).

13.8.4 Lipases

Lipase (triacylglycerol acilhydrolase, EC 3.1.1.3) is responsible for catalyzing the hydrolysis of ester linkages of triacylglycerols to glycerol and to free fatty acids at the oil-water interface, and of not hydrolyzing the substrates that are dissolved in the bulk fluid (Kanlayakrit and Boonpan, 2007; Ozcan et al., 2009; Schreck and Grunden, 2014). Lipases are among the most important hydrolytic enzymes used in a variety of biotechnological applications (Amoozegar et al., 2008). The halophilic enzymes and bacteria require NaCl for its activity and for their growth (Kanlayakrit and Boonpan, 2007). It has been reported that lipases preferentially act on emulsifying substrates with long-chain acids (Moreno et al., 2009). Lipases of microbial origin have many applications in different fields, such as foods, agrochemicals, pharmaceuticals, and cosmetics (Ghasemi et al., 2011). Moderately halophilic bacteria that can grow optimally in media containing 3%–15% NaCl are a valuable source of lipases. A study was performed using halophilic lipases from the *Staphylococcus warneri* PB233 strain to improve the taste and aroma of edible products such as fish sauce (Kanlayakrit and Boonpan, 2007).

It has been reported that *Bacillus cereus* C71, *B. thermoleovorans* ID-1, *B. coagulans* BTS-3, *Geobacillus* sp. TW1, *Bacillus* sp. strain L2, *B. sphaericus* 205y, *B. bogoriensis* sp., *B. salaries* sp., *B. sphaericus* JS1, and B. *vallis-mortis* BCCS 007, among other species of *Bacillus* genus, can produce lipases (Ghasemi et al., 2011). The species of *Bacillus* are taxonomically very diverse and have been isolated from different saline environments. It has recently been reported that *Natronococcus* sp. TC6 (Martin del Campo et al., 2015; Schreck and Grunden, 2014), *Halobacillus*

sp. AP-MSU 8 (Esakkiraj et al., 2016), and *Marinococcus halophilus* JCM 2472 strain (Neagu et al., 2016) are producers of lipase.

13.8.5 Deoxyribonucleases (DNAses)

Many halophilic Archea are commonly polyploids; some species may contain up to 30 genome copies. DNA contains 10% phosphorus by weight, which has allowed its discovery as a phosphorus storage polymer by many Archea. There is a strong relationship between the degree of ploidy and the level of available nutrients. In this way, by degrading DNA outside the cells, halophilic Archea can obtain phosphorus (Oren, 2014). The use of exogenous double-stranded DNA as a nutrient to supply essential phosphorus was demonstrated while studying the extracellular metabolism of *Haloferax volcanii* (Chimileski et al., 2014). By this reason, many Archea have discovered the advantage of DNA. *Vibrio* genus is a large group of Gram-negative bacteria isolated from marine and brackish water as well as human origins that show the requirement of sodium ion for growth (Al-Saari et al., 2015). Strains of this genus show versatile metabolism ability; they are capable of degrading and assimilating complex organic matters such as polysaccharides (Thompson and Polz, 2006). Gao et al. (2016) isolated five alginolytic *Vibrio* strains from seawater samples collected from a coral reef area near Ishigaki Island, Okinawa, Japan, that were capable of producing DNases. These bacteria belong to the *Halioticoli* clade based on the analysis of genomic, phenotypic, genotypic, and phylogenetic data. Besides the production of DNases, these strains are distinguished by traits such as growth temperature range, lipase production, indole production, and assimilation of 10 carbon compounds.

The production of DNases was also reported by Babavalian et al. (2013) from isolates of the seasonal hypersaline lakes in the central desert zone in Aran-Bidgol Lake, Iran. These authors obtained 61 Gram-positive and 22 Gram-negative isolates that were shown to produce a wide variety of hydrolytic enzymes: DNase, inulinase, amylase, lipase, pectinase, protease, chitinase, pullulanase, cellulase, and xylanase. However, the most common enzymes were DNase and inulinase in Gram-positive bacteria, and lipase in Gram-negative bacteria. According to their molecular identification, these are moderately halophilic strains belonging to the *Halobacillus, Idiomarina, Salicola, Halomonas, Thalassobacillus, Bacillus,* and *Salinococcus* genera. When isolates from different hypersaline lakes were compared, it was found that bacteria from each saline lake could produce different hydrolytic enzymes. For example, isolates from Howz-Soltan (also in the central desert in Iran) did not produce pectinase, DNase, amylase, lipase, or inulinase. However, isolates from Urnia Lake (northwest desert) were also good producers of DNase but failed to show chitinase activity (Babavalian et al., 2014).

13.8.6 Other Hydrolases

Pullulanases hydrolyze 1,6-α-glucosidic bonds of maltotriose, so they can act on amylopectin and dextrins (Badui, 1993). Different halophilic pullulanases have been identified. Siroosi et al. (2014) reported an amylopullulanase isolated from *Halorubrum* sp., which was produced extracellularly and was tolerant to organic solvents. The highest production of this enzyme was obtained at pH 7.0 and 40°C. This enzyme had a molecular mass of about 140 kDa and the apparent K_m for the pullulanase activity was 4 mg/mL at 50°C and pH 7.5 while Elyasifar

et al. (2014) mentioned two halophilic bacteria producing pullulanse enzymes that were identified as belonging to the *Bacillus* genus.

Cellulases is a term used to define a complex system of enzymes that hydrolyze the β-1,4 linkages of the glucans, thus producing cellulodextrins. They are used in the extractive industry (obtaining essential oils) or to soften the cellulosic tissues of fruits and vegetables (Badui, 1993). Nagoor-Gunny et al. (2014) indicated production of halophilic cellulase by *Aspergillus terreus* UniMAP AA-6. These enzymes were used for the saccharification of ionic liquid-treated lignocelluloses. These authors mentioned that halophilic cellulase production is affected positively by carboxymethylcellulose (CMC), $FeSO_4{\cdot}7H_2O$, NaCl, $MgSO_4{\cdot}7H_2O$, peptone, agitation speed, and inoculum size. Other substances such as NaCl (2.5 M) or KCl (3 M) can increase cellulase activity (Zhang et al., 2012). The same authors isolated and cloned the *celB* gene, which encodes a halophilic cellulase of the cellulase superfamily and DUF291 superfamily. This recombinant protein had a molecular weight of 62 kDa and was able to hydrolyze soluble cellulose substrates containing β-1,4-linkages.

Xylanases degrade the linear polysaccharide beta-1,4-xylan into xylose, breaking hemicellulose in this way. Kuo-Sheng et al. (2011) isolated the *xynFCB* gene, which encodes 413 amino acids, and the resulting protein belongs to the glycosyl hydrolyase family 10 (GH10). This gene was isolated from *Thermoanaerobacterium saccharolyticum* NTOU1, a thermophilic bacterium, and cloned. The recombinant protein had a molecular mass of 50 kDa and was able to hydrolyze xylan with optimal activity at 63°C and pH 6.4; optimal activity was observed in 12.5% (*w*/*v*) NaCl. Later, Liu et al. (2014) mentioned cloning and expressed the *xynA* gene, which was isolated from *Zunongwangia profunda*. This gene encodes 374 amino acid residues. The recombinant protein had the highest activity at pH 6.5 and 30°C, and its activity was increased by adding 3 M NaCl. Sanghvi et al. (2014) reported an alkali-tolerant halophilic bacterium. This microorganism was able to produce an extracellularly halophilic, alkali-tolerant, organic solvent stable, and moderately thermostable xylanase. Different substrates induced xylanase production, among them corncobs, sugarcane bagasse, and wheat straw, although the maximum xylanase production was obtained with corncobs at pH 9.0 and 37°C. Xylanase activity was increased by adding Ca^{2+}, Mn^{2+}, and Mg^{2+} and this protein was stable in organic solvents such as glutaraldehyde and isopropanol.

Glucose dehydrogenase catalyzes a reversible reaction involving D-glucose plus an acceptor and D-glucono-1,5-lactone plus a reduced acceptor. This enzyme belongs to the medium-chain alcohol dehydrogenase superfamily and requires a zinc ion for catalysis. Esclapez-Espliego et al. (2014) mentioned that the zinc ion is coordinated by a histidine, a water molecule, and two other ligands. These authors reported a glucose dehydrogenase form *Haloferax mediterranei*, which has a zinc ion within the protein surrounded by three ligands.

L-asparaginase and *L-glutaminase* have been used for the treatment of acute lymphoblastic leukemia and tumor cells (Shirazian et al., 2016). L-glutaminase is an amidohydrolase that produces glutamate from glutamine while L-asparaginase breaks down asparagine. Shirazian et al. (2016) reported a bacterial strain that had the highest L-asparaginase production and did not show L-glutaminase production. The activity of this enzyme can be enhanced with an increase in concentration of NaCl up to 2.5 M, and a pH of 8 and 40°C (Gupta et al., 2016).

Under optimal conditions, the production of L-asparaginase and L-glutaminase increased up to 1.5 (61.7 unit/mL) and 2.6 fold (46.4 unit/mL), respectively (Shirazian et al., 2016).

Chitinases break down the glycosidic bonds of chitin, which is part of the cell walls of fungi and the exoskeleton of some animals. Essghaier et al. (2012) mentioned a halophilic chitinase produced by *Virgibacillus marismortui* strain M3–23. This enzyme is characterized by high activity and stability at different pH, temperatures, and salinity and is related to glycosyl hydrolase family 18. While Han et al. (2014) reported two bacteria strains belonging to *Bacillus* and *Paenibacillus* genera that produced chitinase, the *Bacillus* strain showed two chitinase isozymes of 41 and 50 kDa while the *Paenibacillus* strain showed four 4 bands of 30, 37, 45.7, and 50 kDa.

13.9 IMMOBILIZATION OF HALOPHILIC ENZYMES

Enzyme immobilization refers to the retention of the catalytic properties confined in a defined region or space, which can be used repeatedly and continuously while offering protection from some harsh conditions (DiCosimo et al., 2013; Mohamad et al., 2015). Particularly, halophilic enzymes can be used under some harsh conditions such as high salt concentration, alkaline pH, high temperatures, and especially in organic solvents. However, few halophilic enzymes have actually been immobilized and applied in industrial processes. Within the reports of the immobilization of halophilic enzymes, there is an alcohol dehydrogenase (ADH2) from *Haloferax volcanii* immobilized by a covalent method that obtains an increase of enzymatic and thermal stability in a nonaqueous media reaction (Alsafadi and Paradisi, 2014). A halophilic protease from *Bacillus* sp. EMB9 and an α-amylase from *Marinobacter* sp. EMB8 were immobilized by new trends, using silica nanoparticles with an efficiency of 60% and 96%, respectively. Also, in the case of halophilic protease, there was an increase in pH, thermal stability, and specific activity, obtaining a high degree of hydrolysis of whey proteins. However, in the case of α-amylase, the catalytic properties remained equal to the native enzyme, but this was possible due to reuse and repeated cycles (Kumar and Khare, 2015; Sinha and Khare, 2014). Another report of whole cell immobilization involved a haloalkaline mannase from *Bacillus halodurans* PPKS-2, which was immobilized by gel entrapment providing a continuous production of enzyme (Vijayalaxmi et al., 2013) Another case is the use of whole cell immobilization of haloarchaea *Natrinema gari* BCC 24369, used for the histamine degradation of fish sauce. This had 51% histamine removal, and the dark color was reduced using a fixed-bed bioreactor (Chaikaew et al., 2015). Each immobilization method has advantages and disadvantages and the selection of one involves some important aspects, such as method type, support used, enzyme application, reaction media, etc. (Table 13.3) (Datta et al., 2013; Homaei et al., 2013).

13.10 IMPROVEMENT OF ENZYME ACTIVITY THROUGH PROTEIN BIOENGINEERING

Halophiles have competent industrial applications such as being candidates for the expression of soluble recombinant proteins and proper folding of proteins. Studies have been carried out and efforts have been made to develop genetic tools including vectors, mutagenesis, and promoters for halophilic bacteria to express different genes (Yin et al., 2015).

TABLE 13.3 Immobilization Classical Methods

Immobilization Method	Principle	Advantages	Disadvantages	References
Chemical	Covalent	High stability, hyperactive, biocatalysts, major thermal stability	Irreversible, size and shape of support affect activity, enzyme direction	Illanes et al. (2008), Szymańska et al. (2009), Mohamad et al. (2015)
	Noncovalent	Reversible, it is possible the change to fresh enzyme, low cost	Enzyme leakage, from matrix, distortion of kinetics, alter pH stability	Illanes et al. (2008), Datta et al. (2013)
	Carrier free Cross-linked enzyme aggregate (CLEA)	Soluble protein, high specific activity, for multimeric enzymes	Irreversible, control of reagent cross-linking for particle size, temperature, pH	Illanes et al. (2008), Mohamad et al. (2015)
	Carrier free Cross-linked enzyme crystal (CLEC)	High stability to harsh conditions, high specific activity	Purified enzyme, poor mechanicals properties	Illanes et al. (2008)
Containment	Gel entrapment	Biocatalyst robust, easy recover, mechanical stability, minimize enzyme leaching, modification of encapsulated	Irreversible, limitation of mass transfer, enzyme leakage, low loading capacity, abrasion of support material	Illanes et al. (2008), Singh (2012), Shen et al. (2011)
	Microencapsulation (micelles and liposomes)	Adequate microenvironment, minor limitations of mass transfer	Mechanical weakness, water-organic interface, denaturation	Illanes et al. (2008)
	Ultrafiltration	Use a membrane that retains the enzyme and liberates the products	Enzymatic inactivation by interaction by interfaces	Illanes et al. (2008)

Extremophiles are a source of enzymes with high stability and activity under extreme conditions, previously considered to be contradictory to biological molecules (Shafiei et al., 2012).

Recently, the bioengineering of proteins has been used for distinct objectives, including improvement of enzymatic activity. Wei et al. (2013) carried out a study where a gene of the halophilic a-amylase (EAMY) from the *Escherichia coli* JM109 strain was overexpressed in *E. coli* XL10-Gold, and then the recombinant protein was purified and characterized. It was found that EAMY activity varied with the presence of Na^+ and Cl^-, and the maximal activity was obtained with 2 M NaCl at 55°C and pH 7.0. This report on a halophilic α-amylase (EAMY) shows a high salt tolerance, which makes it a good candidate for use in foods such as the processing of seafood and fermented foods, among others. On the other hand, an extracellular lipase produced by marine fish, isolated from *Halobacillus* sp. AP-MSU, was purified and characterized. This lipase is halotolerant and, to obtain the best enzymatic activity, requires a concentration of 2.5 M NaCl (Esakkiraj et al., 2016).

13.11 FUTURE TRENDS

13.11.1 Hydrolases From High Halophilic Microorganisms

Most of the studies on halophilic proteins used low or moderately halophilic groups of microorganisms. For example, Kumar et al. (2012) reported the isolation of different microorganisms belonging to *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus*, *Halomonas,* and *Staphylococcus* genera, which were characterized because they are moderately halophilic bacteria and had a salt requirement in the range of 3–20%. On the other hand, adaptations of individual halophilic proteins at a molecular level are of particular significance for their activity (Munawar and Engel, 2013). Especially, the amino acid composition has an effect on the halophilic enzyme's thermodynamic stability and its catalytic activity (Gabriel Ortega et al., 2011). Another important factor is their high negative surface charge, which makes them more soluble at conditions under which nonhalophilic proteins tend to aggregate and become rigid (Mevarech et al., 2000). For these reasons, it is important to search for halophilic hydrolyzing proteins in microorganisms adapted to environments with high salt concentrations.

13.11.2 Halophilic Gene Sequencing

Halophilic microorganisms have special features to tolerate hypersaline environments, such as avoiding osmotic shock by increasing the ion concentration of their cytoplasm (Gabriel Ortega et al., 2011). The amino acid sequence differences of halophilic in comparison to nonhalophilic enzymes or those containing a zinc ion within the protein surrounded by three ligands can affect their activity and stability (Esclapez-Espliego et al., 2014). Halophilic enzymes have evolved toward a biased amino acid composition to remain folded and active (Gabriel Ortega et al., 2011). The enzymes of extreme halophilic organisms have special characteristics such as cysteine one of the zinc ligands, is replaced by an aspartate residue at position 38 (Esclapez-Espliego et al., 2014). According to these antecedents, it is very important to perform more sequencing of genes and proteins of extremely halophilic microorganisms to better understand how halophilic microorganisms can tolerate hypersaline environments and to search for more applications for these enzymes.

13.11.3 Nonculturable Microorganism

Most halophilic hydrolase enzymes have been studied, cloned, and sequenced using cultivable microorganisms, but less than 10% of all microorganisms can be cultivable. Most of the microorganisms living in extremely salty environments are noncultivable and may possess very interesting hydrolysed enzymes. Therefore, it is very important to study, clone, and sequence noncultivable microorganism's enzymes.

13.11.4 Cloning

More information on recombinant proteins is needed to understand if the general pattern of high negative charge density on the surface of halophilic proteins (Munawar and Engel,

2013) or the coordination of the zinc ion by a histidine, a water molecule, and two other ligands from the protein or the substrate (Esclapez-Espliego et al., 2014) is conserved after cloning in different microorganisms. In this area, new advances in synthetic biology will play an important role.

13.11.5 Enzyme Production Optimization

Munawar and Engel (2013) mentioned that a barrier for the wider adoption of halophilic enzymes at the industry level is the difficulty of producing these enzymes in bulk at the same scale and with the same capacity as that of mesophilic enzymes. Niño-de-Guzmán et al. (2008) optimized the production of a halophilic hydrolase enzyme that showed maximum activity values at pH 9, 30°C, and 1.7 M NaCl. In this context, it is important to use more effective statistical designs for the optimization of enzyme production than those currently used.

13.11.6 Synergistic Effects

Most of the study involves one hydrolase enzyme or one enzymatic activity, but combined hydrolytic activity has been reported. One halophilic bacterial strain combined cellulase and esterase activities while other microbial strains combined two or more hydrolytic activities (Enache et al., 2014). The combined hydrolytic activities of halophilic microorganisms may be used for the bioconversion of organic materials (Gupta et al., 2016).

13.12 CONCLUSIONS

Hydrolases of halophilic origin have traits very different from that of the mesophilic ones. These enzymes are tolerant to extreme conditions such as pH, temperature, and high concentrations of salt. This makes them able to be used in the industry, mainly in food, the production of cured meats, brines, the processing of sea food products, and the treatment of the waste generated during these processes. In this way, it is possible to satisfy the need to have clean processes, industrial profitability, and biotechnological improvements.

Acknowledgments

MDG, SVM, and MRMM want to thank the National Council for Science and Technology (CONACYT) Mexico, for the financial support during their postgraduate studies.

Conflict of Interest

Authors declare no conflicts of interest.

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CHAPTER

14

Fungal Proteases and Production of Bioactive Peptides for the Food Industry

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14.1 INTRODUCTION

Proteins are a fundamental element in foods which can not only provide a wide variety of sensorial characteristics, but also have recently gained attention as sources of functional molecules, like small protein chains. One release from of these from a parental protein can affect biological activities in living organisms after consumption or addition to food, and they can be produced through different techniques, but enzyme technologies can promote their usage in the food industry. Microbial enzymes could be more advantageous than enzymes extracted from animals and plants, because microbial enzymes can be produced in a relatively small space and short time with minimal nutritional requirement, directly impacting their production costs. In this chapter, fungal enzymes will be discussed in detail due to their biochemical characteristics, unique pattern of protein hydrolysis, and the possibility to generate peptides with more accentuated properties.

https://doi.org/10.1016/B978-0-12-813280-7.00014-1

14.2 BIOACTIVE PEPTIDES

Terminus bio-active peptides were first used in 1950 by Mellander, who suggested that a set of peptides with phosphorylated motifs, derived from casein, could promote calcification in infants (Korhonen and Pihlanto, 2003), and at present, much information has been collected on this topic as it has gained scientific and commercial interest. Bio-active peptides are defined as amino acid sequences with lengths from 2 to 20 units, which are encrypted and inactive in parental proteins, but can be released through different methods including enzymatic hydrolysis, and can then be consumed, allowing them to exert influence over biological activities as they act like hormone molecules, promoting and improving human health, or having a prophylactic effect against a set of chronic illnesses (Carrasco Castilla et al., 2012; Hartmann and Meisel, 2007; Mohanty et al., 2016; Sharma et al., 2011). This type of molecule can exhibit miscellaneous bio-functionalities, dependent on various characteristics such as amino acid sequences, extension, hydrophobicity, and charge, also can present multifunctionalities and, therefore, act in different systems (Dziuba and Darewicz, 2007; Meisel and FitzGerald, 2003).

14.2.1 Antioxidant Effect

At low or moderate concentrations, highly reactive molecules derived from reactions arising from organisms' aerobic respiration, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Pisoschi and Pop, 2015), perform beneficial functions such as defending against agents of infection. When their amounts increase, in a series of cellular signaling processes (Valko et al., 2007), they can overwhelm the biological mechanism that regulates presence of these compounds, in a situation known as oxidative stress (Touyz and Schiffrin, 2007), and in this setting, they can generate damage in biological molecules such as lipids, proteins, or DNA, making the organism susceptible to pathologies like diabetes mellitus, cancer, inflammatory or neurodegenerative diseases (Sila and Bougatef, 2016). Oxidative reactions can also deteriorate foods' sensorial and nutritional qualities (Antolovich et al., 2002). For these reasons, the addition and consumption of antioxidant compounds is important in human health and food production, and therefore, protein hydrolysates have gained importance in these fields. Antioxidant activity in protein hydrolysates with diverse enzymes (including fungal enzymes) has been pointed out by diverse authors, from proteins including bovine plasma, sunflower protein, and meat waste products (Bah et al., 2016; Cai et al., 1996; Ryder et al., 2016).

14.2.2 Antihypertensive Effect

Hypertension is a disease in which patients present chronic blood pressure elevation; it contributes to 9.4 million deaths worldwide through cardiovascular complications (Roe and Khong, 2015), but it is a controllable risk factor (Toth et al., 2011). Hypertension is developed by a combination of complex factors ranging from genetic to environmental, and it increases both the risk of organ injury and maternal/fetal health risks and mortality (Ejike et al., 2017; Kurpas et al., 2015) Physiologically, blood pressure is controlled through various mechanisms such as the Angiotensin-Converting Enzyme (ECA), which converts Angiotensin I to Angiotensin II (a potent vasoconstrictor) in vitro, and this molecule releases aldosterone, and

consequently raises sodium concentration, increasing blood pressure (Hernández Ledesma et al., 2011). These mechanisms are targeted with drugs for treatment of hypertension, in which synthetic medications like Captopril or Enalapril are prescribed, but these compounds can induce adverse effects such as cough, allergies, taste disturbances, and skin alterations. Therefore, wide efforts are focused on discovering new antihypertensive molecules to replace them (Harnedy and FitzGerald, 2012). Protein hydrolysates appear to be an alternative to these medications, as short peptides with an extension from 2 to 12 units and with the presence of Tyr, Phe, Trp, and/or Pro in the C-terminal, acting as inhibitors of ECA (Hernández Ledesma et al., 2011).

14.2.3 Antimicrobial Effect

Different authors have detailed activity from food protein hydrolysates with antimicrobial effects (Di Bernardini et al., 2011; Guinane et al., 2015; Théolier et al., 2013), attributed to mass, amino acid composition, charge, or hydrolysis grade (Brogden and Brogden, 2011; Pihlanto-Leppälä et al., 1999). In particular, literature details a composition with hydrophobic amino acid residues that promotes cationic strength (Hancock and Sahl, 2006) acting through a variety of mechanisms such as binding to the cell membrane (which is charged negatively), and then folding, causing permeabilization of the cell. In other cases, they can cross cell membranes and act like targets in a set of crucial functions for pathogens such as enzymatic activity, DNA, membrane protein, or cell wall synthesis (Sibel Akalin, 2014).

14.2.4 Mineral Binding Effect

Adequate bio-ability of minerals like calcium, iron, and others, has health repercussions; deficiencies in these ions can cause health disorders such as dental caries, anemia, or osteoporosis (Huang et al., 2015; Peng et al., 2017); a set of protein hydrolysates can exert a mineral binding action mainly described as being derived from milk proteins like casein phospho-peptides (CPPs), which present a phosphorylated sequence rich in serine, creating a polar acidic domain that traps calcium and other divalent ions in solution at intestinal pH (Phelan et al., 2009; Sharma et al., 2011) preventing their precipitation and promoting intestinal absorption (Meisel and FitzGerald, 2003). Otherwise, peptides from whey proteins (alfa-lactoglobulina) also can exert mineral binding or chelating effects, enhancing their bio-ability but with a higher affinity for iron, in which amino acids like Glu, Asp, Lys, Arg, and His contribute (Kamau et al., 2010).

14.2.5 Hipocholesterolemic Effect

Cholesterol is a steroidal compound, present in mammalian tissues, which can be synthetized in the body but can also be absorbed from dietary sources (Talapatra and Talapatra, 2015). Cholesterol is an important bioactive compound in organisms which acts as a precursor for important molecules like Vitamin D3 in skin, bile salts, and steroidal hormones, and is also an important component in cell membranes (Mathias, 2016). But high amounts of cholesterol in the blood rises the risk for developing or aggravating CVDs (cardiovascular diseases) (Goldstein and Brown, 1990). It has been observed that a set of protein hydrolysates components can promote a decrease in hypercholesterolemia.

The mechanism through which these molecules exert their action has not been well elucidated yet, but it is suggested that they intervene in cholesterol metabolism, acting like competitive enzyme inhibitors that take part in their biological synthesis and the transcription of low density lipoprotein receptors (Lammi et al., 2015), which constitute a system to transport cholesterol to peripheral tissues (Ridker, 2014) and reduce their capacity for absorption in the intestine, disrupting their transporting micelles (Zhong et al., 2007). Amino acid composition executes a crucial function in this activity, and it has been observed that fractions with high hydrophobicity can exert a cholesterol lowering effect, where amino acids like Ala, Tyr, Val, Leu, or Lys are included (Megías et al., 2009). A set of dietary hydrolysates from dietary proteins like soy, rice, salmon, and bovine milk exhibit this class of activity (Lammi et al., 2015; Nagaoka et al., 2001; Wergedahl et al., 2004; Zhang et al., 2012).

14.3 PROTEASES

Enzymes are commonly used in a wide diversity of industrial applications, because they present numerous advantages compared to traditional chemical catalysts. Some of these advantages are the following:

a) Higher reaction rate: The rate of a reaction catalyzed by enzymes is 10^6 to 10^{12} times greater than when uncatalyzed and several orders greater of magnitude than chemically catalyzed reaction.
b) Soft reaction conditions: Enzymatically catalyzed reactions occur at temperatures below 100°C, at atmospheric pressure and a pH close to neutrality. In contrast, chemically catalyzed cells require high temperature, high pressure, and extreme pH.
c) High specificity of reaction: Enzymes have higher specificity than chemically catalyzed reactions on substrates and their products. In enzymatic reactions, secondary products are highly reduced or even avoided altogether.
d) Reaction rates can be easily controlled through adjustment of temperature, pH, or enzyme concentration.
e) Enzymes are easily inactivated once the desired degree of transformation is achieved.

Proteases are present in all living organisms, participating in the hydrolysis of unwanted proteins as well as in the regulation of different physiological processes. Proteases are capable of breaking specific peptide bonds of targeted protein-producing peptides (limited proteolysis) and unable to reduce proteins to their constituent amino acids. There are nonspecific proteases which can reduce a complete protein to amino acids (unlimited proteolysis). The proteases represent the largest group of commercially available enzymes worldwide, accounting for 60% of the industrial enzymes market, due to their wide range of applications in food and beverage, cleaning products, animal feed, pharmacy, and cosmetics. Proteases constitute a broad and complex group of enzymes that differ in properties such as substrate specificity, nature of their active sites, catalytic mechanism, optimum pH, optimum temperature, and stability profile. Study of these properties is fundamental for proteases because knowledge of these enzymes' characteristics will dictate their application at the industrial level (Kumar et al., 2004; Sumantha et al., 2005). Table 14.1 shows a list of some commercial proteases and their sources.

TABLE 14.1 Commercial Proteases (Guadix et al., 2000)

Enzyme	Origin
Alcalase 0.6L	*Bacillus licheniformis*
Neutrase	*Bacillus subtilis*
Protease 600L	*Bacillus subtilis*
Fungal-protease	*Aspergillus oryzae*
P.E.M. 2500 S	*Porcine trypsin, bovine trypsin, bovine chymotrypsin*
Corolase PP	*Aspergillus oryzae*
Corolase PS	*Aspergillus oryzae*
Corolase 7089	*Bacillus subtilis*
Corolase 7092	*Aspergillus oryzae*
Corolase 7093	*Aspergillus oryzae*
Corolase 7107	*Aspergillus niger*
Bromelain Takamina	Vegetal (piña)
Papain Takamina	Vegetal (papaya)
Flavourzyme 500 MG	*Aspergillus oryzae*

14.3.1 Definition and Classification

Proteolytic enzymes, also known as proteases or peptidases, belong to the group of hydrolases, and catalyze the degradation of proteins through hydrolysis of peptide bonds with different degrees of intensity and selectivity. A peptide bond is the link between the carboxyl group of one amino acid and the amino group of another amino acid (Rao et al., 1998). Protease possess great diversity in structure and mechanism of action, and therefore, cannot be classified with the general system of enzymatic nomenclature; hence, their classification may be based on peptide cleavage site, functional group present at the active site, and the optimum pH value. When the cleavage site of the peptide chain is considered, proteases are classified into four groups (Rao et al., 1998; Jisha et al., 2013) (Table 14.2):

1) Exopeptidases (3.4.11): Act on terminal peptide bonds of a protein either at N or C terminal end, resulting in free amino acids.
2) Endopetidases (3.4.21–34): Act at internal peptide bonds of a protein, resulting in short peptide chains.
3) Aminopeptidases (3.4.14): Act on the free N-terminal of the polypeptide chain, releasing an amino acid or a dipeptide or tripeptide.
4) Carboxipeptidases (3.4.16–3.4.18): Act on the C-terminal of the polypeptide chain and are subdivided into four groups depending on their catalytic mechanism and the functional group present in their active site.

However, based on the functional group at the active site, proteases can also be classified into four prominent groups (Whitaker, 1993).

TABLE 14.2 Protease Classification

Protease	EC No.	Mechanism	
EXOPEPTIDASES			The cleave occurs at the peptide bond proximal to the amino or carboxy terminal of the polypeptide
Aminopeptidases	3.4.11		The cleave occurs at the N-terminal from a polypeptide
Dipeptidases	3.4.13		Specific for dipeptides
Dipeptidyl-dipeptidases	3.4.14		With release of a dipeptide from an N-terminal polypeptide
Tripeptidyl-peptidases	3.4.14		With release of a tripeptide from an N-terminal polypeptide
Carboxypeptidases			The cleave occurs at the C-terminal from a polypeptide
Serine type proteases	3.4.16		The serine active center is involved in the catalytic process
Metaloproteases	3.4.17		A metal ion in the catalytic mechanism is needed
Cysteine type proteases	3.4.18		The cysteine active center is involved in the catalytic process
Peptidyl dipeptidase	3.4.15		Releases an C-terminal dipeptide from a polypeptide chain
Dipeptidases	3.4.13		Specific for dipeptides
Omega peptidases	3.4.19		Releases an N-terminal residues linked by isopeptide bonds
ENDOPEPTIDASES	3.4.21–3.4.34		The cleave occurs at the internal bonds of the polypeptide chain
Serine proteases	3.4.21		The serine active center is involved in the catalytic process
Cysteine proteases	3.4.22		The cysteine active center is involved in the catalytic process

TABLE 14.2 Protease Classification—cont'd

Protease	EC No.	Mechanism	
Aspartic proteases	3.4.23		The aspartic active center is involved in the catalytic process
Metaloproteases	3.4.24		A metal ion in the catalytic mechanism is needed
Endopeptidases of unknown catalytic mechanism	3.4.99		Acts on a peptide bond

1) *Serine proteases*: These are characterized by the presence of a serine group in the active site. They belong to the group of exopeptidases and endopeptidases. These enzymes are irreversibly inhibited by 3,4-dichloroisocoumarin (3,4-DCI), diisopropylfluorophosphate (DFP), phenyl sulphonyl methyl sulfonyl fluoride (PMSF), and Tosyl L-lysine chloromethyl ketone (TLCK). Some serine proteases are inhibited by agents inhibiting thiol groups, such as p-Chloromercuribenzoate (PCMB), due to the presence of cysteine residues near the active site. They are generally active at neutral and alkaline pH, with an optimum between 7 and 11 (Rao et al., 1998; Whitaker, 1993).

2) *Cysteine proteases*: The activity of the cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Generally, these enzymes are active only in the presence of reducing agents such as HCN or cysteine. Cysteine proteases have their maximum activity at neutral pH, they are susceptible to sulfhydryl agents such as PCMB but have no effect with DFP and metal chelating agents (Rao et al., 1998; Whitaker, 1993).

3) *Aspartyl proteases*: Commonly known as acid proteases, these belong to the group of endopeptidases; their catalytic activity depends on aspartic acid residues. These enzymes are inhibited with pepstatin. They are also sensitive to diazoketone compounds such as Diazocetyl-DL-Norleucine Methyl Ester (DAN) and 1,2-Epoxy-3 (p-nitrophenoxy) Propane (EPNP) in the presence of copper ions (Rao et al., 1998; Whitaker, 1993).

4) *Metaloproteases*: The main characteristic of these proteases is that they require divalent metal ions for their activity. These enzymes are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP (Rao et al., 1998; Whitaker, 1993).

According to the pH value in which the enzymes present their greatest activity, the proteases can be classified into alkaline, neutral, and acid proteases (Sandhya et al., 2005). Acid proteases have the best activity between pH 2 and 6 and include mainly aspartyl proteases and some cysteine proteases and metalloproteases. They consist of 380–420 amino acids, and different amino acid residues constitute the active site (Rao et al., 1998). Neutral proteases have optimal activity at pH values close to neutrality, and under acidic conditions their activity decreases. This classification includes cysteine proteases, metalloproteases, and some serine proteases (Rao et al., 1998). Alkaline proteases have high activity in a pH range of 8–13 and contain about 420–480 amino acid residues in the chain. In this classification serine proteases can be found (Rao et al., 1998). The study related to such properties is fundamental for proteases, since their application at industrial processes depends on the knowledge of the characteristics of these enzymes (Sumantha et al., 2005).

14.3.2 Source of Proteases

Proteases can be obtained from animal, plant (or vegetable), and microbial sources. Animal proteases include pancreatic trypsin, chymotrypsin, pepsin, and renin. These are produced in small quantities because their production depends on the availability of livestock for sacrifice so that these proteases can be extracted from their organs or tissues. In the group of proteases from plant origin, papain and bromelain are good examples; the first is extracted from the shell of the fruit *Carica papaya*, and the second from the trunk and juice of the pineapple. However, the use of plants as a source of proteases is strongly influenced by factors of viability, cultivation, and climatic conditions and lengthy extraction processes. For the reasons above, most of the commercially important enzymes are produced from a limited number of microorganisms. Additionally, microbial enzymes are preferred over those others, because they can usually be obtained in abundant quantities, on a regular basis and with a uniform quality. Microbial enzymes are generally more stable than their animal and vegetable counterparts and production processes are faster due to their short period of duplication and their relatively simple nutritional requirements. Furthermore, microbes may be genetic and environmental manipulated to obtaining the desired characteristics, to increase activity and yield of the enzyme of interest (Rani et al., 2012).

14.3.3 Methods for Microbial Protease Production

Microbial proteases are produced using Submerged Fermentation (SmF) or Solid-State Fermentation (SSF) technologies.

14.3.3.1 Submerged Fermentation (SmF)

SmF is defined as the growth of microorganisms in a nutrient broth that can contain about 50 g/L of solutes, with a 95% water content. This fermentation system is used to obtain a wide variety of products from a great diversity of microorganisms, because it offers better control of the process (pH, temperature, etc.) and uses well-defined culture media, ensuring that good reproducibility between experiments is obtained. SmF is a method used mainly in Western countries to produce enzymes, antibiotics, and other products. The main factors influencing the production of products through this bioprocess are type of fermenter, inoculum, composition of the culture medium, dissolved oxygen, temperature, and pH.

14.3.3.2 Solid-State Fermentation (SSF)

SSF is defined as bioconversion process involving the growth of microorganisms (typically fungi) on a moist solid substrate in the absence of free water (Ooijkaas et al., 2000). Filamentous fungi are used in this bioprocess because the conditions under which SSF is conducted are similar to those in which these fungi grow in nature, and fungi strains offer several advantages over other types of microorganisms. For example, filamentous fungi are able to produce high amounts of protein and secrete it to the culture medium, which facilitates its recovery (Sandhya et al., 2005). They can also glycosylate proteins, are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), produce a wide range of products of industrial interest such as bio-pesticides, fermented foods, organic acids, etc. (Mitchell et al., 2000), and can be grown on a wide variety of economic substrates as

agricultural byproducts (Li et al., 2000). However, the growth of microorganisms (as well as related activities such as the secretion of hydrolytic enzymes or metabolites) under SSF can be affected by factors such as nature of the solid phase used, composition of the culture media, temperature, particle size of support and substrate used, moisture and water activity, pH of the medium, concentration of O_2 and CO_2 in the gaseous atmosphere, and type and design of the bioreactor. Several reviews about SmF and SSF methods can been found in the literature (Aikat and Bhattacharyya, 2001; Mitchell et al., 2000; Pandey, 2001).

14.3.4 Microbial Proteases

There are several reports on microbial protease production by SmF using filamentous fungi such as *Aspergillus oryzae* (Samarntarn et al., 1999; Sandhya et al., 2005; Wang et al., 2005a, b), *Aspergillus fumigatus* (Wang et al., 2005a, b), *Aspergillus tamarii* (Boer and Peralta, 2000), *Aspergillus flavus* (Hossain et al., 2006), and *Aspergillus niger* (Basu et al., 2008; Yang and Lin, 1998). Similarly, several reports on the production of SSF proteases from filamentous fungi are available. Among the most studied strains are *Penicillium* (Germano et al., 2003), *Aspergillus niger* (Couri et al., 2000), *Aspergillus niger* ANH15 (Villegas et al., 1993), *Rhizopus oryzae* (Aikat and Bhattacharyya, 2001; Tunga et al., 1998), *Rhizopus oligosporus* (Ikasari and Mitchell, 1994), *Mucor bacilliformis* (Fernández-Lahore et al., 1998), *Aspergillus sojae* (Sardjono et al., 1998), *Aspergillus oryzae* (García-Gómez et al., 2009; Sandhya et al., 2005; Sardjono et al., 1998) *Aspergillus flavus* (Malathi and Chakraborty, 1991) and *Asperguillus fumigatus* (Hernández-Martínez et al., 2011). Several factors influencing protease production were analyzed for both types of protease production systems, including the type of reactor used (Aikat and Bhattacharyya, 2001); carbon and nitrogen addition sources to the culture medium (Boer and Peralta, 2000; Yang and Lin, 1998); agitation and aeration (Sardjono et al., 1998; Villegas et al., 1993; Yang and Lin, 1998); the optimization of the protease production process to obtain higher yields (Tunga et al., 1998; Wang et al., 2005a); addition of inducers (Basu et al., 2008; Boer and Peralta, 2000; Malathi and Chakraborty, 1991). In some studies, the proteolytic extract was characterized and purified (Boer and Peralta, 2000; Hossain et al., 2006; Samarntarn et al., 1999; Wang et al., 2005b). In some others' works, the application of crude extracts or partially purified enzymes was tested for detergent additives (Germano et al., 2003), for the extraction of tropical fruit or oilseed pulp oil (Couri et al., 2000), or as depilatory agents (Malathi and Chakraborty, 1991).

Over the last decade, some studies have been motivated by an interest in raising enzyme production yields during fermentative processes. Researchers analyzed several different strategies for increasing protease production yields (Rao et al., 1998). Attempts have been made to increase proteolytic enzyme production yields through the construction of genetically modified strains, either by increasing the copy number of genes encoding proteases (Cheevadhanarak et al., 1991; Frederick et al., 1993; Jarai et al., 1994; Tatsumi et al., 1988; vanKuyk et al., 2000) or through by isolating overproducing mutants (Parekh et al., 2000). The application of modified strains in solid-state fermentation systems attracts attention because it has been demonstrated that the productivity of the proteins in SSF is higher than in SmF (Iwashita, 2002), with the production of homologous proteins often higher than the production of heterologous proteins (Conesa et al., 2001). Conventionally, breeding strains for the production of homologous or heterologous proteins has been accomplished through

genetic mutation, selection, or recombination (Iwashita, 2002). Gene cloning is a technology that has progressed rapidly, as it is an excellent method of gene manipulation and control. SSF and SmF processes have the use of agroindustrial residues as a substrate in common, with exception of Wang et al. (2005a, b) who used a medium of shrimp and crab shells, and Basu et al. (2008) who used fish scale powder. Table 14.3 summarizes various substrates used for protease production along with other production parameters, and Table 14.4 describes the most conventional purification steps used to separate the proteases.

14.3.5 Importance of Microbial Enzymes in Peptide Production

In the field of bioactive peptide production, there is an established set of procedures involving physicochemical techniques such as chemical synthesis, chemical hydrolysis, as well as molecular or biotechnological strategies such as recombinant expression of peptides, fermentative processes, or enzymatic hydrolysis. Enzymatic hydrolysis is one of the most useful techniques for bioactive peptide production from food materials (Bhat et al., 2015) because it is ecologically friendly (avoiding the use of solvents, acid, or alkalis) and it is not a racemization reaction, making enzymatic hydrolysis feasible for implementation in the production of bioactive peptides safe enough for human consumption (Aluko, 2012; Guzmán et al., 2007). Enzymatic hydrolysis presents opportunities to relieve other problems by allowing the reuse of byproducts as raw materials, making the materials relatively cheap (Kim, 2013; Mora et al., 2014). However, this technology includes a procedural bottleneck, necessitating broad study of its critical parameters. A procedure's parameters lead to an optimum process and can affect peptides properties, such as hydrolysis degree (DH), length, molecular weight, and amino acid composition, and consequently, can affect their bio-functionality; these variables can include protein substrate, type of protease, enzyme specificity, and conditions along the hydrolytic process, as well as substrate concentration, pH, temperature, enzyme-to-substrate ratio (E:S), and hydrolysis time (Bah et al. 2013; Li Chan, 2015; van der Ven et al., 2002). In this class of reactions mediated by a biological catalyzer such as a protease, variables such as pH or temperature represent critical points for developing a convenient process, and need to be controlled; due to the enzymes' nature, pH and temperature are strongly linked to their activity. Because of enzymes' protein origin, they are considered ampholytes. They possess dissociation constants for acidic and alkaline groups, so a different pH can affect their solubility, osmotic pressure, viscosity, etc., and can promote ionization changes between enzyme, substrate, or enzyme-substrate complex, affecting their velocity rate (Mayer, 1979). Compared to other chemical reactions, enzyme-catalyzed reactions develop at mild conditions, and increasing temperatures can increase velocity rates. But at high temperatures, enzyme denaturation can occur, resulting in a progressive loss of enzyme activity (Mayer, 1979). Proteolysis can provoke pH changes in the media reaction, and must be compensated for by using buffers or adding a neutralizing solution (Villamil et al., 2017). According to authors such as Capobiango et al. (2007), enzymatic hydrolysis parameters such as protein concentration or E:S ratio are positively affected by a high concentration of protein because the protease is more likely to meet the substrate when substrate concentrations are higher. However, other researchers (Fernandes et al., 2008) studied another type of enzymatic hydrolysis (cellulose enzymatic hydrolysis) to understand how a high concentration of substrate can affect mass transfer due to elevated

TABLE 14.3 Main Characteristics of Fungal Proteases Production

Culture Type	Microorganism	Substrate	Time (h)	Type of de Enzyme	Extract Activity	Optimal		Stability		Ref.
						pH	T (°C)	pH	T (°C)	
SmF	*Aspergillus oryzae*	Mixture of wheat bran and soybean meal	80	NR	≈18 U/L	NR	NR	NR	NR	Wang et al. (2005a)
SmF	*Aspergillus oryzae*	Wheat bran	72	NP	8.7 U/g DM	NR	NR	NR	NR	Sandhya et al. (2005)
SmF	*Aspergillus oryzae*	Defatted soy, lactose and casein	72	AlP	14,100 U/mL	8–9	45	ND	ND	Samarntarn et al. (1999)
SmF	*Aspergillus fumigatus*	2% shrimp and lobster shell powder	144	SP	1 U/mL	8	40	6-10	50	Wang et al. (2005b)
SmF	*Aspergillus tamarii*	1% soy flour	144	NR	160 U/mL	≈7.5	45	6–9.5	30–50	Boer and Peralta (2000)
SmF	*Aspergillus flavus*	NR	NR	SP	NR	8	45	5–9	35–60	Hossain et al. (2006)
SmF	*Aspergillus niger*	Light rice distillate with 4% soybean oil	192	PAc	200 U/mL	NR	NR	NR	NR	Yang and Lin (1998)
SmF	*Aspergillus niger*	Fish scales	168	MP	2776 U	7	50	6–9	30	
SSF	*Penicillium* sp. LPB-5	Defatted soybeans	48	SP	NR	6.5	45	6–9	35–45	Germano et al. (2003)
SSF	*Aspergillus niger*	Wheat bran	42	NR	5.27 U/mL	NR	NR	NR	NR	Couri et al. (2000)
SSF	*Aspergillus niger*	Wheat bran	36	AP	74.3 U/g DM	NR	NR	NR	NR	Villegas et al. (1993)
SSF	*Rhizopus oryzae*	Wheat bran	408	NR	6381 U	NR	NR	NR	NR	Aikat and Bhattacharyya (2001)
SSF	*Rhizopus oryzae*	Wheat bran (ST)	144	NR	341 U/g WB	NR	NR	NR	NR	Tunga et al. (1998)
SSF	*Rhizopus oligosporus*	Rice bran	72	NR	3.9×10^5 PU/g ISS	NR	NR	NR	NR	Ikasari and Mitchell (1994)
SSF	*Mucor bacilliformis*	Wheat bran	72–96 h	AP	700 PU/g ST	NR	NR	NR	NR	Fernández-Lahore et al. (1998)

Continued

TABLE 14.3 Main Characteristics of Fungal Proteases Production—cont'd

Culture Type	Microorganism	Substrate	Time (h)	Type of de Enzyme	Extract Activity	Optimal		Stability		Ref.
						pH	T (°C)	pH	T (°C)	
SSF	*Aspergillus oryzae* *Aspergillus sojae*	Lupine (L) Soya (S)	144		1500 U/gL, 1800 U/g S 1900 U/gL, 1700 U/g S	NR	NR	NR	NR	Sardjono et al. (1998)
SSF	*Aspergillus oryzae*	Wheat bran	72 h	NP	31.2 U/g MS	NR	NR	NR	NR	Sandhya et al. (2005)
SSF	*Aspergillus oryzae*	Rice husk and bran (7: 3)	72–96 h	NP y AlP	1946 PU/g S	NR	NR	NR	NR	Battaglino et al. (1991)
SSF	*Aspergillus flavus*	Wheat bran	48 h	NP	6.54 U/mL	7.5 and 9.5	NR	3–8	NR	Malathi and Chakraborty (1991)

NR, not reported; *AP*, acid protease; *NP*, neutral protease; *AlP*, alkaline protease; *MP*, metaloprotease; *SP*, serine protease; *DM*, dry matter; *WB*, wheat bran; *ISS*, initial solid substrate;
L, lupine; *gS*, gram of soy; *S*, substrate.

TABLE 14.4 Conventional Purification Steps

Step	Step Aim	Peptide Concentration	Applied Techniques	Examples
Insoluble material removal	Remove not-useful molecules	Relatively little peptide concentration	Filtration and centrifugation	Kim et al. (2007) employs ion exchange chromatography with a HiPrep 16/10CMFF column and RP-HPLC for antioxidant peptide from a fish by-product Zhao et al. (2016) use a combination of macroporous adsorption resin, gel filtration chromatography and RP-HPLC for antiinflammatory peptide purification Nimalaratne et al. (2015) employs ultrafiltration, Cation exchange chromatography an RP-HPLC for purify peptides from chicken egg with enzymatic hydrolysate Rodriguez-Illera et al. (2015) used monoliths materials for selective recovery of lact-tripeptide from hydrolysate
Peptides isolation and concentration	Remove molecules with properties which are different in comparison with	Considerable concentration	Salting-out, adsorption, and solvent extraction	
Peptide purification	Removal of impurities with similar physical, chemical, and functional properties	Concentrated	Electro-membrane filtration, membrane chromatography, perfusion chromatography with monolithic columns	
Polishing	Removal of the minimum impurities for safe use of the final product	Elevated concentration	Crystallization, lyophylization	

viscosity in reaction media; Qi and He (2006) mentioned that the reaction's rate can decrease when the initial substrate concentration exceeds the optimum value, and they attributed this behavior to substrate inhibition. A third group (Bizzoto et al., 2006) reported that as the E:S ratio rises, the hydrolysis becomes more efficient, but the cost of the process increases. Turning in a requirement within enzymatic procedures for peptide production, Implementation of optimized designs can make scale-up and modeling more feasible and cost-effective. In a very complex technique such as enzymatic hydrolysis, control of critical parameters results in a process that can generate a product with desirable physicochemical and bio-functional characteristics. One parameter that can used as a response variable in this kind of approach is the HD, although some authors correlate this parameter with bioactivities, that is, when HD increases, the antioxidant capacity increases, too. But an elevated HD can deplete the antioxidant activity, attributable to a higher number of free amino acids, not to peptides. Free amino acids possess an antioxidant effect that is greater in peptides due to

the fact that their structure confers more stability from radicals, avoiding the propagation of oxidative reactions (Gómez et al., 2013). Other strategies are implemented to achieve enzymatic hydrolysis of food proteins, such as enzyme immobilization in membranes or particles, achieving enzyme re-utilization, avoiding the formation of byproducts from enzyme degradation, and avoiding undesirable secondary hydrolysis (Pedroche et al., 2007). The manipulation of the proteolytic reaction conditions can sometimes be used to define the characteristics of the final product. However, the specificity of the enzyme in a proteinase preparation establishes the type of peptide produced, as well as its functional properties, and hence, its application (Neklyudov et al., 2000). It has been reported that the distribution of molecular sizes in the peptides obtained is a function of the enzyme used and the degree of hydrolysis achieved, but it is practically independent of the operating conditions (Guadix et al., 2000; Sumaya-Martínez et al., 2005). Therefore, a proteolytic enzyme to produce the desired functional peptides is required. The selection of this enzyme depends on its origin, optimum and stability values of pH and temperature, specificity, and activity, as well as the production cost (Dumay et al., 2006). Typically, industrial enzyme suppliers present the activity curves, which show activity relative to different pH or temperature values under certain test conditions. Stability curves show residual relative activity after exposure to a given pH or temperature.

As mentioned, the purpose of producing proteases is their potential application as an alternative method within a biotechnological process. One of these processes is peptide production from different protein sources, wherein the use of commercial enzymes produced in academic laboratories has been analyzed. For example, Sumaya-Martínez et al. (2005) optimized the process of enzymatic hydrolysis of golden carp by-products (*Carassius auratus*) with Flavourzyme (exo and endopeptidases commercial complex) for the recovery of peptide fractions with nutritional and functional properties. The optimum conditions for hydrolysis (pH = 5.9, T = 53°C, S: B = 14.7%) were not adequate to obtain high nitrogen recovery values (9.5%). However, within this recovered fraction were found peptides greater than 10 kDa with emulsifying capacity comparable to egg white. Further work was done to evaluate the functionality of peptides obtained by enzymatic hydrolysis of golden carp byproducts using Flavourzyme with optimum conditions for obtaining the hydrolysate (pH = 7, T = 50°C, [E] = 50 LAPU/g, t = 15 min), 44 kDa peptides were obtained, whereby major and minor peptide fractions could be separated from 30 kDa. Although these fractions presented functional and nutritional properties, they had a slightly perceptible bitter taste. Hernández-Rodríguez characterized the physicochemical, rheological, and functional properties of protein hydrolysates obtained from the enzymatic hydrolysis of grouper muscle using Flavourzyme. Peptides were produced favoring the endo- and exo-activities (pH 5 and 7, respectively) of the enzyme complex. In general, the functional properties of the fractions obtained (30-5 <F> 30-5 and 30-7 <F> 30-7) were adequate in food systems requiring high solubility (>80%), low viscosity, such as sparkling wine; however, the fraction 30-7 <F had a high content of hydrophobic amino acids, which is indicative of the presence of bitter taste (Adler Nissen, 1993). Furthermore, proteases play an important role in basic research. Their selectivity with regard to some types of peptide bonds is used to elucidate the structure-function relationship in peptide synthesis and in protein sequencing (Srinubabu et al., 2007).

14.4 FOOD MATRICES FOR PEPTIDE PRODUCTION

Proteins are biopolymers structured by molecular units called amino acids that contains an amino group (−NH_2), a carboxyl group (−COOH), a hydrogen, and a side chain (−R). Each unit has particular properties which confers unique structures and functions (Vaclavik, 2008); in food, these properties influence sensorial, nutritional, and functional features; however, attention has been paid in particular to their potential as substrates in functional hydrolysate production, giving importance to their ability to provide large protein variety and to add value to proteinaceous waste products (Benkerroum, 2010; Sibel Akalin, 2014).

14.4.1 Animal-Derived Proteins

In recent years, animal protein has been a rising source of functional protein hydrolysates, as well as an opportunity for bioremediation in food industry byproducts.

14.4.1.1 Milk

For many years, the milk has been recognized as source of molecules that provide important immunological protection. Milk also has nutritional benefits and is one of the wealthiest sources of bioactive substances (Clare and Swaisgood, 2000). But recently, a particular group of compounds derived from proteins in this rich food has gained attention: bioactive peptides. Milk contains 3.5% of proteins, divided into two principal types: caseins (which represent 80%, divided among α_{s1}-, α_{s2}-, β- and α-casein) and whey proteins (the other 20%, comprised of α-lactoalbumin, β-lactoglobulina, glyco-macropeptide, proteose peptone 3, immunoglobulins, and bovine serum albumin) (Kitts and Weiler, 2003; Pihlanto, 2011). Milk is the most studied source of functional peptides (Clare and Swaisgood, 2000), and it has been well documented that through enzymatic hydrolysis, a wide variety of bioactive peptides produced from this source are antihypertensive, antimicrobial, opioid, mineral-binding, antidiabetic, antioxidant, and immunomodulatory in their effects (Mohanty et al., 2016; Nongonierma and FitzGerald, 2015). Furthermore, studies report the use of many different types of milk in peptide production (bovine, goat, yak, or even camel) and different hydrolysis processes using bacterial or fungal enzymes (Ahmed et al., 2015; Mao et al., 2011; Moslehishad et al., 2013), including combinations of different classes of enzymes, or even a novel procedure using fungal enzymes. But now attention has been focused on whey, a sub-product in the cheese and casein industries, turning in a disposal waste with high water content, 0.8%–1% of proteins, and high oxygen demand and therefore, a pollution problem (González Siso, 1996). Normally, this waste has been used for animal feeding, lactose or protein production in the food industry, and the generation of various bioproducts through fermentative processes, but at present, researchers still seek to discover their potential value in peptide production and to improve their functional power with enzymatic hydrolysis (Corrêa et al., 2014). It has been reported that cheese whey hydrolysis promotes peptides with functionalities such as ECA-I inhibition, mineral binding, or opioid activities (Yadav et al., 2015).

14.4.1.2 Meat

Meat is defined as edible flesh removed from domestic animals (bovines, goats, poultry) or wild game animals. Meat is an important food and one of the first options in animal

protein for consumers (Lafarga and Hayes, 2014). Meat contains water, nitrogenous compounds, lipids, vitamins, carbohydrates, and minerals. Three types of proteins constitute 19% of meat: myofibrillar, sarcoplasmic, and connective. The most abundant myofibrillar proteins are myosin and actin, the principal sarcoplasmic proteins are enzymes and myoglobin (which gives color to the flesh), and the principal proteins in connective tissue are collagen and elastin (Cobos and Díaz, 2015). Blood is another interesting and abundant proteinaceous residue derived from animal slaughter; it is composed of water, proteins, cells, enzymes, and inorganic compounds, and it is divided into two parts: blood cells and plasma, representing 30%–40% and 60%, respectively. Blood is rich in proteins like albumin, fibrinogen, immunoglobulins, hemoglobin, prothrombin, and transferrin (Bah et al., 2013). The products generated through animal slaughter, (depending upon tradition, culture, and religion) include skin, bones, horn, feet, feathers, entrails, meat trimmings, and blood; these are protein-rich products and can be used as substrates in functional peptide production (Di Bernardini et al., 2011), with reported antioxidant and ECA I inhibitory effects (Bah et al., 2016; Castellano et al., 2013; Li et al., 2007a).

14.4.1.3 Marine Proteins

Marine organisms represent half of the world's diverse organisms (Kim and Wijesekara, 2010), and they can serve as raw materials in the production of molecules of protein origin with bioactive potential, which exhibit a wide variety of functionalities due to their amino acid composition: functional peptides (Harnedy and FitzGerald, 2012). It is feasible to use byproducts in peptide production, such as low-standard muscles, viscera, skin, fish heads, bones, fins, and crustacean shells (Pfeiffer, 2003). For both vertebrates and invertebrates, the most abundant proteins are myosin, actin, and collagen (Di Bernardini et al., 2011), and from these, it is possible to generate biofunctional peptides. The most prominent activities present in marine protein-derived peptides are antioxidant, mineral-binding, antihypertensive, anticoagulant, and antimicrobial activities (Kim and Wijesekara, 2010).

14.4.2 Plant-Derived Proteins

Within the context of peptide production, protein substrates are an important subject, given the wide specificity of protease and the search for a wide variety of raw materials. Due to their amino acid profile and their wide availability as waste products from food industry disposal, plant-derived proteins present an alternative opportunity for exploring hydrolysates' bioactivities.

14.4.2.1 Soybean

The soybean is a legume consisting of 36% protein, used as a foundation for many products for human consumption, such as edible oils and Isolated Soy Protein (ISP). ISP contains two principal globulin proteins: β-conglycinin and glycinin (Thrane et al., 2017). As a legume with higher protein content, soybeans have been reported to exhibit bioactivities (Quiroga et al., 2010). Hydrolysates from soybean proteins with in combination with different classes of enzymes (including fungal proteases) demonstrate in vitro antioxidant, antihypertensive, or immunomodulatory effects (Beermann et al., 2009; Gu and Wu, 2013; Kong et al., 2008).

14.4.2.2 Rice

Rice constitutes the most important global crop in human nutrition, providing 20% of caloric intake worldwide, increasing to 50% in Asian countries. In some countries with high consumption rates (like Japan), rice constitutes the most important vegetable protein source (Hoogenkamp et al., 2017). Even when processed into rice flour, the majority of protein in this crop remains, with that from milled rice or broken husk kernels considered hypoallergenic (Amagliani et al., 2016). Various proteins (like albumin, globulin, glutelin and prolamin) constitute different portions of rice's total protein content, based on their solubility (Amagliani et al., 2017), and enzymatic hydrolysis has also been reported to improve their biological functionality, whether from endosperm hydrolysate or liquid by-product from the starch industry with antioxidant activities (Dei Piu et al., 2014; Zhang et al., 2009), in vivo antihypertensive effects (Li et al., 2007b), or the in vitro antihypertensive action of peptides generated during rice fermentation by the fungi *Monascus purpureus* (Kuba et al., 2009).

14.4.2.3 Maize

Maize is a principal crop cultivated worldwide, and it is considered a staple food for the world's widespread population, as well as a major component of animal feed (Pechanova et al., 2013). The grain is suitable for producing alcohol, whiskey, glucose, oil, beer, vinegar, and flour, and the protein content varies in different parts of grain with 76% in the endosperm and 24% in the germ, the majority of which is a low-solubility protein, prolamina (α-zein), representing 60% of the total protein (Gavicho Uarrota et al., 2011). The presence of bioactive peptides in these protein sequences has been reported to demonstrate *in vitro* antioxidant or antihypertensive activities (Dadshahi et al., 2016; Jin et al., 2016; Wang et al., 2014).

14.4.2.4 Nonconventional Protein Sources

Amaranth is a resistant, fast-growing crop capable of adapting to unfavorable climatic conditions and poor soil. Amaranth is a highly nutritional grain with a protein content of 13.2%–18.4% (depending on the species) the majority of which are globulins. Amaranth's protein content is highly hydrolysable and is rich in lysine, methionine, and cysteine (Orona-Tamayo and Paredes-López, 2017). Enzymatic hydrolysis of amaranth proteins has reportedly produced peptides with antithrombotic, immunomodulatory, and antioxidant effects (Fillería and Tironi, 2017; Moronta et al., 2016; Sabbione et al., 2015).

14.4.2.5 Pea

Pea is cool season crop, cultivated principally in Canada, Russia, and the United States for different uses, such as in frozen foods (whether whole or split) flour, protein concentrates, or starch concentrates. Peas are a significant protein source, with protein contents of 18%–30% depending on cultivation factors, and with a methionine-like limiting amino acid. Peas contain diverse types of proteins in varying portions, but the most abundant are globulins, in the form of vicilin and legumin (Tulbek et al., 2017), and their use in functional hydrolysates with ACE I inhibitory effect has been reported (Jakubczyk et al., 2013; Li et al., 2011).

14.4.2.6 Oil Press Cakes

Oil cakes, also called oil meals, are byproducts resulting from seed pressing for oil extraction. Edible oil cakes possess a high protein content, from 15% to 50% depending on the seed variety, environmental conditions, and extraction methods, and are usually used for animal feed or fertilizers (Ramachandran et al., 2007). Researchers are actually searching for a biotechnological improvement of this residue, hoping it will result in an opportunity for peptide production which exploits the potential of oil cakes from sunflowers, rapeseeds, palm kernels, or pumpkin, with antioxidant, antihypertensive, and antimicrobial effects (He et al., 2013; Megías et al., 2009; Popović et al., 2013; Tan et al., 2013; Zarei et al., 2015).

14.5 APPLICATIONS, CHALLENGES AND PERSPECTIVES IN FOOD INDUSTRY

Expanding concern for human nutrition promotes innovation in the food industry. Considering the wide variety of benefits attributed to protein hydrolysates, another challenge present in their production is their incorporation in products like functional food or nutraceuticals.

14.5.1 Peptides as Functional Foods and Nutraceuticals

The phrase "functional food" means foods whose postconsumption nutritional effects improve one's state of health and/or reduces the risk from a chronic disease. An important characteristic of functional foods is that they remain foods and demonstrate their effects in normal consumption amounts (Valls et al., 2013). Nutraceuticals are products generally sold in a pharmaceutical form and derived from a variety of active substances extracted from vegetable or animal foods, which then exerts a pharmaceutical effect beyond the nutritional, employed to prevent or relive some adverse medical condition (Santini et al., 2017). Inside this field, bioactive peptides' present an opportunity due to the broad spectrum of their bioactivities promoting health, their high, targetable bio-specificity, their low accumulation in tissues, high structural diversity, small size (Agyei et al., 2016) and their ability to be produced from inexpensive raw materials. Combined with demand from consumers for more specific nutrition requests creates an opportunity to develop this kind of product in the food sciences. However, despite the benefits of this group of molecules, the bottleneck in producing them at commercial scale is attributed to challenges such as the lack of more conclusive results from in vitro and in vivo trials and a need for more extensive studies on their interaction with food matrices, (Korhonen and Pihlanto, 2006; Samaranayaka and Li Chan, 2011), as well as their gastrointestinal, bloodstream, food processing, and long-term stability (Hernández Ledesma et al., 2011; Kamau et al., 2010; Mulero Cánovas et al., 2011) and posible allergenic responses (Carrasco Castilla et al., 2012; Wang and de Mejia, 2005). Additionally, there needs to be wide study of the mechanisms of their action (Saadi et al., 2014), in order to understand variables that can affect their activities, ensuring their bioavailability in products, and compliance with nutritional intake recommendations. Studies reveal another challenge in their incorporation in foods; production and concentration techniques are not cost effective due to the molecules'

high complexity, and when they are concentrated, they have poor solubility, attributable to their high hydrophobicity. Nevertheless, these problems can be solved with peptide mixtures that can exert multifunctionality or synergism (Li Chan, 2015). Furthermore, by nature, most of these hydrolysates possess a bitter taste (Komai et al., 2007), which has consumer acceptance repercussions; this bitterness can be reversed with an absorption with activated carbon and posterior selective extraction with alcohols, but these can affect bioactivities (Adjonu et al., 2014). Food science is responsible for developing alternatives for the exploitation of bioactive peptides and for removing the barriers to their incorporation in food products, to relieve bottlenecks like stability, where a group of methods (such as modifications in protein to create analogues, modification of dosage forms like lipid vesicles or colloidal carriers, adding enzyme inhibitors, or structural modifications like pegylation, lipidation, or glycoliation) can promote better bioviability in tissues. Nevertheless, the use of methods like microencapsulation is preferred in drug delivery systems with techniques that include interfacial polymerization or spry drying (Segura Campos et al., 2011).

14.6 CONCLUSION

The use of enzymes in peptide production is recognized as an environmentally friendly technique, and is feasible for developing new products consistent with the public's demand for health standards, and a wide and fruitful market. However, a set of intensive studies are needed in fields such as optimization of enzymatic process, the discovery of new enzymes, and the integration process offering the benefits of this kind of molecule for all, scaling up the production and purification processes for an economically accessible product. It is necessary to highlight how enzyme procedures are becoming a key process for this new kind of molecules. There is an opportunity for scientific developments in the biotechnology and food fields in order to achieve improvement and innovation in health, nutrition, and technological issues.

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CHAPTER

15

Application of Proteases for the Production of Bioactive Peptides

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15.1 ENZYME IN FOOD PROCESSING

Enzymes are biological catalysts that function to accelerate biochemical reactions in living organisms. Technology enables enzymes to be extracted from biological materials and purified, and this has extended the use of enzymes elsewhere in the food and nonfood industries. Plants, animals, and microorganisms are the various biological sources for enzymes, among which microbial enzymes account for most of the industrial enzymes (Jisha et al., 2013). Microbial enzymes are the most used, owing to the fast growing ability of microorganisms and their relative consistency in supply. This means that a greater enzyme yield can be obtained without influences from seasonal, agricultural, and other factors. Moreover, biotechnology advancement has allowed recombinant enzymes, the results of enzyme engineering, to be made. Recombinant enzymes are enzymes with desired catalytic characteristic for better processing applications.

Enzymes have been used as food and beverage processing aids in baking, brewing, dairy, flavor enhancement, juice and wine, low-allergenic infant food, meat processing, and oils and fats. Enzymatic modification of foodstuffs helps in achieving the ideal properties of the final product. Not only are products' textures and flavors improved, but shelf life is also extended. Also, a wide range of final products can be fabricated from the raw materials using enzymes (Simpson et al., 2012). The utilization of enzymes in food processing offers many advantages. Enzyme-assisted processing does not give rise to safety and health issues, and it is green to the environment. Chemical modifications can be used to achieve the same effect as enzymes; nevertheless, chemicals are toxic to health and the environment. The action of enzymes on substrates is highly selective and specific, therefore reducing the number of side reactions and by-products. Enzymatic reactions are carried out under mild conditions of temperature and

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https://doi.org/10.1016/B978-0-12-813280-7.00015-3

pH that require only relatively low-cost technology. Also, immobilization of enzymes on support materials permits continuous operation where enzymes are reused to reduce processing costs. Enzymes are readily inactivated after reaction owing to their protein nature whereby inactivation can be done by just applying heat. Commonly used food enzymes include those from the oxidoreductase, transferase, hydrolase, and isomerase groups of enzymes (Simpson et al., 2012). Oxidoreductases catalyze redox reactions in substrate molecules while transferases catalyze the transfer of groups from a donor substrate molecule to the acceptor molecule to form a new product. Isomerases catalyze the intramolecular rearrangement of substrates into their isomers whereas hydrolases catalyze the hydrolytic breakdown of higher molecular weight compounds.

15.2 PROTEASES

Proteases are from the hydrolase family of enzymes. Protease, synonymous with "peptidase" or "peptide hydrolase," is any enzyme that hydrolyzes peptide bonds. The enzymes are further divided into exopeptidases and endopeptidases; the former act only near a terminus of a polypeptide chain while the latter act internally in polypeptide chains. Among the exopeptidases, carboxypeptidases that cleaves amino acids at the carboxyl or C-terminal of the polypeptide chains can be distinguished from aminopeptidases that cleave amino acids at the amino or N-terminal of the polypeptide chains. Meanwhile, the sub-subclasses of endopeptidases–serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases, metalloendopeptidases, and threonine endopeptidases–are grouped on the basis of their catalytic mechanism. It should be noted that previously, in the enzyme nomenclature, "peptidase" was restricted to the enzymes included in sub-subclass exopeptidases. Also, the term "proteinase" which was used previously for the enzymes included in sub-subclasses carried the same meaning as "endopeptidase" has been replaced by "endopeptidase", for consistency (International Union of Biochemistry and Molecular Biology, 2017). Proteases are secreted by all living organisms, including plants, animals, and microorganisms, for their involvement in essential physiological processes. Intracellular proteases play a vital role in the regulation of various cellular and metabolic processes (Rao et al., 1998). These processes include protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes. Extracellular proteases, on the other hand, play an important role in nutrition (Rao et al., 1998). They hydrolyze large proteins to smaller molecules such as peptides and amino acids for subsequent absorption and utilization by the cell.

15.3 MICROBIAL PROTEASES

The microbial proteases market accounted for more than 45% of total market revenue in 2015. Their application is predominantly in the detergent industry (Global Market Insights, 2017). Alcalase, Flavourzyme, Neutrase, and Protamex are just a few examples of microbial proteases employed in different processes. Alcalase, an alkaline bacterial protease obtained

from *Bacillus licheniformis* is an endopeptidase with broad specificities for peptide bonds. Neutrase is a neutral bacterial protease derived from *Bacillus amyloliquefaciens* that has a broad specificity. On the other hand, Flavourzyme, produced by a selected *Aspergillus oryzae* strain, is a fungal protease complex that works under neutral or slightly acidic conditions. Alcalase is a detergent enzyme that works with other substances in the detergent to remove blood, sweat, and other stains. Flavourzyme and Protamex convert meat coproducts into a rich, meat-flavored broth used in meat processing to impart flavor and achieve salt reduction in the final meat products. In beer brewing, Neutrase produces sufficient amounts of free amino nitrogen for consistent yeast growth and therefore optimal fermentation for making quality beer and improving the yield.

Microbial enzymes used in food processing are available in the form of enzyme preparation (Joint FAO/WHO Expert Committee on Food Additives, 2001). Enzyme preparations usually contain an active enzyme, but they may also contain a mixture of two or more active enzymes in some instances. An absolute purity of the enzymes found in the preparation is seldom required. Flavourzyme, for example, has been purified and studied by Merz et al. (2015). The eight key enzymes identified in the preparation were three endopeptidases, two aminopeptidases, two dipeptidyl peptidases, and an amylase. It is interesting to note that Flavourzyme, a well-known peptidase preparation, also contains an amylase that is a carbohydrase. The use of enzyme preparations in food processing has increased tremendously over the past decades. Enzyme preparations help in maximizing the quality and value of processed foods while optimizing the production processes. Moreover, processing using enzyme preparations is sustainable and cost efficient due to the advantages offered by enzymes mentioned earlier. Large-scale production of microbial proteases owing to low production cost and high production yield has promoted the growth of the proteases market. Continuous investments in research relating to optimum operational properties of microbial proteases are necessary for process improvement as well as design and implementation of novel approaches. Noticeably, a remarkable increase in research beyond the conventional uses of microbial proteases has been seen in recent years. Alcalase, Flavourzyme, Neutrase, and Protamex have especially earned attention from researchers on products of protein hydrolysis in other food applications, particularly the production of bioactive peptides having physiological functions. Various food (Table 15.1) and food processing by-products high in protein content (Table 15.2) as the substrates for those proteases have been extensively studied. Materials of plant origin include legumes (beans, peas, and lentils), seeds, grains, tubers, and especially by-products of oil extraction. The materials of animal origin include fish, poultry, dairy, eggs, and especially by-products of fish processing. Microbial proteases have already shown a wide range of applications in different industries, such research extends the current use of proteases for novelty.

15.4 BIOACTIVE PEPTIDES

Bioactive peptides are small protein molecules having less than 20 units of amino acids most of the time. They are amino acid sequences originally found within bigger protein molecules. Bioactive peptides do not exhibit physiological activity or are better described as inactive when they present as part of the native polypeptide sequence in big protein molecules.

TABLE 15.1 Hydrolysate/Bioactive Peptides Produced From Food/Food Proteins as Protein Sources Using Various Enzymes and Their Health-Related Benefits

Food/Food Proteins	Health Benefit	Enzyme	Reference
Chia seed protein	ACE inhibitory activity of hydrolysate and peptide fractions	Alcalase-flavourzyme	Segura Campos et al. (2013)
Winged bean seed	ACE inhibitory activity of hydrolysates	Flavourzyme, alcalase, bromelain, and papain	Wan Mohtar et al. (2014)
Whey protein	Antioxidant activities of hydrolysates	Pepsin, trypsin, alcalase, and flavourzyme	Lin et al. (2012)
Lentil protein concentrate	ACE inhibitory and antioxidant activities of hydrolysate	Alcalase, savinase, protamex, and corolase 7089	Garcia-Mora et al. (2014)
Sea cucumber	ACE inhibitory and antioxidant activities of hydrolysate	Alcalase, papain, bromelain, flavourzyme, pepsin, and trypsin	Ghanbari et al. (2015)
Chicken collagen	Antioxidant and ACE inhibitory functions of hydrolysate	Flavourzyme, neutrase, and alcalase	Soladoye et al. (2015)
Potato protein	In vivo heart protection effect of hydrolysate	Alcalase	Hu et al. (2015)
Velvet bean protein	In vivo hypolipidemic effect and in vitro antithrombotic activity of hydrolysate and peptide fractions	Pepsin-pancreatin, alcalase-flavourzyme	Herrera Chalé et al. (2016)
Tilapia protein	calcium-binding capacity of peptide	Alcalase, flavourzyme, protease, and papain	Charoenphun et al. (2013)

Upon release by hydrolysis via enzymatic or chemical reactions, the freed peptides become physiologically active or bioactive (Aluko, 2012). Bioactive peptides can be found in enzymatic protein hydrolysates and fermented dairy products, and can also be released during gastrointestinal enzymatic digestion of protein. The health-related benefits of bioactive peptides are attributed to their physiological activities exerted in vitro and in vivo, these include lowering blood pressure by inhibiting the angiotensin-converting enzyme (ACE); oxidative stress reduction by neutralizing or scavenging free radicals; enhancing mineral absorption by functioning as carriers for different minerals, especially calcium and iron; and reducing the risk of obesity and type 2 diabetes by inhibiting enzymes associated with the disease development. Being resistant first to digestion by gastrointestinal enzymes and later to degradation by cytosolic and plasma proteases is one of the prerequisites before any effect of bioactive peptides on the targeted organ or system can be seen. Bioactive peptides are just ordinary protein molecules, although shorter in length, susceptible to digestion and degradation by various proteases including gastric and pancreatic proteases, brush border proteases, and cytosolic and plasma proteases. Such digestion and degradation of bioactive peptides limit

TABLE 15.2 Hydrolysate/Bioactive Peptides Produced From Processing By-Products as Protein Sources Using Various Enzymes and Their Health-Related Benefits

Processing Byproducts	Health-Related Benefit	Enzyme	Reference
Corn gluten meal (by-product of corn wet milling)	Antioxidant activities of hydrolysates and peptides	Flavourzyme	Zhuang et al. (2013)
Rockfish skin (by-product from sliced raw fish)	Antioxidant and ace inhibiting activities of hydrolysate	Alcalase, flavourzyme, neutrase, and protamex	Kim et al. (2011)
Defatted rapeseed protein meal (by-product of oil extraction)	Antihypertensive and free radical scavenging properties of hydrolysates	Alcalase, proteinase k, thermolysin, flavourzyme, and pepsin-pancreatin	He et al. (2013)
Northern shrimp by-product	Antioxidant and ace inhibitory activities of hydrolysate	Alcalase, protamex, flavourzyme, papain, and trypsin	Kim et al. (2016)
Defatted corn meal (by-product of corn oil industry) protein	Bile acid-binding capacity of hydrolysate	Alcalase, trypsin, neutrase, protamex and flavourzyme	Kongo-Dia-Moukala et al. (2011)
Ground turkey heads (low value poultry by-product) protein	Bile acid-binding capacity of hydrolysate	Alcalase, flavorzyme, and trypsin	Khiari et al. (2014)
Mackerel processing by-product	Iron-binding capacity of hydrolysate	Trypsin, protamex, flavourzyme, alcalase, and neutrase	Wang et al. (2013)
Scad processing by-product	Iron-binding capacity of hydrolysate	Trypsin, flavourzyme, protamex, and alcalase	Zhang et al. (2016)
Shrimp processing by-product	Calcium binding activity of peptide	Flavourzyme, protamex, alcalase, pepsin, and trypsin	Huang et al. (2011)
Desalted duck egg white (by-product of salted duck egg yolk)	Calcium binding ability of peptide	Protamex	Zhao et al. (2014)
Desalted duck egg white (by-product of salted duck egg yolk)	In vivo calcium absorption regulation of hydrolysate	Protamex	Hou et al. (2017)

their bioavailability. In addition, intestinal cells must be able to absorb bioactive peptides from the gastrointestinal lumen and release the absorbed peptides into circulating blood in order for the peptides to arrive at the targeted organ or system. The products of protein digestion by gastrointestinal enzymes are amino acids and small peptides. Amino acid and peptide absorption at the intestinal brush border membrane are done by several different amino acid transporters and peptide transporters (PEPT1), respectively (Mahan and Escott-Stump, 2008). It is known that considerable amounts of dipeptides and tripeptides are absorbed into

intestinal cells. The absorbed peptides and amino acids are transported to the liver for metabolizing before they are released into general circulation.

Many factors have contributed to the growing interest in bioactive peptide research. Bioactive peptides have shown seemingly promising effects in a significant number of studies conducted. Dissatisfaction with modern medicine because of the adverse effects of pharmaceuticals together with a general tendency to believe that natural remedies possess minimal or no side effects are promoting the search for alternative remedies derived from food materials. Bioactive peptides are generated from food and food processing by-products that are high in protein content by using proteases extracted from natural sources. This makes bioactive peptides one of the good options as alternative remedies. With increasing health consciousness and rising personal disposable income coming along when education has become a high priority, people are showing great interest in taking charge of their own health and willing to pay the costs of alternative remedies. Bioactive peptides can also be regarded as nutraceutical. Nutraceutical is a term coined from "nutrition" and "pharmaceutical", being defined as parts of a food or a whole food that have medical or health benefit including the prevention and treatment of disease. People nowadays lead sedentary lifestyles and indulge in unhealthy foods and bad eating habits, which are all directly linked to the rising noncommunicable diseases (NCDs) statistics. Prevalence of NCDs can be reduced by incorporating nutraceuticals in the daily diet coupled with regular physical activity and healthy food choices. The nutraceuticals market has grown substantially due to the shift from treatment to prevention in healthcare. Nutraceuticals are thought to be an essential part of preventive healthcare in the future. The production of bioactive peptides from various protein sources using microbial proteases is aimed at reducing the worldwide epidemic of NCDs which had accounted for millions of premature deaths annually, as well as correcting some nutritional deficiency problems. It is estimated that, of the 56.4 million global deaths in 2015, 39.5 million or 70% were due to NCDs (World Health Organization, 2017a). The four main NCDs are cardiovascular diseases, cancers, diabetes, and chronic lung diseases. On the other hand, in 2011, 3.5 billion people worldwide were at risk of calcium deficiency due to an inadequate dietary supply, with the prevalence highest in Africa and Asia (Kumssa et al., 2015). In developing countries, every second pregnant woman and about 40% of preschool children are estimated to be anemic due to iron deficiency (World Health Organization, 2017b).

15.5 FEASIBILITY AND POSSIBLE CHALLENGES IN INDUSTRY APPLICATION AND RELATED ISSUES

All works described later in the chapter involved the production of bioactive peptides using microbial enzymes in the research laboratory. To the best of our knowledge, none has had its application go beyond the research laboratory for industrial-scale production yet, not even those with a long history of research that are considered well established. Successful application in the industry for large-scale production to cater to market demand requires an imperative step of process scale-up. The research laboratory can collaborate with industrial producers for an advantageous position. The research laboratory has strong research expertise that can go as deep as getting the bioactive peptides characterized. The industrial

producer, on the other hand, has strong expertise in large-scale production but is lacking in detailed scientific research. The industrial producer with vast experience in product formulation and consumer research also helps in ensuring the production of more consumer-oriented products. Product strategy, compliance, and marketing are probably the areas necessary to look into for product commercialization. Bioactive peptides made from such collaborations can be marketed as manufactured from a scientific and research-based perspective. They can also claim health-related benefits, but this should not be purported to be a cure. Ongoing research provides updates and papers describing the latest findings on bioactive peptides can be published from time to time. Collaboration between the research laboratory and the industrial producer to complement each other might make sense, but there has not been a significant one yet. Products made from the tryptic hydrolysis of the milk protein that promote relaxation and reduce allergenicity are commercially available; trypsin is protease from the pancreas of animals. Fermented products containing peptides resulting from the action of probiotic secreted proteases on milk protein during fermentation are marketed as products for the reduction of blood pressure. However, none is from the direct hydrolysis of protein using microbial proteases.

The greatest challenge in launching a new product is probably consumer acceptance. Even though enzymes have a long history of usage, a recent consumer study conducted in the United Kingdom (Kantar Public, 2017) reported that participants' awareness of enzyme use was low, particularly among the lower socioeconomic groups. However, the use of food enzymes is more accepted by participants than the use of other food additives such as sweeteners and preservatives. Information is scarce to better understand the consumer response toward enzymes used in novelty food. Factors driving consumers' willingness in consuming food manufactured using new technologies provide useful insight instead. As reviewed by Aleksejeva (2014), consumers' willingness to consume food made using genetic engineering, nanotechnology, and cloning was influenced by consumers' perceived risk about the food. The researcher also highlighted regional difference in readiness toward food produced using new technologies, with the United States and China being more positive while EU countries and Switzerland were rather negative. Moreover, both studies witnessed an increase in consumer interest for more reliable and consistent information that would give consumers greater confidence to make informed decisions about their food choice. In the case for bioactive peptides, consumers' knowledge about enzymes, bioactivity, and peptides is essential in increasing their acceptance and reducing their perceived risk. This makes it urgent to educate the public about bioactive peptides, even before the product is made commercially available in the market.

As mentioned earlier, bioactive peptides must show resistance to digestion by gastrointestinal enzymes and degradation by cytosolic and plasma proteases. Furthermore, whole bioactive peptide absorption by the intestinal cells is essential. Nevertheless, research on bioactive peptides has generally failed to consider these two aspects. Most frequently included in the research of bioactive peptides is an in vitro simulated gastrointestinal enzyme digestion analysis where bioactive peptides are subjected to hydrolysis by pepsin and pancreatin. Pepsin is an endopeptidase secreted by the stomach to normally cleave big proteins and large peptides into oligopeptides while pancreatin is a mixture of enzymes secreted by the pancreas to preferentially cleave the C-terminal and N-terminal of big proteins and oligopeptides. However, the in vitro analysis is unable to mimic all physiological factors affecting food digestion in the human gastrointestinal tract such as pH variation, the relative amount of the

enzyme, and interaction with other constituents. Therefore, those results obtained may not correspond to the actual stability of bioactive peptides against gastrointestinal proteases. A pepsin-pancreatin sequential hydrolysis of food protein or food processing by-products under investigation is also suggested in all studies on bioactive peptides. The pepsin-pancreatin hydrolysate obtained represents a pool of peptides resembling those generated during digestion of the protein in the human gastrointestinal tract. Very limited study included this and thus rendered unfair comparison of the results collected, especially in the case when only enzymes of interest are used. Proteins in the lima bean (Chel-Guerrero et al., 2012), for example, were hydrolyzed with Alcalase and pepsin-pancreatin sequential hydrolysis. Pepsin-pancreatin hydrolysate turned out to have a higher ACE inhibitory activity though the degree of hydrolysis was higher for Alcalase hydrolysate. This simply implied that hydrolyzing the protein in the lima bean prior to ingestion is not necessary. The protein can be cleaved into peptides with even higher biological activity by the proteases in the gastrointestinal tract.

Microbial proteases hydrolyze protein in food and food processing by-products into bioactive peptides. Protein hydrolysis in the materials may also give rise to pH, taste, flavor, color, and textural changes in the materials, mainly due to the products of hydrolysis, their interaction with other food constituents, and the destruction of food constituents. Some of these changes may not be favorable to the materials. Unless purification steps are performed after hydrolysis to purify bioactive peptides from other constituents in the materials, the adverse changes in the materials will otherwise leave them unacceptable to consumers. Additional costs are incurred when purification steps after hydrolysis are needed, and the burden will fall on the consumers who bear the cost of additional processing. Understanding the effects of microbial protease hydrolysis on food and food processing by-products is deemed crucial for product realization in the aspect of product quality. However, this area seems to be less addressed. Studies have shown the use of food processing by-products as substrates for microbial proteases for the production of bioactive peptides. Microbial proteases transform food processing by-products into coveted products providing health benefits. With by-products of food processing as one major form of waste produced other than liquid and gaseous wastes, it makes economic and environmental senses to obtain value from processing by-products and minimizing waste. The meaningful utilization of food processing by-products creates value from items that would otherwise be wasted and mitigates the impact of waste on the planet, which includes an increase in food prices, loss of biodiversity, and greenhouse gas emissions, among many other effects.

15.6 PRODUCTION OF BIOACTIVE PEPTIDES USING MICROBIAL PROTEASES IN THE LABORATORY

Food and food processing by-products that are high in protein are the materials used for microbial protease hydrolysis. In most cases, proteins are isolated from the materials in the form of protein isolate or protein concentrate that will be subjected to hydrolysis. Hydrolysis is sometime done directly on the materials. The protein isolation step is probably to reduce interference from other constituents during hydrolysis which may cause adverse effects. It should be also highlighted that the product of protein hydrolysis, also known as hydrolysate or purified bioactive peptide from hydrolysate is used to evaluate the health-related benefits

of bioactive peptides. Hydrolysate is obtained through protein hydrolysis by enzymes, it is a rather crude mixture primarily composed of peptides and amino acids. Bioactive peptides, on the other hand, are purified from hydrolysate and usually have known amino acid sequences. Researchers also work on peptide fractions, which are the pools of peptides from hydrolysate now being fractionated according to molecular weight, polarity, or charges. The fractionation of peptides into different ranges of molecular weight is often done. Temperature affects both the enzyme's activity and stability while pH affects the activity. Protein hydrolysis by microbial proteases is thus allowed to take place at the known temperature and pH optimum of the proteases used. The variable factors of protein hydrolysis commonly studied include the enzyme:substrate ratio and the hydrolysis time for the maximum yield of bioactive peptides or attainment of peptides with the greatest biological activity. Sequential hydrolysis using two proteases was sometimes employed by researchers to achieve a more complete hydrolysis of the protein because each protease has its specificity. For instance, Flavourzyme (an exo- and endopeptidase) is used after Alcalase (an endopeptidase). However, this does not guarantee the production of more potent bioactive peptides. One common parameter evaluated in the studies of bioactive peptides is the degree of hydrolysis of the hydrolysate. Bioactive peptides must be small protein molecules, only then they can escape hydrolysis by gastrointestinal enzymes during passage through gastrointestinal tract and their absorption in intact form by intestinal cells can be enhanced (Aluko, 2012). The degree of hydrolysis analysis is a measure of the extent of protein hydrolysis carried out. The higher the degree of hydrolysis, the more the proteins have been cleaved into peptides or amino acids, and ideally, the more of the potentially active peptides can be obtained. Nonetheless, the highest biological activity is not always reflected in the smallest peptides.

15.6.1 Angiotensin-Converting Enzyme Inhibition

The ACE inhibition capacity is by far one of the most studied biological activities of bioactive peptides. Chia seed protein (Segura Campos et al., 2013) that was hydrolyzed by Alcalase-Flavourzyme sequentially produced hydrolysate with moderate ACE inhibition activity. Subsequent ultrafiltration of hydrolysate yielded a very low molecular weight peptide fraction (less than 1 kDa) that gave the highest ACE inhibition activity. Based on the amino acid composition of the chia seed protein, the researchers suggested that hydrophobic residues of chia seed peptides contributed substantially to ACE inhibition activity. Using winged bean seed (Wan Mohtar et al., 2014) as the protein source, Flavourzyme, Alcalase, bromelain, and papain produced hydrolysates with ACE inhibitory activity, among which the hydrolysate obtained from papain hydrolysis demonstrated exceptionally high inhibition of ACE. However, this doesn't mean plant proteases such as papain and bromelain are more superior to microbial proteases. The action of proteases on different protein sources will not be the same.

15.6.2 Antioxidative Activity

Alcalase hydrolysate of whey protein concentrate (Lin et al., 2012) had consistently shown the highest activity in ferricyanide reduction, ferric reducing, and diphenyl-picryl hydrazinyl radical scavenging when compared to the pepsin, trypsin, and Flavourzyme

hydrolysates. The increase in antioxidant activity of Alcalase hydrolysate was coincidental with the increase in the degree of hydrolysis of the hydrolysate. Meanwhile, the peptide fraction with molecular weight less than 10 kDa that was separated via ultrafiltration from Flavourzyme hydrolysate of corn gluten meal (Zhuang et al., 2013) exhibited the highest free radical scavenging, metal ion (Fe^{2+}/Cu^{2+}) chelating, and lipid peroxidation inhibition capacities.

15.6.3 Angiotensin-Converting Enzyme Inhibition and Antioxidative Activity

Hydrolysates with multiple functions, most frequently ACE inhibition coupled with antioxidative activity, have been produced from various food protein and food processing by-products. Rockfish skin gelatin (Kim et al., 2011) was hydrolyzed with Alcalase, Flavourzyme, Neutrase, and Protamex. Subsequently, the Alcalase hydrolysate with the highest antioxidative activity measured by Metrohm Rancimat and superior ACE inhibiting activity was further hydrolyzed with Flavourzyme. Glycine, proline, alanine, and glutamic acid detected in the hydrolysate of sequential (Alcalase-Flavourzyme) hydrolysis were postulated to have contributed to the activities observed. Thermolysin, Proteinase K, Alcalase, Flavourzyme, and pepsin-pancreatin hydrolysates of rapeseed protein isolate (He et al., 2013) were separated into different peptide fractions by membrane fractionation. Higher oxygen radical scavenging ability was seen in peptide fractions with molecular weight less than 3 kDa. Alcalase hydrolysate had the highest renin and ACE inhibitory activities among all hydrolysates. When Alcalase hydrolysate was orally administered to spontaneously hypertensive rats, it also appeared to be the most effective in reducing blood pressure. However, the pepsin-pancreatin hydrolysate which resembled those generated during digestion in the human gastrointestinal tract had the most prolonged effect on blood pressure reduction. The researcher postulated that small peptides penetrated better into the ACE protein structure to cause a reduction in enzyme activity. Effective absorption of bioactive peptides from the gastrointestinal tract by the rats was also shown by the strong blood pressure lowering effect of Alcalase and pepsin-pancreatin hydrolysates. Northern shrimp by-product (Kim et al., 2016) hydrolysates were prepared by Alcalase, Protamex, Flavourzyme, papain, and trypsin hydrolysis. Protamex hydrolysate exhibited the most potent ACE inhibitory activity and was further fractionated by ultrafiltration membranes. Fractions with small peptides (molecular weight less than 3 and 3–10 kDa) showed more potent 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity while the peptide fraction with molecular weight of more than 10 kDa demonstrated significant reducing power.

Garcia-Mora et al. (2014) examined the potential of Savinase, another commonly used microbial protease in liquid and powder laundry formulations. When compared to Protamex and Corolase hydrolysates, the Alcalase and Savinase hydrolysates of lentil protein concentrate (Garcia-Mora et al., 2014) showed the highest degree of hydrolysis resulted in a higher yield of peptides. Hydrolysate produced by Savinase also displayed the highest ACE inhibitory and oxygen radical absorbance capacity. After simulated in vitro gastrointestinal digestion, the ACE inhibitory and antioxidant activity of the peptide fraction with molecular weight 3 kDa improved significantly. The researchers attributed the dual bioactivity observed to the presence of 32 peptides rich in hydrophobic amino acids

identified in the peptide fraction. Alcalase hydrolysate of the sea cucumber (Ghanbari et al., 2015) also showed the highest ACE inhibitory activity and the highest antioxidative activities measured by 2,2-diphenyl 1-1-picrylhydrazyl radical scavenging and ferrous ion-chelating methods among all hydrolysates, including those prepared from papain, bromelain, Flavourzyme, pepsin, and trypsin. Strong positive correlations between ACE inhibitory and antioxidative activities were also observed. Flavourzyme, Neutrase, and Alcalase were utilized for the hydrolysis of chicken collagen (Soladoye et al., 2015). Flavourzyme hydrolysate showed the highest antioxidant activity measured by oxygen radical absorbance capacity. Further purification by size exclusion chromatography was conducted to obtain peptide fractions from Flavourzyme and Neutrase hydrolysates. Peptide fractions with molecular weight of 170–776 and 450–1200 Da had the highest antioxidant capacity and the highest ACE inhibitory activity, respectively. The researchers also reported a weak or no linear relationship between the degree of hydrolysis and ACE inhibition and antioxidant activity of peptide fractions.

15.6.4 Bile Acid-Binding

Flavourzyme hydrolysate of defatted corn meal protein (Kongo-Dia-Moukala et al., 2011) manifested stronger bile acid binding capacity than all other hydrolysates obtained from hydrolysis by Alcalase, trypsin, Neutrase, and Protamex. Furthermore, the bile acid binding capacity of Flavourzyme hydrolysate was almost preserved after simulated gastrointestinal enzyme hydrolysis, suggesting that the Flavourzyme hydrolysate may be resistant to digestion in the gastrointestinal tract. In another study, the hydrolysis of ground turkey head protein (Khiari et al., 2014) by Alcalase, Flavorzyme, and trypsin produced hydrolysates that were able to bind bile acid about 2.5-fold lower than that of cholestyramine, a reference drug. The researchers proposed that the binding of bile acid to peptides in hydrolysate was mainly due to hydrophobic interactions. Various hydrophobic amino acid residues (Ala, Iso, Leu, Phe, Val, Pro, Met, and Tyr) were found in the poultry gelatin.

15.6.5 Heart Protective Effect, Hypolipidemic Effect, and Antithrombotic Activity

Alcalase hydrolysate of potato protein (Hu et al., 2015) reduced serum triglyceride, total cholesterol, and low density lipoprotein levels in aging rats on high fat diets to the normal levels expressed in the control group. Noteworthy, the hydrolysate reduced serum lipids without affecting high density lipoprotein levels, unlike Probucol, an antihyperlipidemic drug that was found to lower high density lipoprotein in subjects. Besides, the hydrolysate enhanced activation of the compensatory IGF1R-PI3K-Akt survival pathway which is known to increase the cardiac cell survival rate in heart disease animal models. In a separate study, velvet bean protein (Herrera Chalé et al., 2016) was hydrolyzed by pepsin-pancreatin and Alcalase-Flavourzyme to obtain hydrolysates. Ultrafiltration fractionation of the hydrolysates was subsequently performed to obtain peptide fractions which demonstrated greater in vitro inhibition of human platelet aggregation and cholesterol micellar solubility than the hydrolysates. The hydrolysates and peptide fractions also reduced cholesterol and triglyceride levels in alloxan-induced diabetic rats.

15.6.6 Mineral-Binding and Regulation of Mineral Absorption

Alcalase hydrolysates of mackerel processing by-products (Wang et al., 2013) and scad processing by-products (Zhang et al., 2016) showed the highest iron-binding capacity compared to hydrolysates obtained from trypsin, Protamex, Flavourzyme, and Neutrase hydrolysis. In another study using tilapia protein (Charoenphun et al., 2013), Alcalase hydrolysate demonstrated the greatest calcium-binding capacity compared to Flavourzyme, Protease GN, and papain hydrolysates. Specifically, in the study using tilapia protein, the calcium-binding peptide was successfully purified and found to possess essential residues Glu and His for calcium-binding capacity. Contrary, the trypsin hydrolysate of the shrimp processing by-product (Huang et al., 2011) showed the most potent calcium-binding activity compared to the hydrolysates of Flavourzyme, Protamex, Alcalase, and pepsin. A tripeptide containing histidine with calcium binding activity was purified and identified.

Desalted duck egg white peptides (Zhao et al., 2014), the product of Protamex hydrolysis on desalted duck egg white, enhanced bone tissues (increased bone calcium content, bone mineral density, and bone mineral content) and bone mechanical properties (improved bone strength) in rapidly growing rats. The effects observed in the in vivo model were attributed to the calcium-binding ability of five peptides that were purified and identified, forming desalted duck egg white peptides-calcium complexes. It was also found that phosphorylated desalted duck egg white peptides were more effective than their nonphosphorylated counterparts in improving calcium absorption and bone strength. In another in vivo study by Hou et al. (2017) which was a continuation from Zhao et al. (2014)'s work, the researchers reported and confirmed structural folding and aggregation of amino acids or oligopeptides during the chelation process of desalted duck egg white peptides-calcium complexes. Moreover, an investigation on gene and protein expressions of calcium receptors in the study suggested that desalted duck egg white peptides regulated the proliferation and differentiation of enterocytes through the interaction with the transient receptor potential vanilloid 6 calcium channel.

15.6.7 Other Health-Related Benefits

Bioactive peptides have also been investigated for antiobesity and antidiabetic properties. Hydrolysates prepared from defatted salmon backbones (Slizyte et al., 2016) using trypsin, bromelain-papain, and Protamex displayed cellular glucose transporter inhibitory activity, contributing to decreased glucose uptake by intestinal cells and therefore a reduced risk of overweight and obesity. Trypsin, Flavourzyme, Proteinase K, Thermolysin, Alcalase, pepsin, papain, and chymotrypsin were used to hydrolyze black beans (Mojica and de Mejía, 2016) and the Alcalase hydrolysate inhibited the activities of α-amylase and α-glucosidase. Molecular docking analysis to predict individual peptide biological potential revealed that the black bean peptides inhibited α-amylase and α-glucosidase through hydrogen bonds, polar, and hydrophobic interactions. Papain and Protamex hydrolysates of fish water-soluble protein (Liu et al., 2013) also showed pancreas lipase and α-amylase inhibitory activities.

15.7 CONCLUSION

The production of bioactive peptides from food and food processing by-products using microbial proteases is believed to have great potential in combating NCDs and improving the problem of nutrient deficiency. Among the microbial proteases, Alcalase, Flavourzyme, Neutrase, and Protamex with superior performance for commercial exploitation should be given priority. Such applications of microbial proteases in a large industrial-scale production of bioactive peptides is deemed necessary. Bioactive peptides can be regarded as nutraceutical, and the ever-growing nutraceutical market demands efficient and economic processes. Consumer education on bioactive peptides as a novelty is going to play a crucial role in the success of product realization and commercialization. Peptide purification, and in vivo analysis on peptide stability against gastrointestinal enzymes and peptide absorption by the intestine are perhaps components that must be included in future studies on bioactive peptides. Turning food processing by-products into bioactive peptides reveals the prospect of microbial proteases in waste management and mitigating environmental issues for a sustainable environment.

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CHAPTER

16

Development of Functional Food From Enzyme Technology: A Review

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16.1 INTRODUCTION

Currently, the term "functional foods" is implicit in food or food components with more health benefits than simply meeting the nutritional needs of the human body. Increased health awareness has made people pay special attention to their diet and thus the development of new and convenient food products with the use of new technologies, such as bioprocess technology, has increased in recente years (Fuentes-Zaragoza et al., 2010). The most important bulk industrial enzymes traditionally used in bioprocessing are proteases, carbohydrases, and lipases. More recently, new enzymes have been developed such as tannase, phytases, and L-asparagine. Their uses in the production of bioactive compounds for functional foods have been enhanced because these new substances present some characteristics such as stability, solubility, and bioavailability that the original bioactive molecule frequently does not have (Draelos, 2008). Often, these enzymes are capable of inactivating/disabling antinutritional compounds. Therefore, the objective of this chapter is to highlight the research conducted on the application of these enzymes in the production of functional foods. The chapter begins by presenting some results of applications of proteases for the production of bioactive peptides. Following that are some carbohydrate results for resistant starch production. Lipase studies show the use of these enzymes for the production of structured lipids and functional fatty acid esters. The results obtained with tannases indicate the potential of this enzyme in the hydrolysis of polyphenols with the production of more bioactive molecules while phytase was used to reduce the phytic acid content in foods and to increase the mineral content. Finally, we will present some results that have used L-asparaginase to hydrolyze asparagine and reduce acrylamide levels in food.

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00016-5

16.2 PROTEASES

16.2.1 Potential Proteases as Biocatalysts for Development of Functional Foods

Proteases are universal enzymes present in innumerable biological systems ranging from microorganisms to higher organisms (Zeeb et al., 2017). The hydrolysis of proteins by proteases conducts to change the sensory, functional, and nutritional properties of the foods comprising gelation, solubility, foaming, emulsifying, flavor, texture, reduction of protein allergy, and bioactive peptide liberation (Fernández and Riera, 2013; Luo et al., 2014; McSweeney and O'Mahony, 2016). Proteases that use food-grade proteins and/or polysaccharides as substrates have acquired an increased interest among food scientists while microbial proteases are the enzymes that are largely employed in many established industrial processes (Verma et al., 2017; Zeeb et al., 2017). Microorganisms with specific hydrolytic enzymes, like proteases, have the ability to develop important processes for improving functional properties in bioprocesses and metabolites with the potential of commercialization (Chopra et al., 2015; Rai et al., 2016; Salim et al., 2017). Protein hydrolysis during an enzymatic or fermentation treatment, for instance, generates peptides that provide a wide range of functional properties (Balakrishnan et al., 2011; Rai and Jeyaram, 2015). Enzymatic hydrolysis with isolate enzymes is also an efficient and reliable method to produce peptides with antithrombotic, antihypertensive, anticancer, antioxidant, opioid, immuno-modulatory, and anti-inflammatory activities (El-Fattah et al., 2017). Furthermore, hydrolysis employing enzymes is accomplished under mild conditions that can be easily managed, allowing the obtainment of products with well-defined features (Zambrowicz et al., 2013) depending on the protein substrate (De Castro and Sato, 2014), protease type (Zhao et al., 2012), enzyme concentration (Yao et al., 2013), hydrolysis time (Luo et al., 2014), temperature, and pH (Le Maux et al., 2016). Modification of the food functional properties should be economically interesting and also have processing significance (Qian et al., 2010). The choice of enzymatic hydrolysis conditions must be realized, considering heat stability, taste, and specific application properties of the product (Tavano, 2013). With the estimation by the World Health Organization that health problems such as stroke and heart disease will overtake infectious diseases to become the principal cause of death and incapacity throughout the world by 2020, researchers have confirmed that dietary habits and numerous food components can prevent or even reduce the risk of many diseases. Thereby, the production of foods enriched with bioactive ingredients (peptides, isoflavones, etc.) has become increasingly significant in the development of functional foods (Groziak and Miller, 2000; He and Chen, 2013; Rai and Jeyaram, 2015).

16.2.2 Bioactive Peptides Obtained With the Use of Proteases and Their Health Benefits

One of the most important bioactive compounds cited above is the bioactive peptide that consists of protein fragments generated by the action of proteases, which have a positive effect on body functions and could influence health in addition to their nutritional purpose (Rai and Jeyaram, 2015). Bioactive peptides, after oral administration, can affect the major body systems. Thereafter, the association of distinct dietary peptide sequences to improve human health has stimulated great scientific interest in recent years because these peptide

sequences can reduce the risk of chronic diseases or boost natural immune protection. When encrypted in parent proteins, bioactive peptides are inactive, but once released during hydrolysis, they could behave, with hormone-like activity, as physiological modulators (Rai and Jeyaram, 2015). For the purpose of exerting the bioactivity function, food peptides have to be ingested and then reach the intestine in their intact form or be released in situ from their parent proteins to act locally, that is, in the gut, or even systemically, for example, through the blood stream (Panchaud et al., 2012). The action of bioactive peptides formed by protease hydrolysis will be dependent on their size, which could range from 2 to 20 amino acids, and their composition. Some are multifunctional and may perform more than one of the functional properties (Saito et al., 2000). Animal studies have revealed that larger peptides (with 10–51 amino acids) produced from the diet can be absorbed unbroken through the intestinal tract and generate biologic effects at the tissue level. The strength of bioactive peptides reduces as the chain length enhances (Roberts et al., 1999). Curiously, small di- and tripeptides are absorbed faster from the small intestine than free amino acids as they are transported by two independent systems. Most of the di- and tripeptides, after intracellular uptake, are quickly hydrolyzed, except very resistant proline-containing peptides that reach the portal venous system intact. Subsequently, these peptides exert health benefits in several body systems such as cardiovascular, immune, endocrine, and nervous. According to the amino acid sequence, bioactive peptides display many biological activities such as antimicrobial (Sila et al., 2014), antioxidant (Perna et al., 2013), hypocholesterimic (Hartmann and Meisel, 2007; Khaled et al., 2012), antithrombic (Ren et al., 2014), hypotensive (Balti et al., 2015), and immunomodulatory properties (Qian et al., 2011). Moreover, a role for food-derived peptides has been described by many reports, and shows the increasing gut absorptive and secretory ability or gut tissue growth besides an effect on satiety as well as stimulation of hormone secretion. Lastly, many peptides also exhibit opioid activity either in the agonist or antagonist mode (Moughan et al., 2007; Panchaud et al., 2012). Bioactive peptides may also be produced by in vitro hydrolysis of protein sources using suitable proteolytic enzymes. The nature of the protein substrate is influenced by the molecular weight and amino acid composition of bioactive peptides as well as by the specificity of the enzyme used for the proteolysis, by the conditions used during hydrolysis (time and temperature),and by the enzyme/substrate ratio, which finally influences their biological activities (Sila and Bougatef, 2016; Van der Ven et al., 2002).

16.3 CARBOYDRASES

16.3.1 Potential Carboydrases as Biocatalysts for the Development of Resistant Starch

The main source of carbohydrate in most diets is starch, a carbohydrate storage plant such as legumes, roots, and cereals (Cummings and Stephen, 2007). Starch occurs in foods that naturally contain polysaccharides that can be digested by the enzymes present in the human gut. However, scientific data have shown that a significant amount of starch is not fully digested, called “resistant starch” (RS) (Topping and Clifton, 2001). RS encompasses those forms of starch that are resistant to in vitro digestion by a-amylase and pullulanase and may be fermented in the colon (Ashwar et al., 2016). From 1992, the definition for RS assumed

a character more related to its biological effects: "the sum of the starch and products of its degradation that are not absorbed in the small intestine of healthy individuals" (Walter et al., 2005). It can be said that resistant starch is the fraction that will not supply glucose to the body, but which will be fermented in the colon to produce mainly short-chain fatty acids and gases. Due to this characteristic, the effects of resistant starch are considered to be, in some cases, comparable to those of dietary fiber and, for this reason, it is usually considered a component of starch. In this way, the digestibility of starch is a crucial topic, as starch needs to be resistant to digestion in the small intestine. The chemical composition and the way the starch is processed and stored modify its digestibility (Moraes et al., 2016). Crude starches are poorly digested and their digestibility can be improved through cooking, especially in the presence of water, which gels the starch and ensures better access to amylases. Degradation of the food structure by grinding and other processes (e.g., mastication) also increase digestion, allowing access to the matrix by digestive enzymes. While cooking increases the digestibility of the starch, subsequent cooling leads to the formation of crystallites resistant to digestion. This process is called retrogradation and the RS content of food seems to increase when subjected to a growing number of heating and cooling cycles (Topping and Clifton, 2001). Physiological factors may also interfere with the amount of RS in a food (Topping and Clifton, 2001). For example, chewing can dictate the particle size of an ingested food. Large particles travel faster through the gut than smaller ones, so higher chewing would be expected to increase digestibility. This wide range of RS determinant strongly suggests that all RS types may not have the same effects on the colon microflora (Ashwar et al., 2016). RS can be prepared by using different treatments. However, currently, RS is mainly manufactured by physical methods, including the heating-cooling and chemical modification processes, with an unsatisfactory yield of 20%–30%. This process may also have food safety issues. In contrast, the enzymatic method or a combination of the enzymatic and physical method can reduce the amount of substrate for RS production and help avoid safety problems in the production of RS-rich foods (Li et al., 2017). Studies have indicated that the use of debranching enzymes contributes to the enzymatic production of RS by reducing substrate molecular weight and increasing amylose yield (Li et al., 2017). Some authors have found that RS can be produced from high amylose starch by gelatinization followed by treatment of the pulp with debranching enzymes such as pullulanase. The product obtained can be isolated by drying/extrusion. Products having at least 50% RS content were fabricated into water-starch slurry, then the slurry was heated until complete starch gelatinization, followed by cooling to allow amylose retrogradation. RS was also prepared from the gelatinization of starch (common corn starch and waxy corn starch), followed by treatment with debranching enzymes (isoamylase or pullulanase), and precipitation of the shelled starch. Higher RS yields were obtained by subjecting the starch to enzymatic hydrolysis (pullulanase, 40 U/g/10 h), autoclaving (121°C/30 min), and refrigerated storage (4°C/24 h) followed by lyophilization (Pomeranz and Sievert, 1990). In a study investigating the debranching pattern of a thermostable isoamylase and its application to resistant starch production, the enzyme presented efficient, specific, and complete ability to dismantle starches from various sources. The smaller branches that can be recognized by this enzyme were composed of 1 and 3 glucose residues, which differ from the substrate specificity of all other isoamylases already reported. This feature, combined with its thermal resistance, contributes to the enzymatic production of RS by reducing molecular weight and improving linear dextrin formation. In addition, a novel strategy combining amylase and isoamylase has been developed to produce RS (Li et al., 2017).

16.3.2 Health Benefits of Resistant Starch

Several physiological effects have been ascribed to RS that have been proved to be beneficial to health. Foods rich in RS, because of low digestibility, were able to reduce the glycemic response, guaranteeing a more controlled release of glucose in the plasma (Ashwar et al., 2016). Reader et al. (1997) found that RS-rich foods reduced postprandial glycemia in humans and therefore could be used to control type II diabetes. However, in order to obtain this effect, RS should contribute at least 14% of the total dietary intake of starch (Ashwar et al., 2016). RS digestion occurs 5–7 h after food consumption whereas normal starch is digested immediately. This slower digestion reduces the glycemic response and the release of postprandial insulin in addition to increasing the period of satiety (Ashwar et al., 2016). Due to the large anaerobic community and low availability of oxygen in the colon, the metabolism of the bacteria is dominated by fermentation and anaerobic respiration. Under these conditions, RS can be fermented and release a number of fermentation products, such as short-chain fatty acids (SCFAs), organic acids, and alcohols, among other products. However, most of the studies investigating the influence of RS on intestinal health have focused on the production of short-chain fatty acids and intestinal bacterial composition (Lockyer and Nugent, 2017). The SCFAs resulting from RS fermentation are considered signaling molecules that bind the intestines to peripheral tissues, with implications for insulinemic responses and glucose homeostasis. Studies indicate that the mechanism involves differentiation, regulation, and metabolism of adipocyte cells (Bach Knudsen, 2015; Moraes et al., 2016). Some studies suggest that the satiety effects of the fibers may be due to the binding of SCFA to the free fatty acid receptor located in the colonic L cells that are involved in the control of anorexic hormones (Sleeth et al., 2010). In addition, these fermentable substrates can not only alter these hormones, but also exert a more effective action on body weight control, indirectly altering the expression of hypothalamic neuropeptides (Moraes et al., 2016). Studies also indicated the potential beneficial effects of SCFAs resulting from RS fermentation in the chronic kidney disease (CKD) population. According to Vaziri et al. (2014), SCFAs can participate in the integrity of the intestinal epithelial barrier, attenuating local and systemic inflammation. SCGAs were related to the stimulation of intestinal blood flow and their effects may contribute to the prevention of intestinal ischemia. Prebiotics are nondigestible foods that benefit the host by selectively stimulating the growth and/or activity of beneficial microorganisms in the gastrointestinal tract (Scholz-Ahrens et al., 2007). To exert prebiotic beneficial aspects, the products resulting from RS fermentation have to reach the colon and serve as substrate for the probiotic microorganisms, stimulating their growth (Moraes et al., 2016). RS-fermented products increased the growth of *Bifidobacterium* in the gastrointestinal tracts of rats and promoted the growth of *Lactobacillus* (Ying et al., 2013). A diet rich in RS significantly increased the populations of *Lactobacillus*, *Bifidobacterium*, and *Streptococci*, decreased the population of Enterobacteria, and altered the metabolism of microbial enzymes in the colon of rats. Several studies have shown that RS has great potential to inhibit fat accumulation. The SCFAs inhibit the lipolysis of adipose tissue and, in the liver, particularly the acetate-inhibited glycogenolysis, with the result of sparing carbohydrates and increased fat oxidation (Robertson et al., 2003). Studies have been conducted to elucidate how RS can influence lipolysis at the level of transcription. There are some indications of increases in genes coding enzymes involved in adipocyte differentiation and mobilization (Lockyer and Nugent, 2017).

16.4 LIPASES

16.4.1 Potential Lipases as Biocatalysts for the Development of Functional Foods

Lipases (triacylglycerol acyl-hydrolase, E.C. 3.1.1.3) are one of the most advantageous enzymes for the food industry (Lopes et al., 2011). Their potential as biocatalysts is huge for the production of fatty acid derivatives, such as structured lipids, flavoring esters, and fatty acid esters of antioxidants, essentially because they have high regio- and stereoselectivities (Ferreira-Dias et al., 2013). A very important field that uses lipases as catalysts is the production of fatty acid esters of antioxidants. The spontaneous reaction of atmospheric oxygen with lipids is known as autooxidation (Gordon, 2001), and the autooxidation of lipids begins with the formation of free radicals in an initiation reaction. This is followed by a series of propagation reactions of such radicals, and lastly a termination reaction occurs that includes the arrangement of two radicals with the development of stable products. The prevention of oxygen access, the inactivation of certain redox enzymes, the use of low temperatures, and appropriate packaging contribute to reducing oxidative damage. Oxidation may also be inhibited by the usage of specific additives (antioxidants) that could vary in their mode of action and chemical structure (Ferreira-Dias et al., 2013). The acylation of an antioxidant by transesterification or esterification can transmit new properties to the molecule, both in terms of biological or physical properties. Biocatalysts, as enzymes, become a greener alternative to traditional stochiometric methods, giving appropriate tools for the industrial conversion of synthetic and natural materials with low energy requirements and mild reaction conditions. Furthermore, the undesirable isomerization and rearrangement side reactions are minimized (Sabally et al., 2007). Lipases represent the most important group of enzymes that catalyze the formation or transference of ester bonds (Plou et al., 2003), and have been efficaciously used for the acylation of antioxidants. In these synthetic applications, the immobilization of the enzyme commonly favors the substrates to reach the catalytic site, reducing protein-protein contacts presented with the use of enzymes suspended in organic solvents (Torres-Salas et al., 2011). Esters of L-ascorbic acid with long-chain fatty acids (generally stearic or palmitic) are employed as additives in place of L-ascorbic acid (Vitamin C) for the stabilization of oils, fats, and fatty products because they retard the autooxidation of unsaturated fatty acids, due to the low miscibility of L-ascorbic acid in oily products. Lipases have been employed with great success to catalyze the synthesis of these ascorbyl esters using different solvents (Song and Wei, 2002), and applying saturated and unsaturated free fatty acids and vinyl and alkyl esters as acyl donors (Ferreira-Dias et al., 2013). Recently, Xu and Tang (2015) produced 6-O-L-ascorbyl-stearate using Novozyme 435 as the catalyst and stearic acid methyl ester as the substrate using an environmentally benign downstream process, obtaining a product with 99.8% purity. Costa et al. (2014) also used the lipase Novozyme 435 to catalyze the synthesis of ascorbyl palmitate under microwave irradiation. The reactions were performed in different organic solvents (acetone, tert-amyl alcohol, cyclohexane, and acetonitrile) and results demonstrated that tert-amyl alcohol was the best solvent for this reaction. Other parameters such as molar ratio, temperature, and enzyme loading were studied through a central composite design. They obtained, in the optimized conditions, 71% yield of ascorbyl palmitate after 1 h, using 15% of the nzyme (*w*/w in relation of ascorbic acid weight) with a

molar ratio of 1:5 and 70°C. Esters of tocopherol can be also produced by enzymatic reactions using lipases as catalysts as an alternative to the chemical production of tocopherols, also known as Vitamin E. This is important in the animal nutrition market where it is applied in high doses to upgrade the shelf life and quality of meat (Valentin and Qi, 2005). Torres et al. (2008) studied the enzymatic acetylation of Vitamin E, employing vinyl acetate as the acylating agent for the first time. They concluded that just the lipase B from *Candida antarctica* was a powerful biocatalyst for this reaction, out of 15 lipases, esterases, and proteases screened. Plou et al. (2012) also studied the enzymatic synthesis of vitamin E acetate by lipase-catalyzed transesterification. Similarly, they tested several lipases, esterases, and proteases for these syntheses; nevertheless, once again, only lipase B from *C. antarctica* catalyzed the acylation.

Finally, other alternative antioxidants used in food and as functional ingredients are the natural phenolic compounds, or phenolic antioxidants, found in fruits and vegetables (Medina et al., 2010; Shahidi, 2012). They are used to protect food-based products from oxidation and also to increase the shelf life of lipid-containing products that have nutritional and pharmaceutical relevance (Figueroa-Espinoza and Villeneuve, 2005). Another choice is the application of natural phenolics, although that is restricted due to their poor solubility in hydrophobic media, which shows poor absorption and results in a very low concentration in the circulatory system (Biasutto et al., 2009). To address this, several papers on the lipophilization of natural phenolics to prepare lipophilic antioxidants have been published (Kanjilal et al., 2008; Mbatia et al., 2011; Sabally et al., 2006). Lipases are important in the production of structured lipids, which can be defined as triacylglycerols modified or restructured to transform the fatty acid composition and/or their positional distribution in glycerol molecules by chemical or enzymatic processes (Lee and Akoh, 1998). Consequently, novel triacylglycerols can be obtained by the incorporation of new fatty acids by changing the positions or profile of fatty acids from the natural state, or by the synthesis of completely new triacylglycerols (Xu, 2000). The interest in the production of structured lipids presenting specific functional properties by lipase-catalyzed reactions in the field of edible oils and fats has greatly increased due to the advantages of the enzymatic route compared to chemical processes. Also, when sn-1,3 regioselective lipases are used, the original fatty acid at the sn-2 position in the triacylglycerol is preserved, and the obtained structured lipid presents a novel structure and properties not attainable by chemical catalysis. The synthesis catalyzed by lipases of distinct sorts of SLs has been widely described in the literature, either with organic solvents or in solvent-free media, and in bioreactors operating batchwise or in continuous mode (Álvarez and Akoh, 2015; Faustino et al., 2016; Gruczynska et al., 2015; Liu et al., 2016; Ribeiro et al., 2017; Teh et al., 2016; Zheng et al., 2017). High-yield sn-1,3 regioselective immobilized commercial microbial lipases have been employed in most of these studies. Nevertheless, the low operational stability of some of them and the high cost of commercial lipases have been identified as the main limitations to the usage of lipase-catalyzed processes for SL generation in the food industry (Ferreira-Dias et al., 2013). Speranza et al. (2015) studied for the first time the enzymatic interesterification of Amazonian oils using two lipases in three different enzymatic systems-crude lipase from *Rhizopus* sp., commercial lipase, and a mixture of both lipases—(*Rhizopus* sp. and commercial one) to check any synergistic effect. *Rhizopus* sp. lipase was specific in the annexation of oleic acid at the sn-1,3 positions of the triacylglycerol, which results in an oil abundant in saturated fatty acid in the sn-2 position. The commercial lipase from *Thermomyces lanuginosus*, in the second enzyme process, was not specific in the conditions examined.

There was no alteration in the distribution of unsaturated and saturated fatty acids in the three positions of the triacylglycerol, occuring as just a replacement of the type of fatty acid at the same position. And, in the third enzyme system, both lipase mixtures displayed no synergic effect.

16.5 TANNASE

16.5.1 Potential Tannase as Biocatalysts for Development of Functional Foods

Phenolic compounds are abundant in our diet, being present in a wide variety of plants, teas, wine, fruits, and nuts. They are considered bioactive compounds due to their beneficial effects in health promotion and prevention (Martins et al., 2011), for example, a decrease in the incidences of degenerative diseases such as cancer and diabetes (Sun et al., 2012) and a reduction of cardiovascular risk factors (Oboh and Ademosun, 2012). However, the efficacy of polyphenolics may not have reached the maximum potential in food products because they are tightly bound to cellulose matrices (Arnous and Meyer, 2010; Zheng et al., 2009), generally present in polymeric, glycosidic, or esteric forms, and interfere in bioavailability and bioefficacy (Holst and Williamson, 2008). Evidence shows that monomeric phenolics and some oligomers are absorbable (Shoji et al., 2006), but the polymeric forms have a low absorption rate (Donovan et al., 2002; Gonthier et al., 2003). Enzymatic hydrolysis of polyphenols to simpler and smaller compounds is a strategy to increase the bioavailability of bioactive compounds. This has great potential in the exploitation of biologically active compounds from natural sources (Georgetti et al., 2009). Tannase (tannin acyl hydrolase) is an extracellular enzyme produced by fungi, bacteria, and yeasts in the presence of tannic acid. Tannase have been generally identified by its action on hydrolysable and complex polyphenols, showing the ability to hydrolyze the depside bond (galloyl ester of gallic acid) and the ester bond (galloyl ester of an alcohol component) of tannins, for example, epicatechin gallate, epigallocatechin gallate, chlorogenic acid, and tannic acid (García-Conesa et al., 2001). Tannase also has the ability to degrade the plant cell wall, cleaving cross-links between cell-wall polymers (García-Conesa et al., 2001). Furthermore, this enzymatic hydrolysis is expected to potentially increase total phenols and help to release smaller and simpler phenolic compounds, therefore facilitating their absorption in the small intestine (Georgetti et al., 2009; Shoji et al., 2006). All these modifications may be connected to increases in antioxidant, anti-inflammatory, antiproliferative, immunomodulatory, and anticarcinogenic activities. Advances in the application of microbial tannases during recent decades are mainly related to the production of gallic acid, the synthesis of propyl gallate, the hydrolysis of tea cream, reducing the bitterness of fruit juices, and the degradation of tannery effluents and tannin from food and animal feed (Govindarajan et al., 2016; Zhang et al., 2016). However, the knowledge about tannase optimal expression, some properties, large-scale applications, and related costs is still insufficient, restricting its practical uses (Yao et al., 2014). Recently, food residues were submitted to enzymatic treatments with the aim of exploring these underutilized materials, rich in bioactive compounds bonded to cell wall structures. The main tannase applications in food products and beverages and the resulting functional improvements are presented in Table 16.1. Tannase-treated grape pomace and grape seeds showed enhanced total polyphenolics, antioxidant activity (AA), an increased amount of gallic acid (GA), and other phenolic acids, monomeric polyphenolics, and aglycones (Chamorro et al., 2012; Martins et al., 2016). Also,

TABLE 16.1 Potential Functional Foods and Ingredients Derived From Tannase Biotransformation

Product	Enzyme Treatment	Results	Reference
Grape pomace	50 U/g, 40°C, 5 h pH 5.0	• ↑ TP, ↑ AA • ↑ quercetin, ↓ rutin, • ↑ GA, caffeic acid, resveratrol, vanillic acid, p-coumaric acid, myricetin, 3,4-dihydroxyphenylacetic acid, procyanidin B2 • ↑ anti-inflammatory effect	Martins et al. (2016, 2017)
Grape pomace and grape seed	Grape seed (2000 U/g) and grape pomace (1000 U/g) 35°C, 24 h, pH 5.5	• ↑ TP, ↑ AA • ↑ GA, EC, procyanidin B2 • ↓ ECG, ↑ EGC • changed the galloylated form of catechin to its free form	Chamorro et al. (2012)
Citrus residues	Fermented by *Paecilomyces variotii* (tannase producer) 10 g residue, 32°C 48 h	• ↑ hesperetin and naringenin • ↑ lipolysis on fat tissue (3T3-L1 adipocytes)	Nakajima et al. (2016)
Citrus residues	Fermented by *P. variotii* (tannase producer) 10 g residue 32°C, 48 h	• ↑ hesperetin, naringenin and ellagic acid • ↑ AA	Madeira et al. (2014)
Castor bean residue	Fermented by *P. variotii* (tannase producer) 5 g of residue +5 mL of saline +10% tannic acid + inoculum 30°C, 96 h	• ↓ hydrolysable tannin • ↑ GA • ↓ cytotoxicity (RAW 264.7 cells) • Detoxification of residue—↓ ricin	Madeira et al. (2011)
Black, green, white, and mate tea	300 mL infusion pH 5.5 + 2.35 g immobilized tannase 60°C, 2 h, 300 rpm	• ↓ galloylated catechins in green, white and black tea • ↓ lipid accumulation in adipocytes • ↑ GA, CAT, EC • ↓ EGCG, ECG • ↑ inhibition of digestion enzymes	Roberto et al. (2016)
Tea by-products and tea infusion	Fermented by *Aspergillus niger* (tannase producer) 5 g tea residue, 30°C, 96 h 1 mL tea infusion +3.44 U/mL tannase 30°C, 20 min	• ↑ gallic acid • ↓ EGCG, GCG, ECG • ↑ AA • ↑ tea clarity	Ni et al. (2015)
Green tea	500 U/g 35°C 2 h	• ↑ GA, ↓ EGCG • ↑ AA • ↑ immunomodulatory activity	Baik et al. (2014, 2015)
Green tea	200 g extract +1 g tannase 35°C, 20 min	• ↓ EGCG, ↓ ECG • ↑ inhibition of elastase and tyrosinase activities • antiwrinkling or depigmenting agent	Hong et al. (2014)

Continued

TABLE 16.1 Potential Functional Foods and Ingredients Derived From Tannase Biotransformation—cont'd

Product	Enzyme Treatment	Results	Reference
Green tea	500 U/g 35°C	• ↑ EGC, EC, and GC • ↑ GA and EGC	Noh et al. (2014)
Green tea and yerba mate	5 mg extract +5 mg tannase 40°C, 30 min, pH 7.4	• ↑ AA • ↓ EGCG, ↑ GA • ↓ toxicity without affecting antiproliferative effects (PG100 and HT29 cells) • Modulated the expression of genes related to carcinogenesis	Macedo et al. (2011, 2012)
Green tea	250 U/g, 35°C 20 min, pH 6.0	• ↑ AA • ↓ EGCG, ↓ ECG, ↑ GA, EGC, and EC • ↓ protein binding ability • Better color appearance, less cream • ↑ viability of RAW 264.7 cells • ↑ protective ability to cells from Cu^{2+} damage • ↓ nitrite-mediated N-nitrosation • ↓ formation of *N*-nitrosodimethylamine (NDMA)	Lu et al. (2009) and Lu and Chen (2007, 2008)
Orange juice	5 U/mL 40°C 60 min	• ↑ naringin and hesperidin (aglycones) • ↓ naringenin and hesperetin (glycosides)	Madeira et al. (2015)
Orange juice	5 U/mL 40°C 60 min	• ↑ naringin and hesperidin (aglycones) • ↓ naringenin and hesperetin (glycosides) • ↑ AA • ↑ antiproliferation activity in HepG2 and HT29 cells	Ferreira et al. (2013)
Soymilk	1.8 U/mL 40°C 30 min	• ↑ TP, ↑ AA • ↓ daidzin, ↓ genistin (glycosides) • ↑ daidizein, ↑ genistein (aglycones) • ↑ equol content	Queirós et al. (2016)
Oil palm fruit	600 U/g 50°C 1 h	• ↑ TP, ↑ AA, ↑ carotenoids, ↑ phenolic compounds • ↑ oil extraction	Teixeira et al. (2013)

Abbreviations: AA, antioxidant activity; CAT, catechin; EGC, epigallocatechin; EC, epicatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallate; GA, gallic acid; GC, gallocatechin; GCG, gallocatechin gallate; HepG2, human liver cells; HT29, human colon adenocarcinoma cells; PG100, human gastric adenocarcinoma cells; RAW 264.7, murine macrophages; TP, total phenol.

the extracts presented in vitro anti-inflammatory effects associated with decreases in reactive oxygen species and inflammatory biomarkers in Caco-2 cells (Martins et al., 2017). The reduction of hydrolysable tannins, the increase in gallic acid content, and the reduction of in vitro cytotoxicity in macrophages were observed in castor bean residue fermented by *Paecilomyces variotii*, a tannase-producing fungi. The fermented product was also detoxified, with reduced levels of ricin (Madeira et al., 2011). Citrus residues submitted to the same fermentation

process resulted in a residue richer in hesperetin, naringenin, and ellagic acid (phenolic aglycones), with enhanced antioxidant activity and an in vitro antilipogenic effect on adipocytes (Madeira et al., 2014; Nakajima et al., 2016).

The main tannase-treated beverage studied is green tea. This biotransformation process can develop teas with a better color appearance, less cream formation, and better clarity (Lu et al., 2009; Ni et al., 2015). Also, the teas showed higher AA, GA, and immunomodulatory activity. Reductions in epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), and epicatechin gallate (ECG); inhibition in the formation of *N*-nitrosodimethylamine (NDMA), a possible carcinogenic compound; less protein binding ability; lower toxicity; the modulation of carcinogenesis genes; and a reduction in lipid accumulation in adipocytes were also demonstrated after enzymatic treatments (Baik et al., 2015; Hong et al., 2014; Lu and Chen, 2007; Noh et al., 2014; Roberto et al., 2016). These results demonstrate the wide range of beneficial actions of tannase treatment on phenolic-rich food beverages. In recent studies, tannase also showed the ability to change the phenolic composition of orange juice, transforming glycosides in aglycones (acting on hesperidin and naringin) and generating a product with higher functional activity due to increases in antioxidant and antiproliferative activities on human cells (Ferreira et al., 2013; Madeira et al., 2015). Tannase activity can also be interesting for the enhancement of oil extraction from oleic matrices, increasing phenolic compound extraction and AA (Teixeira et al., 2013). Soybeans and soybean-derived products are attracting attention for their health-promoting effects, mainly related to isoflavones, which can also act as phytoestrogen compounds (Xiao, 2008). Soymilk isoflavones (daidzin and genistin) were biotransformed by tannase, producing daidzein and genistein (aglycones), compounds with greater bioavailability. Also, the treatment increased the AA and total phenolic content of soymilk samples. Furthermore, the enzymatic production of equol from soy isoflavones was observed for the first time (Queirós et al., 2016). Equol is a daidzein metabolite produced in our body exclusively by intestinal bacteria, with superior nutraceutical health effects compared to other soy isoflavones (Yuan et al., 2007).

16.6 PYTASE

16.6.1 Potential Pytase as Biocatalysts for the Development of Functional Foods

Phytic acids, also known as myo-inositol-1,2,3,4,5,6-hexakisphosphates (InsP6) or phytates when in salt form, are antinutritional factors in food products due to their hability to form complexes with essential minerals (cations of Ca, Fe, K, Mg, Mn, and Zn). They also interact with proteins in food and during digestion. Phytates are the major forms of organic phosphorus in plant-derived food, mainly in edible legumes, nuts, oil seeds, and cereals (Kumar et al., 2010). The dephosphorylation of phytate is crucial for increasing the nutritional value of plant-based food, decreasing the mineral chelating power. Some techniques for food preparation, for example, soaking, germination, cooking, and fermentation, are capable of reducing the phytate in food. Besides, some enzymes (phytases) are capable of sequentially dephosphorylating phytic acid to products with higher solubility and lower chelating capacity, decreasing its inhibition on mineral absorption (Greiner and Konietzny, 2006). Phytases are normally present in plants and produced by microorganisms, but the application of exogenous enzymes can also be a strategy in the food industry. This is normally used to improve

the nutritional value of animal food, especially for monogastric animal diets, and to reduce phosphorus pollution in animal waste (Jain et al., 2016). Recent reviews show the activity and importance of phytase supplementation in fish, shrimp, broilers, pigs, and poultry feed (Dersjant-Li et al., 2015; Kavitha, 2016; Lemos and Tacon, 2016). However, there is a great potential for the use of these enzymes for the development of human functional food, being an alternative to reduce the risk of mineral deficiency in susceptible people (Jain et al., 2016). Recently, the decrease of phytate was also correlated to the reduction of oxalate in food, which may help in reducing the development of kidney stones (Israr et al., 2017). As shown in Table 16.2, over the last two decades there have been numerous studies monitoring the effects of phytases added to food products. The inclusion of phytases during food processing is suggested for the increment of phytate dephosphorylation. This bioprocess may be a good strategy for the development of new low-phytate vegetable products and, consequently, larger nutritional and functional significance for humans and animals (Gupta et al., 2015).

TABLE 16.2 Potential Functional Foods and Ingredients Derived From Phytase Treatment

Product	Study	Phytase Treatment	Results	Reference
Fermented soy drink	In vitro	*Lactobacillus casei* phytases 50 mL soy drink +6×10^7 CFU/mL *L. casei*, 37°C 0, 3, 5, 7, 16, 20, 24 h	• ↓ phytate • ↑ InsP3 • ↑ Ca, Fe and Zn bioavailability • New probiotic fermented vegetable products	García-Mantrana et al. (2015)
Soy protein isolate	In vitro	19.86 mg phytase/g soy 25°C, pH 5.0 5, 10, 20, 40, and 60 min	• ↓ phytate, ↑ protein content • ↑ Zn and Ca content/ ↑ Zn bioavailability • Better gelling and nutritional properties • Better in vitro digestibility	Wang et al. (2014)
Soymilk	In vitro	Immobilized phytase versus native phytase 7 h, 60°C	• Immobilized enzyme: ↓ 92.5% phytate • Native enzyme: ↓ 98% phytate	Çelem and Önal (2009)
Rye bread	In vitro	Commercial preparations of 6-phytase A and phytase B 5000 U/kg of flour pH 4.5, 35°C, 150 min	• ↓ phytate content • In vitro bioavailability of phytate: 9% (bread with phytase B), 7% (6-phytase A) and 50% control bread. • ↑ bioaccessibility of InsP3 (Caco-2 cells)	Duliński et al. (2016)
Whole-grain sourdough bread	In vitro	*L. casei* producing phytases starter in bread making process	• Not satisfactory ↓ phytate in bread, with residual InsP6 • ↓ expression bifidobacterial phytases • ↓ accessibility of phytate	García-Mantrana et al. (2016)

TABLE 16.2 Potential Functional Foods and Ingredients Derived From Phytase Treatment—cont'd

Product	Study	Phytase Treatment	Results	Reference
Whole quinoa flour bread	In vitro	*Bifidobacterium* phytases added during mixing stage	• quinoa: ↑nutritional value, raising fiber and minerals (Ca, Fe, and Zn) • ↓ phytates: below the detection limit • ↑ mineral bioavailability • the breads were accepted by consumers	Iglesias-Puig et al. (2015)
Amaranth flour dough bread	In vitro	Purified phytases from *Bifidobacterium* added during mixing concentration = phytase endogenous activity (flour)	• ↑ nutritional value (slight depreciation in the quality) • ↑ phytate degradation (compared to commercial phytases)—without affecting quality • InsP6 levels ↓ threshold of mineral bioavailability inhibition (Fe and Zn)	García-Mantrana et al. (2014)
Brown rice flour-added bread	In vitro	*Aspergillus niger* phytase added to water (3000 U/bread)	• ↓ bread InsP6 content • No adverse effects on the rising of the bread	Matsuo et al. (2010, 2012)
Whole-wheat bread	In vitro	20 U phytase/g of wheat flour 30 or 60 min pH 7.0 or 5.0	• ↓ phytate • ↑ mineral availability • Biggest increase in Fe^{2+} • Phytate degradation enhanced the mineral availability of bread	Park et al. (2011)
Whole-wheat bread	In vitro	Lactic acid bacteria producing phytases added during mixing stage 37°C, 60 min	• Bread with similar technological quality than the control • ↑ freshness • ↓ levels of inositol phosphates	Palacios et al. (2008)
Wheat, oat, barley bran and red kidney and white beans	In vitro	0.12 U/100 mg 37°C, 75 min, pH 5.5	• ↑ phosphate concentration in all samples • Wheat and oat bran: ↓ soluble oxalate • Red kidney and white beans: ↑ soluble oxalate	Israr et al. (2017)
Rapeseed meal	In vitro	0.4–1.6 U/g 55°C, pH 5, 200 rpm 2 or 4 h	• ↑ alkaline extraction yield of proteins from rapeseed meal • Production of rapeseed protein concentrates with very low levels of phytic acid • ↓ phytic acid in the remaining rapeseed meal	Rodrigues et al. (2017)

Continued

TABLE 16.2 Potential Functional Foods and Ingredients Derived From Phytase Treatment—cont'd

TABLE 16.2 Potential Functional Foods and Ingredients Derived From Phytase Treatment—cont'd

Product	Study	Phytase Treatment	Results	Reference
Pea flour	Animal study	800 U/kg of feed pH 5.5, 37°C, 60 min, 350 rpm	• ↑ Fe content after soaking, ↑ total P • ↑ Fe absorption by growing rats • ↓ phytic acid • No ↑ digestive utilization of protein	Urbano et al. (2003, 2007)
Lupin (legume)	Animal study	750 U/kg Growing rats	• ↑ bioavailability of P, Ca, and Mg • Supplementation of phytases: did not cause any further improvement	Porres et al. (2006)
Phytic acid (PA), soymilk and wheat bran (WB)	Animal study	Endogen phytases—produced by microbiota	• Soymilk perfusions: ↑ phytate hydrolysis • PA and WB groups: ↑ Ca absorption • intake of pure PA: ↑ phytase in the upper parts of the small intestine • WB diet: ↑ ileal phytase	Lopez et al. (2000)
Maize meal + RUTF + micronutrient powder	Clinical trial	*Aspergillus niger phytase* (190 U) *Women	• ↑ Fe absorption • No interaction between phytase and RUTF	Monnard et al. (2017)
Millet-based porridge	Clinical trial	Cereal porridge with phytase (20.5 U)—added before consumption *Children	• ↑ fractional absorption of Zn • ↑Zn absorption in young children	Brnic et al. (2016)
Maize or sorghum porridges	Clinical trial	fortified with Zn and phytases pH5.0, 55°C, 60 min *Men and women	• Phytase degraded phytic acid during digestion or during food preparation • ↑ Zn absorption • sorghum polyphenols + phytic acid: inhibited Zn absorption	Brnic et al. (2014)
Teff flour bread	Clinical trial	10 mg/100 g 15 mg /100 g of flour *Women	• ↑ bioavailability of Fe • Help maintain serum Fe levels, especially when phytase is added	Bokhari et al. (2012)
Meals containing white wheat rolls and wheat bran	Clinical trial	*A. niger* phytase 40,000 U/mL, 37°C, pH5.0 *Men and women	• ↑ Fe absorption • Complete degradation of phytate occurred in the stomach • ↑ activity of microbial phytase at physiological stomach pH	Sandberg et al. (1996)

Abbreviations: InsP6, myo-inositol hexakisphosphates; InsP3, myo-inositol triphosphates; RUTF, ready-to-use-therapeutic food; Ca, calcium; P, phosphorus; Zn, zinc; Mg, magnesium; Fe, iron; E/S, enzyme protein/substrate mass.

One of the most studied food products in this field is bread. The production of bread with the incorporation of different cereals and grains is a growing trend in the food industry, mainly because consumers are more interested in food and nutritional health effects. Whole grains have larger amounts of vitamins, minerals, fibers, and other biologically active compounds than refined grains (Jonnalagadda et al., 2011). Studies applying phytase during the bread-making process (rye bread, whole quinoa bread, brown rice bread, teff bread, wholewheat bread, and amaranth-flour bread) showed efficient decreases of phytate in the final product. They also showed enhanced nutritional value by raising fiber and mineral bioaccessibility without effecting bread quality (Bokhari et al., 2012; Duliński et al., 2016; García-Mantrana et al., 2014; Iglesias-Puig et al., 2015). In vitro bioavailability of Fe and Zn was increased after phytase treatment in wheat seed, rice, and sorghum (Abid et al., 2016; Nielsen and Meyer, 2016). In vivo studies confirmed the increase in the digestive utilization and absorption of Fe from pea flour protein and faba bean flour after phytase treatment. The higher Fe availability was correlated to higher levels of this mineral in the sternum of rats (Luo and Xie, 2012). Consuming supplemental dietary phytase or dietary Zn additively enhanced Zn status and increased bone mineral density in rats fed a low-Zn diet. This study confirmed that bone health can be disrupted by Zn deficiency, and if dietary Zn is limited, supplemental phytase may be valuable (Scrimgeour et al., 2010). Also, the enhanced bioavailability of Ca, Mg, and P was observed after consumption of lupin treated with phytase (Porres et al., 2006). These enhanced mineral bioavailability effects can also be related to the increase in endogen phytase production by the microbiota in the rat's small intestine (Lopez et al., 2000). A possible advance in phosphorus digestibility, lower phosphorus excretion, and superior biochemical indices for glucose and cholesterol was observed in rats fed sorghum treated with tannase and phytase (Schons et al., 2011). Controversially, the presence of phytate in food products can provide protection against cancer through antioxidation, by the suspension of cellular signal transduction, and by affecting the cell cycle. The solubility advantage of InsP3 over InsP6 could enhance phytate's future use as antioxidants for food safety, nutrition, and therapeutics (Phillippy and Graf, 1997). Studies show the therapeutic use of phytates against diabetes mellitus, atherosclerosis, and coronary heart disease (Kumar et al., 2010). Considering this, the use of phytases can also make a balanced and controlled degradation of phytate, producing food with a known content of myo-inositol phosphate esters with antioxidant effects, health benefits, and lower antinutritional effects. Microbial phytase preparations are commercially available, being a feasible technique to be use in food processing. Even with this great potential for human consumption, no phytase-treated food product for human application has been developed yet.

16.7 L-ASPARAGINASE

16.7.1 Potential L-Asparaginase as a Biocatalyst for the Development of Functional Foods

Acrylamide is easily generated and found in fried and baked foods, found mainly in food derived from plants, for example, potato products, grain products, and coffee, as a result of the reaction between asparagine and reducing sugars via the Maillard reaction (Xu et al., 2016). These food products normally show large acrylamide levels, which have

been considered a presumable carcinogen for animals and humans (Mottram et al., 2002). Technological and agronomical strategies have been studied and suggested that aim to diminish acrylamide in baked and fried foods, for example, reducing acrylamide precursors, controlling conditions during the process (reducing pH, reducing temperature, shortening time), and the use of additives (Palazoğlu and Gökmen, 2008; Zyzak et al., 2003). An enzymatic approach for acrylamide reduction is the transformation of the main precursor of acrylamide. L-Asparaginase can hydrolyze asparagine into aspartic acid and ammonia. This process may be considered a promising way to effectively mitigate acrylamide in food products, especially because of the minor or negative impact on product taste, flavor, and appearance (Hendriksen et al., 2009). An overview of the enzymatic mitigation of acrylamide in food products using asparaginase was recently published (Xu et al., 2016). Studies evaluated the effects of asparaginase on potatoes, gingerbread, French fries, biscuits, crispbread, fried dough, bread, potato chips, coffee, rotilla chips, Lebkunchen, cookies, and wheat-oat bread. L-Asparaginase from different sources was effective in reducing acrylamide levels in these products (Xu et al., 2016). During the last 2 years, the main studied food products were French fries and potato chips, which are consumed and appreciated worldwide by different populations. A novel asparaginase from *Aquabacterium* sp. A7-Y (14.6 U/mL, 45°C, 30 min) was able to reduce 88.2% of the acrylamide content from potato strips (Sun et al., 2016). The addition of a purified asparaginase from *Pseudomonas oryzihabitans* (2.8 U/g dried potato, 40°C, 15 min) reduced 90% of the acrylamide in fried potatoes (Bhagat et al., 2016). L-Asparaginase from *Aspergillus oryzae* CCT 3940 reduced 72% of the acrylamide concentration in fried potatoes (50 U/mL, 50°C, 30 min), with the advantage of not reducing the glutamine levels in the sample, contributing to the better quality of the final product (Dias et al., 2017). A novel L-asparaginase gene from *Paenibaeillus barengoltzii* was cloned and expressed in *Escherichia coli*. The treatment (80 U/mL, 45°C, 20 min) was effective in mitigating acrylamide in potato chips and mooncakes by 86% and 52%, respectively (Shi et al., 2017). The production of L-asparaginase was reported in different organisms, including animals, plants, and microorganisms (actinomycetes, algae, bacteria, fungi, and yeasts) (Batool et al., 2016). The main commercial asparaginases are Acrylaway from *A. oryzae* (Novozymes) and Preventase from *Aspergillus niger* (DSM Food Specialities), used industrially in more than 30 countries in different products such as biscuits, snacks, French fries, and coffee. In 2008, it was announced that the first commercially available "acrylamide-free" product, biscuits treated with Preventase, could be found in Germany (Foodingredientsfirst.com, 2008). In 2013, Novozymes reported the development of a thermostable solution, the Acrylaway HighT, capable of reducing acrylamide concentration in food products processed at elevated temperatures (Foodingredientsfirst.com, 2013). However, no new acrylamide-free food products are available on the market, probably because their implementation is still costly in comparison to other methods, and also due to commercial L-asparaginase products having been only recently available to the food industry. Mitigation procedures that affect the Maillard reaction may have negative influences in the flavor and color of baked and fried food products. These novel L-asparaginases can be potential candidates for applications in the food processing industry, but additional studies are needed to develop new sources with low-cost, large-scale production and methods to minimize flavor and color interferences, keeping the quality of the final product and consumer acceptance.

16.8 CONCLUSION

The use of enzymes for the production of functional foods has presented very promising results. This chapter presents papers from the literature on food production with functional properties using different classes of enzymes that meet consumer demand for healthier foods.

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CHAPTER

17

Current Development and Future Perspectives of Microbial Enzymes in the Dairy Industry

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17.1 INTRODUCTION

Although enzymes are proteins, some RNA molecules are also considered as enzymes that act as catalysts and increases the rate of biochemical reactions in all biological systems, including microorganisms, plants, and animals (Alberts et al., 2002). These enzymes are so potent that in very small quantities they can increase the rate of reactions up to a million times. A great example is orotidine-5′-phosphate-decarboxylase, which enables a reaction to happen in milliseconds that would otherwise take millions of years (Callahan and Miller, 2007; Radzicka and Wolfenden, 1995). Due to this fact, enzymes have evolved as an important molecule that is essential to sustain life, as it allows all metabolic processes to occur at a fast rate. More than 5000 types of biochemical reactions are known to be catalyzed by enzymes (Schomburg et al., 2012). An enzyme's specificity and activity is a result of its unique three-dimensional structure, which can easily be affected by environmental surroundings. Hence, enzymes can operate well only under a narrow set of conditions such as pH and temperature. The activity of enzymes is also affected by the presence of inhibitors and activators (Athel, 2014). At an industrial scale, enzymes are even utilized for the synthesis of antibiotics. Some enzymes have found their application in household products such as detergents, which break down a variety of stains on clothes. Many other enzymes are employed in the dairy industry for the manufacturing of yogurt, cheese, and other dairy products as well as in the enhancement of flavor and texture (Neelam et al., 2015).

Millions of Indians depend on milk and its products for their nutrient supply, as milk contains all the essential nutrients except iron and Vitamin C. Thus, after the white revolution,

https://doi.org/10.1016/B978-0-12-813280-7.00017-7

India has turned into the biggest milk producer with the creation of 110 tons in 2010. The request of handled dairy items has ascended with developing urbanization, which is especially high for different types of cheeses and low-lactose milk as a result of the expansion in lactose-intolerant people. A spectacular increase in milk production has brought the dairy sector to a high position. More than 400 dairy plants are now able to handle as much as 20 million liters per day (lpd). To meet this requirement, the responsibility of producers has increased to satisfy the expectations of consumers as well as the world market in respect to producing high-quality dairy products. The approximate figure for the milk requirement is ~130–152 MT by 2030. This may be a difficult task that could not be completed without improving the present technologies in the dairy sector (Francesco and Andrea, 2002). India has to employ advanced technologies to achieve the desired target and to become the number one producer in the worldwide milk industry. This can only be achieved by the significant contribution provided by innovations in biotechnology. Since biotechnology provides lots of newer opening for dairy industry in terms of amplifying production of milk and milk products through which everyone needs can be fulfilled. Instead of contributing to improving the safety and overall quality, biotechnology has elevated the commercial value both for local consumption and exports. It is a universal fact that in the production of dairy products, microorganisms play a significant role both in positive and negative ways, largely due to the enzymes they produce. It is now clear by the finding of the studies conducted until now that microorganisms have diverse functions and microbial enzymes have not been utilized up to the mark in the production of dairy products. Keeping all these points in mind, the role of microbial enzymes in the dairy sector with their current developments is compiled here, with their applications.

17.2 DAIRY PRODUCTS OR MILK PRODUCTS

Dairy products are the kinds of foods that are obtained primarily from or contain milk of mammals such as cattle, goats, sheep, etc. Dairy products include a variety of foods such as cheese, butter, yogurt, and many more. They are consumed throughout the world, excluding some parts of central Africa and some countries in East and Southeast Asia (Neelakantan et al., 1999). Lactose intolerance in people varies according to their society and exists as a major problem. The intolerance index shows that 5% of North Americans, North Europeans and Australians, about 17% of the British, and more than 50% of South Americans, Africans, and Asians suffer from lactose intolerance. Practically lactose can be removed from the milk by partial hydrolysis, either by the enzyme β-amylase (EC 3.2.1.2) or complete hydrolysis by lactase (EC 3.2.1.23), even though, biotechnology focus for the development of low-lactose or lactose-free products for lactose intolerance people such as lactose fermented milk, ice creams and milk drinks (Clark et al., 2009; Mohan and Jaishankar, 2008; Shinjini and Rakesh, 2007).

17.3 ENZYMES IN DAIRY TECHNOLOGY

Enzymes play an important role in the reduction of energy in several technologies needed in dairy processing. This result can be ascribed to their properties that boost color, flavor, and aroma along with an increasing yield of milk products. Other functions of enzymes include as

coagulants and bioprotective agents that are responsible for enhancing the shelf life as well as safety of the dairy products are also in high demand in the dairy sector. The production of yogurt, cheese, and several other dairy products can be ascribed to the dairy enzymes (Pai, 2003; Neelam et al., 2015). Cheese production took place by coagulating milk into solid curds by a combination of enzymes named chymosin and pepsin, together known as rennet. Globally, around 33% of cheese is produced using microbial rennet. Several proteases are also known that accelerate the production of cheese and reduce lactose intolerance belonging to milk products (Neelam et al., 2015). Lipases, which are another class of enzymes, are responsible for the enhancement of flavor, the fast production of cheese, and fat lipolysis as well as the manufacturing of other customized milk and milk products (Ghosh et al., 1996; Sharma et al., 2001). To change the functional properties of dairy products, one of the important enzymes—transglutaminase—plays a significant role in carrying out the polymerization of proteins present in milk (Afroz et al., 2015; Rossa et al., 2011). Similarly, lactase (β-galactosidase, EC 3.2.1.23) is popularly known for catalyzing the hydrolysis of lactose to glucose and galactose, which is utilized as an aid for digestion in people suffering from lactose intolerance while further improving the flavor and aroma in milk products (Soares et al., 2012; Neelam et al., 2015). Enzymes can be obtained both from microbial and nonmicrobial (plants and animals) sources. However, microbial enzymes have gained much attention due to several significant characteristics, including their stability, their excellent catalytic activity, and their production and optimization, which is easier in comparison to those enzymes obtained from plants and animals. They also have reduced processing time, are cost effective, nontoxic, require low energy input, and are ecofriendly. This was just a brief overview about various applications of different enzymes. However, in this chapter a detailed discussion on the significance of microbial enzymes in the dairy sector is highlighted under a separate heading.

17.4 ROLE OF MICROBIAL ENZYMES IN THE DAIRY INDUSTRY

Microbial enzymes possess several important characteristics such as ease of production and availability that make them suitable candidates for successful use in food processes. They are utilized by the food industry to improve quality, shelf life, and flavor as well as for specific use related to every processed food. At the food industry scale, several enzymes play crucial role: lactase is involved in eliminating lactose from milk and milk products, lipases are mainly employed for cheese ripening, proteases for meat tenderness, and groups of pectinase, transglutaminase, and asparaginase are involved in clarification and minimizing acrylamide formation. Other enzymes such as cellulase, laccase, phytase, and a complex of xylanase are also important in food process technologies to enhance the bioavailability of minerals, increasing aroma in processed food, prevent destabilization of beverages, and make nutraceuticals more effective. For manufacturing of superior quality cheeses with pleasant flavors and aromas as well as good texture, animal rennets (bovine chymosin) are traditionally used as the milk-clotting agent in the dairy industry. Due to increasing demand for cheese worldwide, calf rennets are not sufficient, which forces the researcher to search for new sources for the production of chymosin such as microbial rennets (Stanley, 1998). In the current scenario, almost one-third of cheese production depends on microbial rennets throughout the world. The mechanism of rennin action for milk coagulation occurs in two steps: enzymatic and

nonenzymatic. In the enzymatic phase, the sample milk acquires gel-like consistency due to the presence of Ca^{+2} ions at optimized temperatures, also called milk curdling (Bhoopaty, 1994). Microbial rennets used here are basically proteases and act as an alternate for calf rennets. They are successfully isolated from several species such as *Mucor* sp., *Aspergillus oryza*e, *Rhizomucor pusillus*, *R. miehei*, *Irpex lactis*, and *Endothia parasitica.* The most prominent rennet producer is found to be a *R. pusillus* when grown on a solid-state medium using 50% wheat bran. *R. miehei* and *E. parasitica* produce a good quantity of rennets when the medium is supplemented with 4% potato starch, 3% soybean meal, and 10% barley. Here one limitation is observed, as lipase is also secreted by microbes during growth, which may hinder the activity of rennets. Therefore, it must be deactivated before adding to cheese production by lowering the pH.

Several microbial rennets for the production of cheese have been commercialized since the 1970s under business names such as Rennilase, Hanilase, Fromase, and Marzyme. A very famous company called Novo Nordisk successfully isolated one protease for cheese preparation from the fungus *Rhizomucor miehei*, which was expressed in *A. oryza*e. This host organism produces a single potent rennet that splits the casein into a glycol-macropeptide and paracasein by hydrolyzing only at the phe^{105}-met^{106} peptide bond. This monocomponent enzyme product has the trade name Novoren (Law, 1997). Besides the several advantages, microbial rennets face few shortcomings such as the development of a bitter taste and unpleasant flavor in ripened and nonripened cheeses. Microbial rennets have more proteolytic efficiency as compared to calf rennets and thereby generate small peptides during cheese ripening, which are volatile in nature and give rancidity. The best way to overcome this problem is that the calf rennet gene should be cloned and expressed in a selective microbe (bacteria, yeasts, and molds). The resulting enzymes must be used in cheese ripening. Several microorganisms that are successfully using for dairy product preparations are summarized in Table 17.1.

TABLE 17.1 List of Microorganisms Used in Dairy Product Manufacturing

Dairy Products	Microorganisms	Type of Microorganism
Smear-Ripened Cheese	*Arthrobacter arilaitensis* *Arthrobacter bergerei* *Arthrobacter globiformis* *Arthrobacter variabilis* *Brevibacterium casei* *Brevibacterium linens* *Corynebacterium casei* *Microbacterium gubbeenense*	Bacterium
	Thrichosporon beigelii *Torulaspora Delbrueckii* *yarrowia lipolytica*	Fungus
Surface-Ripened Cheese/Tilsit Cheese	*Arthrobacter nicotianae* *Microbacterium foliorum* *Proteus Vulgaris*	Bacterium
	Candida kefyr *Candida krusei* *Penicillium commune*	Fungus

TABLE 17.1 List of Microorganisms Used in Dairy Product Manufacturing—cont'd

Dairy Products	Microorganisms	Type of Microorganism
Dairy	*Bifidobacterium animalis* *Bifidobacterium bifidum* *Bifidobacterium breve* *Bifidobacterium infantis* *Bifidobacterium lacti* *Bifidobacterium longum* *Bifidobacterium pseudolongum* *Bifidobacterium thermophilum* *Carnobacterium maltaromaticum* *Propionibacterium freudenreichii* *Lactobacillus gasseri*	Bacterium
	Yarrowia lipolytica	Fungus
Beaufort Cheese	*Brachybacterium alimentarium* *Brachybacterium tyrofermentans*	Bacterium
Gruyère Cheese	*Brachybacterium alimentarium* *Brachybacterium tyrofermentans*	Bacterium
Limburger Cheese	*Candida mycoderma* *Debaryomyces kloeckeri*	Fungus
	Microbacterium gubbeenense	Bacterium
Reblochon Cheese	*Candida zeylanoides*	Fungus
Cheese	*Brevibacterium aurantiacum* *Carnobacterium divergens* *Corynebacterium ammoniagenes* *Corynebacterium flavescens* *Corynebacterium mooreparkense* *Corynebacterium variabile* *Hafnia alvei* *Kocuria rhizophila* *Geotrichum candidum* *Lactobacillus coryniformis* *Lactococcus lactis* *Lactococcus raffinolactis* *Micrococcus luteus* *Propionibacterium acidipropionici* *Propionibacterium jensenii* *Propionibacterium thoenii* *staphylococcus equorum* sp. *linens* *Staphylococcus Fleurettii* *Streptococcus thermophilus*	Bacterium
	Candida colliculosa *Candida utilis* *Cystofilobasidium infirmominiatum* *Fusarium domesticum* *Mucor plumbeus* *Mucor racemosus* *Penicillium nalgiovense*	Fungus

Continued

TABLE 17.1 List of Microorganisms Used in Dairy Product Manufacturing—cont'd

Dairy Products	Microorganisms	Type of Microorganism
Cheese/Cream	*Enterococcus faecalis*	Bacterium
Manchego Cheese	*Enterococcus faecium*	Bacterium
Yogurt	*Lactobacillus acidophilus* *Lactobacillus delbrueckii* sp. *bulgaricus* *Pseudomonas fluorescens* *Streptococcus salivarius* *Streptococcus thermophilus*	Bacterium
Idiazabal Cheese/Manchego Cheese/ Roncal Cheese/Yogurt	*Lactobacillus casei* *Leuconostoc mesenteroides* sp. *dextranicum*	Bacterium
Grana Padano Cheese	*Lactobacillus casei* sp. *pseudoplantarum*	Bacterium
Parmigiano-Reggiano Cheese	*Lactobacillus casei* sp. *pseudoplantarum*	Bacterium
Cacio Di Fossa Cheese	*Lactobacillus curvatus*	Bacterium
Canestrato Pugliese Cheese/Pecorino Romano Cheese/Pecorino Sardo Cheese	*Lactobacillus curvatus* *Lactobacillus delbrueckii* sp. *lactis* *Lactobacillus fermentum*	Bacterium
Cheddar Cheese	*Lactococcus lactis* ssp. *Cremoris*	Bacterium
Tilsit Cheese	*Microbacterium gubbeenense*	Bacterium
Cheese/Sausage	*Penicillium chrysogenum*	Fungus
Emmental Cheese	*Propionibacterium Freudenreichii* sp. *shermanii*	Bacterium
Tomme Cheese	*Verticillium lecanii*	Fungus
Raclette Cheese	*Yarrowia lipolytica*	Fungus

17.4.1 Rennets and Recombinant Rennets in the Dairy Industry

The successful cloning of the calf rennet gene and its expression was carried out very early in the 19th century, becoming the first mammalian gene to be cloned (Foltman, 1993). The structure of the rennet gene and its characteristics were analyzed when cloned in *E. coli* (Meisel, 1988). It was studied that *E. coli* synthesized this protein in the form of inclusion bodies, which are insoluble and inactive. Inclusion bodies are harvested by centrifugation after disintegration of the cells. This recombinant enzyme possesses identical properties to the native calf rennet and was further confirmed by ELISA and immunodiffusion techniques (Kawaguchi et al., 1987). This calf gene was also cloned in *Saccharomyces cerevisiae*, where the level of expression was 0.5%–2.0% of the total yeast protein (Aikawa et al., 1990). But here, only ~20% os recombinant rennet is obtained in solubilized form while the remainder remains associated with cell debris (Mellor et al., 1983). Another competent host, *Kluyveromyces lactis* (Yeast), has been found for industrial-scale production of rennet (Teuber, 1990; Novoren, 1994). In India, the scenario is different. Here, the major milk source is buffalo instead of cow,

which has a different composition from the cow. In this regard, the National Dairy Research Institute (NDRI), Karnal, has taken an initiative in cloning the gene of the buffalo chymosin/rennet. As was done with the calf rennet, this was also cloned in *E. coli*. Sequencing the product confirms the homogeneity with the cattle rennet (Dalgleish, 1992). Recombinant rennets are fruitfully used for cheese preparation at the experimental level and pilot scale, and no significant differences have been observed in terms of flavor, aroma, quality, and yield (Mohanty et al., 2003).

17.4.2 Lactase in the Dairy Industry

Lactase, or the β-galactosidase enzyme, has been given tremendous attention during the past decade (Alberts et al., 2002). Lactase works on lactose sugar (milk sugar) and hydrolyzes it to glucose and galactose. Due to some genetic imbalance, the absence of the lactase enzyme in some people creates lactose intolerance. They face difficulty in consuming milk and dairy products. For such people, low-lactose or lactose-free food is essential to prevent any unavoidable situations such as severe tissue dehydration, diarrhea, and sometimes even death. Lactase also offers several other advantages such as it increases the sweetness in the resultant milk and thereby avoids the addition of artificial sweeteners in the preparation of milk products. Lactase further improves the scoop and creaminess and minimizes the sandiness in preparations of ice creams, frozen desserts, and yogurt, which arises due to crystallization of the concentrated lactose. Lactase hydrolyzed milk also enhances the cheese ripening process as compared to normal milk for cheese production. Lactase is most appropriate for the dairy industry for removing the crystallization of lactose, but its high cost minimizes its utilization. Moreover, a large amount of cheese-whey produced as a by-product also pollutes the environment. But researchers also find the solution to this problem by utilizing whey as an additional source of lactose in the fermentation medium for making lactic acid by *Lactobacillus bulgaricus*, *Aspergillus niger*, *A. oryzae,* and *K. lactis* (Gekas and Lopez-Leiva, 1985). All these mentioned strains are found to be safe as they have already passed several levels of safety tests, whereas most researched *E. coli* lactase is not considered safe for food processing due to its high cost and toxicity issues.

17.4.2.1 Characteristics of Lactase

The characteristics of any enzyme depend on several factors such as its source, the technique applied for commercial production, and the end-product inhibition attribute, if exists. It has been noticed that when lactase is isolated from fungi, it shows an optimum pH in the acidic range 2.5–4.5, but when isolated from bacteria and yeast, then pH optima falls in the neutral pH range, that is, 6.5–7.5 and 6–7, respectively. Optimum temperature and pH also change when immobilized enzymes and a variety of carriers are used. This property of variation in pH is very useful for several specific applications, such as fungal lactases can be used for acid whey hydrolysis while yeast and bacterial lactases are suitable for milk (pH 6.6) and sweet whey (pH 6.1) hydrolysis. In product inhibition, things are running in different manners such as galactose strongly inhibits lactase when isolated from *A. niger* but can't inhibit the same enzyme when isolated from *A. oryzae*. This end-product inhibition can be reduced, to some extent, by using immobilized enzymes for hydrolyzing lactose at low concentrations or by recovering the enzymes using ultrafiltration after batch hydrolysis.

As reported in the literature, lactase isolated from *Bacillus* species is better in many characteristics in terms of thermostability, optimum pH, higher activity toward skim milk, less sensitive for end-product inhibition, and for high-substrate concentration (Fox et al., 2004). Therefore, it plays a very important role in the dairy industry. At industrial level, lactase is used in a free form or immobilized form or by immobilized whole cells producing intracellular lactase for enzymatic hydrolysis of lactose in the batch fermentation process. Although numerous hydrolysis systems have been investigated, only a few of them have been scaled up with success; even fewer have been applied at an industrial or pilot scale. Several commercial immobilized systems have been already developed for commercial exploitation. One such successful system, called the "Snamprogretti process," is running in Italy for industrial-scale milk processing. In this process, fiber-entrapped yeast lactase is used for already sterilized skim milk in batch fermentation. The processing of the whey by-product is accomplished by the system (ultrafiltration-permeate) developed by Corning Glass, Connecticut, Lehigh, Valio, and Amerace Corp. The technique applied by Corning Glass is used at the commercial scale for baker's yeast production using hydrolyzed whey (Afroz et al., 2015).

17.4.3 Proteinases and Peptidases in the Dairy Industry

For good flavor and textural development, proteolytic activity is required to a great extent. Proteinases used in cheese processing include rennet, plasmin, and proteinases of the starter and nonstarter bacteria. Approximately 6% of the rennet added to raw milk for cheese ripening remains in curd form and offers proteolysis during ripening. When used with a combination of microbial peptidases, microbial rennets, and lipases, neutral proteinases are able to minimize bitterness that is produced in milk products by the action of acid proteases. An even more appropriate approach is to use attenuated starter cells or cell-free extracts (CFE) for the acceleration of cheese ripening (Krause et al., 1998). Proteolytic strains (*Lactobacillus acidophillus*, *L. plantarum*, *L. lactis*, and *L. helveticus*) secrete extracellular proteinases, aminopeptidases, endopeptidases, carboxypeptidases, tripeptidases, and proline-specific peptidases, all belonging to the serine proteases class. Lactic acid bacteria have a complex proteolytic system capable of converting milk casein to the free amino acids and peptides necessary for their growth. This contributes significantly to flavor development in fermented milk products.

17.4.4 Other Dairy Enzymes

Besides the main enzymes used at a large scale, some minor enzymes also play a crucial role in the dairy sector, out of which lipases have a very significant contribution in the development of flavors, especially in cheese production. Lipolysis makes an important contribution to Swiss cheese flavors, as lipase is already present in the starter culture. The typical peppery flavor of blue cheese is due to the presence of short-chain fatty acids and methyl ketones, which are generated during lipolysis catalyzed by lipase isolated from *Penicillium roqueforti*. Very recent EMC (enzyme modified cheese) technology has been developed at an industrial scale to generate a variety of characteristic cheese flavors in Swiss, blue, cheddar, provolo-nemor, or romano and many others. In this EMC technology, homogenized and emulsified medium-aged cheese is used, which is pasteurized in the presence of lipase from *R. miehei* and proteinase. This mixture is ripened at a high temperature for 1–4 days, which

results in a paste that is well suited for inclusion in dressings, soups, dips, or fast foods. This is a quite effective method for accelerating ripening but there are some limitations, such as its uniform distribution in curd, high price, and the possible risk of overripening the cheese.

Some other enzymes have limited but important applications in the dairy industry, including glucose oxidase, catalase, superoxide dismutase, sulfydryl oxidase, lactoperoxidase, and lysozymes. Glucose oxidase and catalase are frequently used together for the preservation of some dairy products. Superoxide dismutase is found to be an effective antioxidant for packaged foods but is more effective when used in combination with catalase. Sulfydryl oxidase is also used for removing toxic and volatile sulfydryl groups that are produced during the processing of milk by the ultra high temperature (UHT) technique. Lactoperoxidase is a natural inhibitor present in raw milk but in a slightly inactive form that can be further activated by the addition of traces of H_2O_2 and thiocyanate. Lysozyme is also added in milk and milk products as antibacterial agents, so it becomes especially suitable for infant milk. This lysozyme enzyme made no inhibitory action on *Lactobacillus bifidus* used for processing cow milk (Puranik and Kanawjia, 1998). The demand for microbial enzymes in the dairy sector is significantly outsized globally but is being limited by few enzyme producers with satisfactory yields. However, in India, in the past two to three years, several manufacturers have made varieties of milk products such as state dairy federations, cooperatives, and private dairy product manufacturers such as Amul, Vijaya, Verka, Dynamix, Nestle, and Smith Kline Beechem due to the advent of biotechnological techniques. Still, several enzymes important for the dairy sector such as rennets, lipases, and lactases are imported for commercial purposes. So, in India, there is great need for finding microbial enzymes that can be used at an industrial level after crossing all safety tests, including being made suitable for consumption by infants. In the future, the requirement for these enzymes is bound to increase by leaps and bounds, due to the huge demand for value-added dairy products in the country.

17.5 ROLE OF MICROBIAL ENZYMES IN CHEESE RIPENING

Cheese ripening gets completed in several steps where numerous biochemical and biophysical changes occur, all done by enzymes. In the first step, tasteless curd is developed from raw milk using rennets. Then, specific taste, aroma and flavor for the designated cheese is generated by the partial and gradual breakdown of carbohydrates, lipids, and proteins by specific enzymes during ripening. These enzymes are added either in free form or as whole microorganisms as a starter culture, nonstarter culture, secondary inoculum, and/or residual coagulants. Proteolysis occurs in all the cheese varieties and is a prerequisite for characteristic flavor development that can be regulated by the proper use of the above enzymes. Accelerated cheese ripening can also be achieved by using high temperatures, but this is associated with generating undesirable, imprecise reactions that are responsible for off-flavor development. So the use of the enzyme in cheese ripening is a better and safe option for flavor development, aroma enhancement, and a longer shelf life for packaged foods. The pathways leading to the formation of flavor compounds are largely unknown, and therefore the use of exogenous enzymes to accelerate ripening is mostly an empirical process (Krause et al., 1998). Various microbial enzymes and their sources used to accelerate cheese ripening are presented in Table 17.2.

TABLE 17.2 Microbial Enzymes Used to Accelerate Cheese Ripening

Enzyme	Microbial Source
Microbial lipase	*Aspergillus niger, Aspergillus oryzae, Rhizomucor miehei*
Lactases	*Streptococcus lactis, Kluyveromyces* sp., *Escherichia coli*
Microbial serine proteinases	*Aspergillus niger*
Neutral proteinases	*Bacillus subtilis, Aspergillus oryzae*

17.6 MODERN TECHNOLOGIES USED IN DAIRY SECTORS

To meet the demand of growing dairy industries for selected development and to obtain innovative foodstuffs for either direct consumption or as functional ingredients, several technologies have been developed that are successfully adapted at an industrial scale; a few are discussed here in brief.

17.6.1 Membrane Technology

For nearly 30 years, this membrane filtration technology (microfiltration) has been functional in the dairy sector but developments in the last 10 years offer immense possibilities for new products, either for direct consumption or specific use in milk processing. This technology offers filtering of microorganisms from milk, sterilizing it. The presence of different membranes at different levels also helps in separating milk ingredients from milk and whey. Various membrane separations include microfiltration (removes suspended particles), ultrafiltration (removes macromolecules), nanofiltration (removes sugars, divalent salts, and dissociated acids), and reverse osmosis (removes salts, undissociated acids) technologies in many steps; all are pressure-driven membrane processes. Maubois, a well-known researcher and initiator of membrane filtration technology, reported in his research that by using different types of membranes (Fig. 17.1), many functions can be achieved in a single step such as separation of proteins and other components from the milk (Maubois, 1999).

17.6.2 Ultrafiltration for Standardization of Milk Protein Content

The natural protein content in milk ranges from 28 to 40 g/L and is a very vital factor to know the quality of milk. In several countries, milk producers get the payment of liquid milk in view of their protein content in milk. To standardize the protein content in different valuable forms of milk, either condensed or solids (milk powder), several measures are applied continuously to increment the economic value of milk both for producers as well as for processors. Recently, the Codex Committee of Milk and Milk Products (CCMMP) passed the standard protein content in skim milk that should be at least 34%. And this content should be adjusted before drying, so that the composition of the liquid phase does not get altered. This can be achieved by using ultrafiltration (UF)

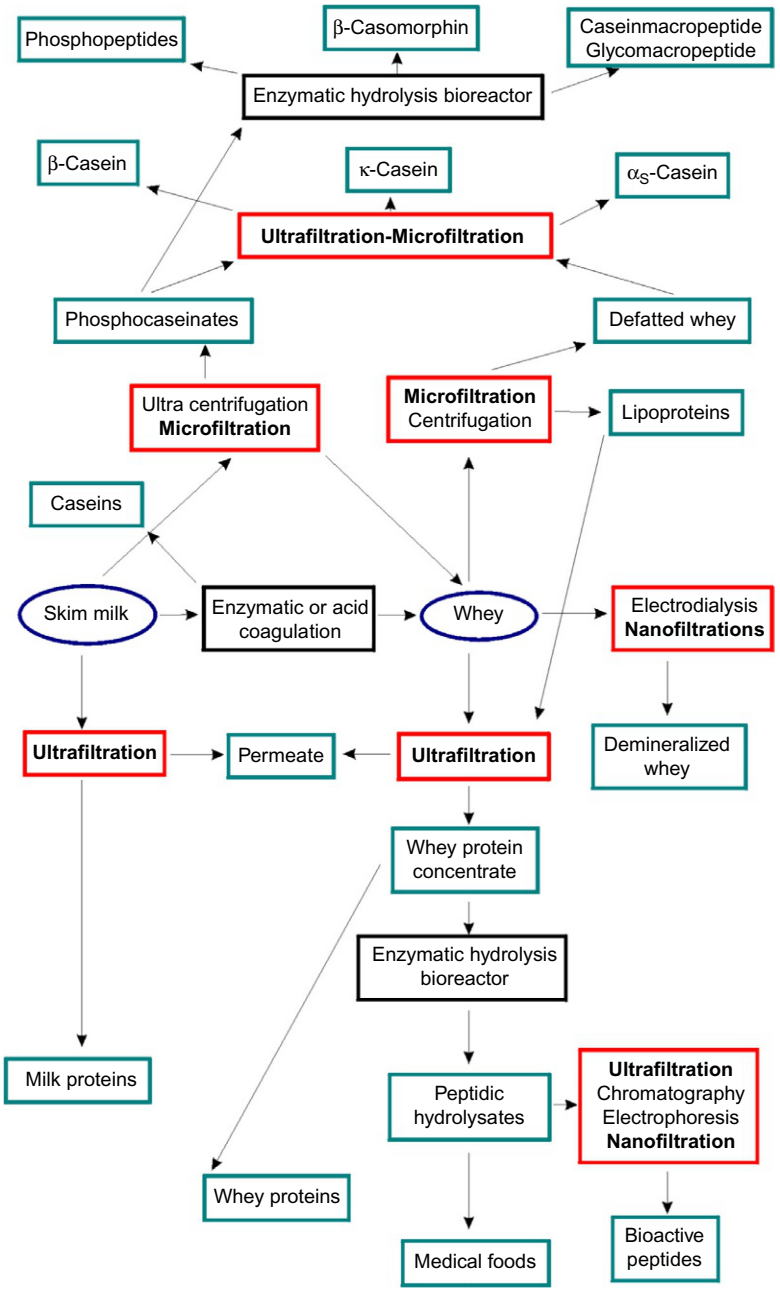

FIG. 17.1 Pathway showing products obtained from milk and whey by membrane separation *Modified from Maubois, J.L., 1999. Fractionation of milk proteins. In: Proc. 25th Int. Dairy Congress, Aarhus, Denmark, pp. 74–86.*

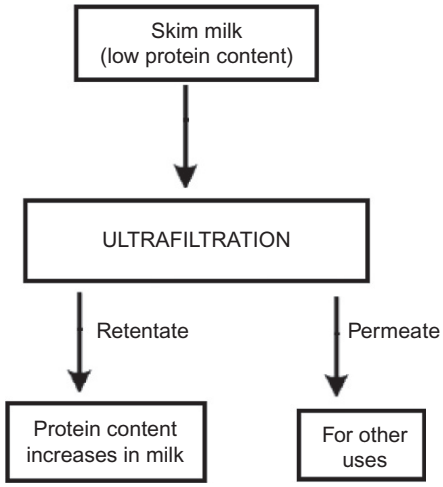

FIG. 17.2 Ultrafiltration for protein standardization of liquid milk.

technology. Increased consumption of milk forces the request by industry to assimilate protein content for the liquid. This practice already exists in some countries, whereby the legitimate actions are not clear. Rather, in the coming years, it will become a mandatory checkpoint for significant milk-producing countries (Le et al., 2014). The process is simple and outlined in Fig. 17.2.

17.6.3 Ultrahigh Temperature Processing (UHT)

Ultrahigh temperature (UHT) processing, ultraheat treatment, or ultrapasteurization is a technique used for the sterilization of milk. Here, a high temperature of 135°C (275°F) is applied to raw milk for 1–2 s to kill the microorganisms and their spores. This is the most common method employed in dairy industries, but it may also be extend for fruit juices, wine, creams, soups, soy milk, yogurt, honey, and stews.

17.6.4 Microfiltration for Extended Shelf Life of Milk

Microfiltration is another technique used in the dairy sector for extended shelf life (ESL), an alternate to UHT. Recent improvements here pose an implausible significance for the future. Here, the shelf life of milk increased by 14–45 days, but unlike UHT, the milk does not get sterilized by microfiltration and therefore needs refrigeration for storage. As per the claims of producers, microfiltration is preferred over UHT because (a) it tastes comparable or surprisingly better than condensed milk, (b) the taste stays consistent for the entire shelf life, and (c) it is more economical (Fig. 17.3). Bactofugation, also called steam infusion, is also a technique applied for killing microorganisms in milk. Bactofuge is similar in action to a centrifuge, which removes bacteria and their spores and is applied before heat treatment/UHT (Le et al., 2014).

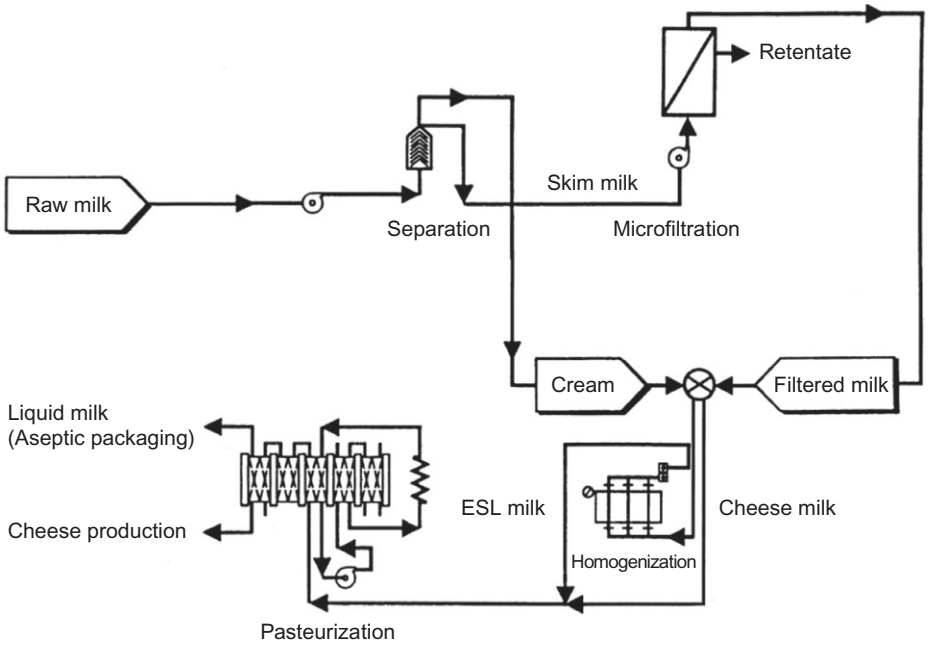

FIG. 17.3 Microfiltration for removal of microorganisms from skim milk.

17.6.5 Ultrahigh Pressure Technology

In addition to present biosystems, ultrahigh hydrostatic pressure (UHP) technology is found to be quite effective for milk treatment, as has been recognized by Hinrich and his team (Hinrich et al., 1996). This is far more valuable than UHT and is found to reduce the microbial count by the magnitude of 5–6 decimals when pressure of 6.8 bars is applied for 10 min at ambient temperatures. The present machine allows pressure ranges from 0 to 10 kbar at 0–80°C for batch-wise treatment of milk and milk products (Fig. 17.4A). This range is not up to the mark for use on an industrial scale and even its price makes it unsuitable for large-scale production. In case of biotechnology, future amendments can't be ignored and we hope to see positive and approachable changes that will be in limit of common man. Fig. 17.4B demonstrates the impact of this treatment on milk products.

17.6.6 Modern Biotechnology

Several available biotechnological treatments offer several significant outcomes to transform milk products, impacting better action of cultures, creating good yield of metabolites,and increasing the shelf life of dairy food. During the last 30 years, biotechnology has offered tremendous up-gradation and as a result, such genes have been identified as producing the desired enzymes, which play a crucial role in milk processing. This is called metabolic engineering. Here, for example, the gene and thereby the enzyme for lactic acid bacteria, which leads to flavor production and flavor stability, is improved. This is a determinant for the

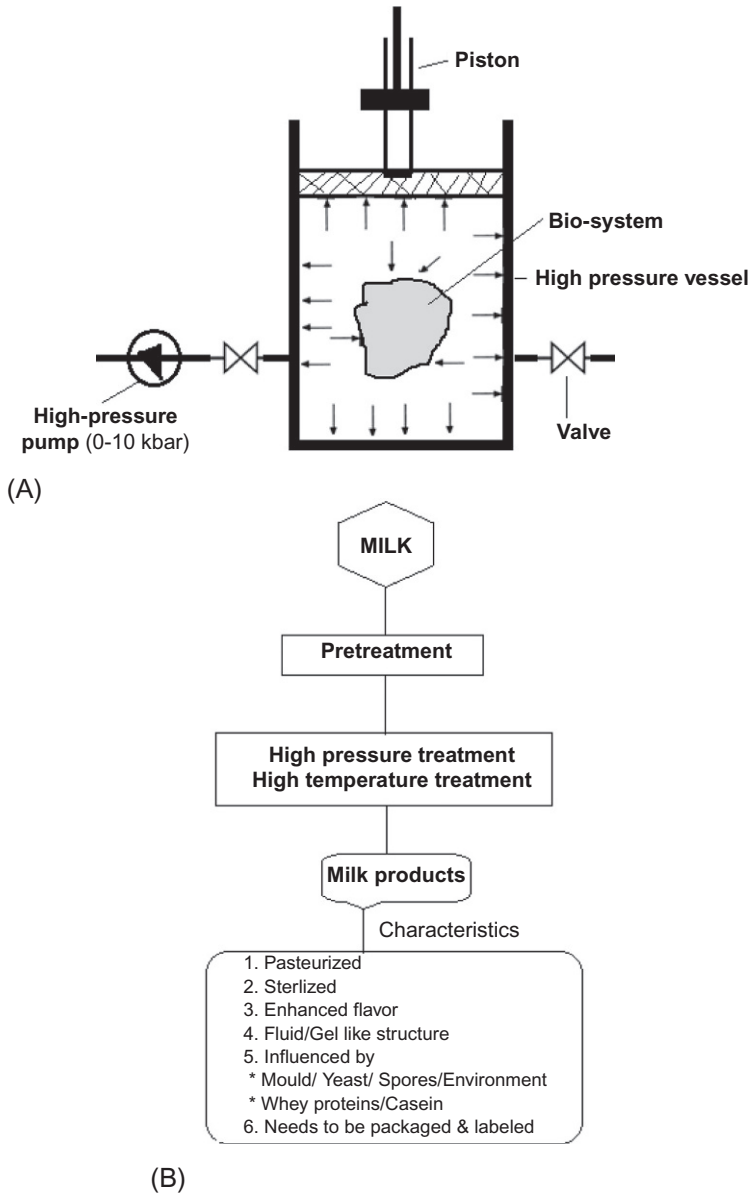

FIG. 17.4 (A) Illustration of ultrahigh pressure (UHP) treatment. (B) Influence of UHP technology on milk products.

quality of various fermented milk products. Genetic engineering also offers great possibilities for constructing strains with peculiar properties. Such strains are focused, having good levels of proteolytic and lipolytic enzymes; they can be used as starter cultures for cheese ripening. For fermented dairy products, the presence of probiotics makes it more demanding and valuable. This can be achieved by using such engineered bacterial cultures having potential for the production of probiotics (Pai, 2003). The biotechnological technique that produces the

genetically modified foods (GMO) is under serious consideration from the consumer's point of view. Numerous consensus are associated with GMOs like the engineered strain do not possess any antibiotic-resistant gene, the change at the genetic level should be well characterized, and the utilization of homologous DNA (originating from cells of the same species) is more worthy than utilizing DNA from cells of other species.

17.7 CONCLUSION AND FUTURE PROSPECTS

Commercially used fungal rennet preparations contain complex mixtures of protease, esterase, lipase, cellulase, and other enzymes that are involved in improving cheese quality. It has been experimentally found that the purified and blended form of fungal rennets are better as compared to calf rennet in terms of faster ripening and flavor development of cheese. The potential role of animal rennets is not explored in most publications due to its limited use. More focused research is required to obtain appropriate strains that have better control in the production of cheese ripening and minimizing by-products. A recombinant chymosin is developed by genetic recombinant technology as a substitute to calf rennet, but as of yet, it has not made a mark in the marketplace. Until the recombinant rennet becomes commercial, the standardization of calf rennet is requisite. It has been reported that a high level of steadiness and more precise proteolytic activity is achieved during cheese ripening when pure chymosin is used rather than traditional calf rennet. Recent research is focused on protein engineering technology where amino acid sequences of rennets are altered, which improves its overall functionality. The structure–function relationship of certain calf chymosins has been figured out with the help of site-directed mutagenesis. It also helped in improving the proteolytic activity. Protein engineering can easily alter microbial rennets that will result in products comparable to calf chymosin.

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CHAPTER

18

Enzymes for Fructooligosaccharides Production: Achievements and Opportunities

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18.1 INTRODUCTION

Fructooligosaccharides (FOS), also called oligofructose or oligofructan, are small dietary fibers with low caloric value and prebiotic effects. Nowadays, they are considered natural food ingredients in most countries because of their multiple beneficial effects on human and animal health (Panesar et al., 2013). Currently, the industrial production of fructooligosaccharides is growing rapidly due to different aspects, including pharmaceutical application of FOSs (Mutanda et al., 2014), a philosophy of zero waste in some agricultural industries such as the sugar industry, converting waste feedstocks into nutraceutical products (Rehman et al., 2016), and increasing the healthy-sugar market because of FOS's prebiotic function (Surin et al., 2012). By this last reason, FOSs and other oligosaccharides can be used during the selection of probiotic bacterial strains and (Kaplan and Hutkins, 2003). FOSs have been granted generally recognized as safe (GRAS) status by the US Food and Drug Administration (Dominguez et al., 2013). Different biological activities have been attributed to FOSs, and when these compounds are consumed in adequate dosages, they offer benefits to human health (Mutanda et al., 2014) and are worth about 150€ per kilogram (Dominguez et al., 2013).

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00018-9

FOSs can be extracted from fruits and vegetables, most prominently bananas, onions, chicory root, garlic, asparagus, barley, wheat, tomatoes, leeks, *Stevia rebaudiana*, Jerusalem artichokes, and yacon (de Oliveira et al., 2011; Panesar et al., 2013). Several reviews about their benefits and properties have been published so far, but little on the enzymes involved in the process.

18.1.1 Enzymatic Production of Fructooligosaccarides

It is well known that FOSs can be synthetized in nature by the catalytic action of enzymes with transfructosylating activity, but in small amounts, or they can be prepared by the degradation of inulin or polyfructose. Mutanda et al. (2014) stated that *exo*-inulinases and *endo*-inulinases acting alone or synergistically hydrolyze inulin-producing FOSs. *Exo*-inulinases cleave the nonreducing β-(2,1) end of inulin, releasing fructose, while *endo*-inulinases randomly break the inulin internal bonds, releasing inulotrioses (F3), inulotetraoses (F4), and inulopentaoses (F5). In addition, these enzymes elongating sucrose may produce FOSs. On the other hand, it has been reported that fructosyltransferases break a sucrose molecule, and after that can transfer the releasing fructose to an acceptor molecule such as sucrose. Also, this enzyme could transfer the fructose to another oligosaccharide elongating the short-chain fructooligosaccharide, as products of this enzyme action are 1-kestose (GF2), nystose (GF3), and fructofuranosyl nystose (GF4). The action of inulosucrase is similar. Díez-Municio et al. (2013) mentioned that an inulosucrase isolated from *Lactobacillus gasseri* transferred fructose moieties of sucrose to C-1 of the reducing end. This transference was also detected at C-6 of the nonreducing end of maltose, with the most important products of this reaction being trisaccharide erlose and neoerlose. Other extracellular enzymes of bacterial origin acting on sucrose have been mentioned, namely levansucrases. These enzymes produce β-2,6-linked fructans of different chain sizes (Alama et al., 2012). MFOS of a high polymerization degree can be obtained by transference of more fructose residues to C-1 of the β-2,1-linked fructose as well as transference of more fructose residues to C-1 of the β-2,6-linked fructose to maltose (Díez-Municio et al., 2013). Alama et al. (2012) indicated that is possible to obtain potentially prebiotic fructooligosaccharides using sucrose or raffinose.

Production of FOSs has been reported using different enzymes, fermentation methods, microbial sources, and methodologies for the optimization of nutritional and culture parameters (Dominguez et al., 2013) (Table 18.1). There are reports using purified and commercial enzymes. Sirisansaneeyakul et al. (2000) reported a study using a stirred tank reactor under controlled conditions. In this case, a mixture of two enzymes, β-fructofuranosidase (*Aspergillus niger* ATCC 20611) and glucose oxidase, was used. Ghazi et al. (2006) employed a membrane reactor with immobilized enzymes (Pectinex Ultra SP-L) and pretreated molasses as the substrate while Silva et al. (2013) reported a study where an aqueous-organic system and free, immobilized, and pretreated immobilized inulinase were used. Similarly, Alves-Risso et al. (2012) indicated production of FOSs using aqueous and aqueous-organic systems and sucrose as the substrate. In this case, an immobilized inulinase was employed. Another fermentation method used for FOS production is solid-state fermentation. Mussatto et al. (2012) indicated a high yield of FOS using coffee silverskin as the support moistened with a 240 g/L sucrose solution and inoculated with *Aspergillus japonica* spores. However, it is still a technical challenge to produce high yields of oligosaccharides from inulin and sucrose. Most

TABLE 18.1 FOS Produced Using Different Enzymes, and Sources

FOSs Produced	Source	Enzymes	Enzyme Source Organism	FOS Yield	Reference
FOS	Sucrose	b-Fructofuranosidases	*Aureobasidium pullulans* DSM 2404	62%	Yoshikawa et al., 2008
FOS	Sucrose	b-Fructofuranosidases	*Aureobasidium* spp.	(53%–59%)	Yoshikawa et al., 2008
FOS	Sucrose	b-Fructofuranosidases and glucose isomerase (GI)	b-Fructofuranosidases from *Aureobasidium pullulans* DSM 2404, commercial GI	69%	Yoshikawa et al., 2008
Kestose and nystose	Sucrose	Fructofuranosidase and glucose oxidase (GO)	Fructofuranosidase from *Aspergillus niger* ATCC 20611, Commercial GO	4.97 and 5.44 g/Lh	Sirisansaneeyakul et al., 2000
FOS	Sugar molasses	Pectinex Ultra SP-L	*Aspergillus aculeatus*	63%	Rehman et al., 2016
scFOS	Sucrose	Sucrose: sucrose fructosyltransferase	*Helianthus tuberosus* L.	54.46%	Ngampanya et al., 2016
FOS	Sucrose	Inulinase	*Kluyveromyces marxianus* NRRL Y-7571	14.6 ± 0.9 wt%	Alves-Risso et al., 2012
Nystose and 1-kestose	Longan syrup	Pectinex Ultra SP-L, glucose oxidase	*Dimocarpus longan* Lour	Nystose 30.27 g/L) and 1-kestose (123.36 g/L	Surin et al., 2012
FOS	Sugar syrup Beet Molasses	Pectinex Ultra SP-L	*Aspergillus aculeatus*	56 and 49%	Ghazi et al., 2006
GF2 (kestose), GF3 (nystose) GF4 (fructosyl nystose)	Inulin	Inulinase	*Kluyveromyces marxianus* NRRL Y 7571	11.89% of GF2; 20.83% of GF3	Silva et al., 2013
GF2 (kestose), GF3 (nystose) GF4 (fructosyl nystose)	Inulin	Inulinase	*Aspergillus niger*	77.19% of GF2; 14.03% of GF3; 0.07% of GF4	Silva et al., 2013
FOS	Sucrose	Inulosucrase	*Lactobacillus gasseri* DSM 20604	45%	Díez-Municio et al., 2013
Maltosylfructosides (MFOS)	Sucrose-maltose mix	Inulosucrase	*Lactobacillus gasseri* DSM 20604	13% (10:50 sucrose/maltose) to 52% (30:30 sucrose/maltose)	

enzymes used for FOS production have a microbial origin, but there are reports using vegetal enzymes. Ngampanya et al. (2016) reported the use of a crude extract from tubers of the tropical Jerusalem artichoke (*Helianthus tuberosus* L.), which had high transfructosylating activity.

Other commercial enzymes used for FOS production are glucose isomerase at an activity ratio of FFase and GI of 1:2 (Yoshikawa et al., 2008), Pectinex Ultra SP-L, and glucose oxidase (Surin et al., 2012).

18.2 FOS PRODUCTION FROM SUCROSE

Fructooligosaccharides (FOS) from sucrose are produced by a synthesis performed by the action of the enzymes β-fructofuranosidase (EC 3.2.1.26), or β-D-fructosyltransferase (EC 2.4.1.9). These enzymes catalyze the hydrolysis of sucrose, releasing glucose and fructose transferring the fructosyl moiety to another acceptor molecule of sucrose or FOS (Vega and Zuniga-Hansen, 2014).

FOS produced from sucrose belongs to inulin-type FOS and the enzymes used for its production are commonly fungal enzymes. It is reported that filamentous fungi from the genera *Aureobasidium*, *Aspergillus*, and *Penicillium* provide enzymes with the best yields of FOS production (Dominguez et al., 2013; Maiorano et al., 2008; Muñiz-Márquez et al., 2016; Mussatto and Teixeira, 2010; Sangeetha et al., 2004; Yoshikawa et al., 2006).

Seeking to optimize FOS production, several authors have modeled, explained, and attempted the dynamic of the FOS production phenomenon. Jung et al. (1989) described one of the most well-known mechanisms for FOS formation via the transfructosylation of *β*-Fructofuranosidase from *Aureobasidium pullullans:*

$$GFn + GFn \rightarrow GFn-1 + GFn+1$$

A disproportionate reaction in which FOSs are produced, subsequently having sucrose (GF) as the initial substrate, the enzyme acts on sucrose where one molecule of sucrose serves as a donor and another acts as an acceptor, resulting in the formation of 1-kestose (GF_2) and glucose (GF_0). A similar chain of reactions occurs to generate nystose (GF_3) and 1-Fructofuranosyl nystose (GF_4):

(1) $GF + GF \rightarrow GF_2 + G$
(2) $GF_2 + GF_2 \rightarrow GF_3 + GF$
(3) $GF_3 + GF_3 \rightarrow GF_4 + GF_2$

Strategies employed in improving FOS yield and purity. To reach a higher purity, different strategies have been attempted, including ultrafiltration, successive or coculture fermentations, enzyme immobilization, and genetically modified strains.

Nobre et al. (2016) purified FOS produced from sucrose by simulated moving bed (SMB) chromatography. In order to improve the efficiency of the purification, a microbial treatment was tested, a coculture by *Aureobasidium pullulans* (FOS producer strain) and *Saccharomyces cerevisiae* to remove the small saccharides. Compared to a successive fermentation, FOS were produced first by *A. pullulans* and then the small saccharides were metabolized by *S. cerevisiae*, giving as result that this kind of two-step fermentation is most efficient and productive with high levels of FOS produced of high purity (81.6 ± 0.8% *w*/w), on a dry weight basis.

Genetic engineering has proved to be an efficient tool to improve FOS production. In a recent paper (Zhang et al., 2017), an optimized (PEG)-mediated protoplast transformation system was established in *A. niger* ATCC 20611 and used for strain improvement. The strain was evaluated and showed an increase in specific β-fructofuranosidase activity (up to 507 U/g), compared to the parental strain (320 U/g). This also reduced the time needed for FOS formation, proving the strain enhancement in terms of FOS production by genetic engineering.

Other techniques used to remove sugars from the medium are nanofiltration and microfiltration systems, which have been proposed in different jobs to remove low molecular weight carbohydrates of the oligosaccharide mixture (Goulas et al., 2007). These techniques have reached high blends of FOS above 90% (w/w) (Nishizawa et al., 2001) and above 80% (w/w) (Sheu et al., 2002). These systems have demonstrated good production yields of FOS but unfortunately these make the process cost rise.

18.3 FOS PRODUCTION FROM INULIN

FOS can be produced by (1) extraction from inulin-rich plant materials, (2) enzymatic synthesis from sucrose, and (3) enzymatic degradation of inulin (Panesar et al., 2014; Rastall, 2010). Within the enzymes used for FOS production, fructosyltransferases (FTases) and B-D-fructofuranosidases (FFases) are mainly used (Sangeetha et al., 2005). FOS can be produced using high sucrose levels as the substrate. The maximum theoretical yield achieved by this process ranges between 0.55 and 0.60 g FOS/g sucrose (Nishizawa et al., 2001).

Another substrate for FOS production is inulin, the second most abundant polysaccharide found in nature after starch. FOS can be obtained from inulin hydrolysis through the action of *endo*-inulinases. Generally, higher content FOS are reached than with the transfructosylation process of sucrose. However, *endo*-inulinases have been not extensively used in the scale-up production of FOS (Wang et al., 2016). In both cases, production of FOS from inulin or sucrose, glucose, fructose, and sucrose is also obtained, which decreases the prebiotic activity of FOS. For this reason, removing the nonprebiotic sugars is required and technologies such as nanofiltration, microbial treatments, activated charcoal systems, and ion-exchange chromatography have been used (Crittenden and Playne, 2002). A less expensive alternative is the use of mixed enzymes or microorganisms, which is why the recombinant production of *endo*-inulinases has attracted much attention.

Molds are the most prominent group of microorganisms producing *endo*-inulinase. Some microorganisms that produce considerable amounts of this enzyme are *Bacillus* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Aspergillus* sp., *Penicillium* sp., and *Yarrowia* sp. (Kango and Jain, 2011; Singh and Singh, 2010). The production of FOS using commercially available *endo*-inulinases achieved yields of only 65% while also requiring further purification steps (Singh et al., 2016). Using *endo*-inulinase from *Pseudomonas* sp., yields of 75.6% and 80% have been reported, obtaining a mixture of FOS of DP ranging from 2–7 and 3–4, respectively (Hyun Kim et al., 1997; Park et al., 1998). In contrast, Wang et al. (2016) achieved a high FOS yield (90%) by implementing a one-step bioprocess by yeast fermentation of chicory inulin. The recombinant *S. cerevisiae* expressed a heterologous *endo*-inulinase gene and the inherent invertase gene *SUC2* was disrupted. Maximum titer achieved was 180.2 0.8 g/L at 24 h, and FOSs with different DP were obtained: 12% DP_3, 38% DP_4, 33% DP_5, and 17%

DP_6. Previously, Chen et al. (2015) also reported a recombinant *endo*-inulinase with yields of 3860 U/mL, which represents great potential for the scale-up production of FOS from inulin.

18.4 MICROBIAL SCREENING FOR ENZYMATIC FOS PRODUCTION

Due to the recently reported benefits of FOS such as prebiotic activity, considerable research has been carried out to select microorganisms with high transfructosylating activity and fructosyltransferase (FTase) or β-fructofuranosidases (FFase) production (Mussatto and Teixeira, 2010; Nascimento et al., 2016). The microorganisms considered as potential producers of FOS are *Aspergillus oryzae*, *A. niger*, *Aspergillus japonicus*, *Aspergillus foetidus*, and *A. pullulans* (Ganaie et al., 2013; Mussatto et al., 2013). There are also some bacteria *Bacillus macerans*, *Lactobacillus reuteri*, *Zymomonas mobilis* (Michel et al., 2016) and *Microbacterium paraoxydans* (Ojha et al., 2016).

18.4.1 Fungal Strains

Ganaie et al. (2013) reported a screening of microbial strains to produce FOS where they found that the strains with the most activity were *Aspergillus flavus* NFCCI 2364 (33.73 U/mL) at 120 h and *A. niger* (SI 19) produced 35.64 U/mL at 72 h. However, he states that these ratios can be optimized by the parameters of temperature and pH. Nascimento et al. (2016) reported on the work with eight fungi of the genus penicillium, of which the *P. citreonigrum* strain (UMR 4459) obtained the highest activity of FFase 227.56 U/mL. Therefore, they optimized the process to obtain 301.84 U/mL of activity FFase and used it to produce FOS, because it is much larger than that reported by other authors. However, *A. niger* ATCC 20611 is considered one of the most suitable strains for FOS production, as reported by Zhang et al. (2017). However, *A. niger* ATCC 20611 is considered one of the most suitable strains for FOS production as reported by Zhang et al. (2017) who demonstrated that an efficient genetic transformation system was developed when the egfp reporter gene was co-transformed with ptrA and the efficiency reached approx. 82% while the β-fructofuranosidase variant FopA (A178P) was overexpressed in *A. niger* ATCC 20611 the engineering strain CM6 exhibited a 58% increase in β-fructofuranosidase production.

The use of *A. pullulans* to produce FOS through the production of sucrose managed to obtain 64.7 g FOS/g sucrose with fermentation conditions of 32°C to 385 rpm (Dominguez et al., 2012).

On the other hand, reported by Nobre et al. (2016), using *A. pullulans* produced 0.63 ± 0.03 g of FOS per gram of sucrose. *S. cerevisiae* is a microorganism that can produce invertase and that has the ability to ferment sucrose and from this synthesize FOS. A recent study showed that the maximum synthesis of FOS was with the concentration of sucrose at 250 mg and 2.5 U invertase in 1 mL reaction at pH 5.5 and 40°C (Chand Bhalla et al., 2017).

18.4.2 Bacterial Strains

Ojha et al. (2016) studied the synthesis of FOS using cells of *M. paraoxydans*, where the best method was using sucrose to induce the process, achieving a maximum yield of

155 g/L of FOS. There are not many studies to produce FOS using bacteria; however it may be a viable alternative such as the *Bacillus licheniformis* where Levan-type FOS is produced. The gene encoding the endolevanase LevB1 of *B. licheniformis* was isolated, cloned, and expressed as a heterologous protein. Endolevanase was applied to levan obtained from different sources and thereby obtaining a yield of 97% FOS (Porras-Domínguez et al., 2014).

18.4.3 Techniques to Eliminate Nonprebiotic Sugars

The products generated from the enzymatic catalysis to produce FOSs include a mixture of fructose sucrose and glucose, therefore no very high yields are obtained. However, there are reports of 180.2 g/L, 0.9 g FOS/g inulin (Wang et al., 2016). Fructose, glucose, and sucrose are cariogenic, caloric, and have no prebiotic activity, which affects the value of the product. In recent studies, commercial (cationic and anionic) resins have been used for the separation of FOS from salts and other nonprebiotic sugars, obtaining a 92% recovery using a column loaded with Diaion UBK535Ca with 90% purity (Nobre et al., 2014).

Other processes have been carried out for the purification of FOSs such as the activated carbon column. There are reports describing high yields in the separation of oligosaccharides. Nobre et al. (2012), used an activated carbon column for the separation where fermentation was realized with Aureobasidium Sp. to produce FOS giving as result, a sugar mixture containing 56% (w/w) FOS. Once purified with the activated carbon column allowed a FOS recovery of 74.5% (w/w) to 92.9% (w/w) purity, obtaining a product with a higher value.

Otherwise, authors have applied microbial treatments for the elimination of small sugars released during FOS production. *S. cerevisiae* is highly efficient to remove mono- and disaccharides from the fermentation using two-step fermentation system as reported by Nobre et al. (2016) using *A.pullulans* to biosynthesis FOS and *S. cerevisiae* to reduce the content of small sugars. The field of protein purification is highly studied. However, the field in systematic studies for the purification of oligosaccharides is still little studied.

18.5 PROPERTIES OF FUNGAL FRUCTOSYLTRANSFERASE AND FRUCTOFURANOSIDASE

Fungal fructosyltransferases and fructofuranosidases are known to be the best option in the production of fructooligosaccharides (FOS), due to their high fructotransferase and hydrolytic activities, respectively. According to Chuankhayan et al. (2010), the structure of these enzymes has two folded domains, comprised by 632 residues. These residues constitute an N-terminal five-blade-propeller (residues 21–468) and a C-terminal sandwich domain (480–653), and both structures are linked by a 9-residue short-helix (469–479).

Fructosyltransferase has been reported with a dimeric domain reaching molecular weights of 85 kDa (Chuankhayan et al., 2010), 135 kDA (Ghazi et al., 2007), and even 150 kDa (Hernalsteens and Maugeri, 2008). This is compared to fructofuranosidase produced from

Rhodotorula dairenensis (Gutiérrez-Alonso et al., 2009) weighing up to 680 kDa and formed by a homotetramer. Also, the isoelectric point reported from these fungal enzymes goes from 3.8 to 5 (Alméciga-Díaz et al., 2011;Ghazi et al., 2007).

The stability of this enzyme can vary. The optimal conditions depend on the microorganism producing the enzymes. These enzymes can work at different conditions defined as optimal to promote the growth of the microbial source (Guio et al., 2012; Muñiz-Márquez et al., 2016).

Fructosyltransferases can have activity even with high sugar concentrations. Many of the enzymes produced by fungi have optimal catalysis activity from 400 to 600 g/L sugar concentrations. This sugar concentration in the culture medium is sucrose in a high concentration, glucose, and fructose. This substrate conditions provide a high amount of fructose to the enzyme. The enzyme can link it to glucose or sucrose and produce short-chain FOS (Dominguez et al., 2006; Huang et al., 2016; Nemukula et al., 2009).

Most of the fructosyltransferase enzymes produced by fungi increase or decrease its enzymatic activity as pH increases or decreases in the culture medium. When the pH goes too acid or too basic, the enzyme activity decreases, as is shown in some studies (Farid et al., 2015; Gutiérrez-Alonso et al., 2009); Michel et al. (2016) mentioned that the optimal pH activity for these enzymes goes from 4.5 to 8 based on different studies but optimal conditions of fructosyltransferase activity. The optimal conditions for different fungi to produce fructosyltransferase and fructofuranosidase are mentioned in Table 18.2.

Due to all these properties affecting the enzyme activity, it needs to be evaluated even if the source genre and species is known. Sometimes the isolation source affects the enzyme and it is adapted to different conditions than other enzymes.

18.6 OPTIMIZATION OF FRUCTOSYLTRANSFERASE AND FRUCTOFURANOSIDASE FOR FRUCTOOLIGOSACCHARIDES PRODUCTION

Obtaining FOSs from natural sources has been reported from conventional extractions. The use of conventional extraction as heating or employing new technologies such as ultrasound has been reported. However, the yields of FOS after treatments are very low, obtaining 0.22 ± 0.01 g per g of raw, as Machado et al. (2015) reported.

TABLE 18.2 Optimal Condition for Optimal Enzyme Activity From Different Fungi Sources

Source	pH	Temperature (°C)	Enzyme	Reference
Penicillium aurantiogriseum	6.0–6.5	–	Fructosyltransferase	Farid et al., 2015
Aspergillus oryzae KB	5	55	β-Fructofuranosidase	
Aureobasidium pullulans	5–6.5	50–60	Fructosyltransferase	Antošová et al., 2008
Aureobasidium pullulans	–	47.5	Fructosyltransferase	Onderková et al., 2010
Rhodotorula dairenensis	5	55–60	β-Fructofuranosidase	Gutiérrez-Alonso et al., 2009

The use of enzymes in FOS production has increased through the years, and all of this because of what the great properties of FOS provide to human health. During this time, scientists have tried to produce high yields of FOS from enzymes produced from different natural sources, such as fungus or bacteria.

As is known, fungi can produce intra- and extracellular fructosyltransferase. In most of the studies producing FOSs, the enzyme used to produce them is the extracellular fructosyltransferase while the intracellular enzyme is removed inside the microorganism. Lateef et al. (2007) use some emergent technologies to release the intracellular enzyme and use it in the production of FOS.

Dominguez et al. (2006) proved that some *Aspergillus* have transfructosylation activity, changing some conditions on the Czapek-Dox culture medium. Also, Fernandez et al. (2007) evaluated different temperatures and pH conditions on *A. oryzae*, *A. niger,* and *A. pullulans* strains. They showed that these two conditions affect in different ways all the strains evaluated, but proved that the *A. oryzae* strain has the best potential in fructosyltransferase activity so it can be used to produce FOS in different conditions.

Another study using *Aspergillus aculeatus* commercial enzyme Crude Pectinex Ultra SP-L [Novozyme, Pty (Ltd.,)] compared with another *Aspergillus* strains proved its capacity to produce enzymes with fructotransferase with hydrolytic properties. These enzymes can be used to produce FOS (Ghazi et al., 2007; Nemukula et al., 2009). These works show an optimal pH between 5 and 6 in FOS production; it needs to be in a temperature range between 50 and 60°C degrees so it can work at optimal conditions. Muñiz-Márquez et al. (2016) showed an *A. oryzae* strain producer of fructosyltransferase with an optimal temperature lower than the other enzymes (32°C). All the enzymes were used on a high sugar concentration culture medium and the optimal activity on each condition was between 12 and 24 h. The FOS conversion can reach between 50% and 60% FOS yield conversion using all these enzymes.

There are other fungi strains such as *Penicillium aurantiogriseum* reported from Farid et al. (2015). This *Penicillium* strain AUMC 5605 can produce fructosyltransferase using a high sugar medium content in fermentation, but the difference with this strain and *Aspergillus* strains is the enzyme production time while the highest enzyme activity was at 96 h. For example, comparing the *A. oryzae* from Muñiz-Márquez et al. (2016) with the highest enzyme activity at 24 h, *Penicullium* needs almost a fourfold time to produce high activity.

As some macromycetes can produce these enzymes, some yeast can produce them too. Jiang et al. (2016) reported that the *Aureobasidium* sp. P6 strain isolated from a mangrove ecosystem can produce fructofuranosidase with a high hydrolyzing activity, reaching 30.98 U/mL in optimal conditions and using a Czapek-Dox culture medium at 60 °C degrees in pH of 4.5. However, the time this yeast needs to produce the highest enzyme activity is 108 h, which is even later than the *Penicillium* strain.

The other way to optimize the production of fructosyltransferase and fructofuranosidase is by genetic modification. Spohner and Czermak (2016) used an *Aspergillus terreus* strain producer of fructosyltransferase and made it express an optimized codon with optimal conditions. They then modified a strain of *Kluyveromyces lactis* so this yeast can recombinantly express this codon-optimized version. After these changes in the yeast, *K. lactis* was able to produce fructosyltransferase activity in optimal conditions (50–72°C, pH 4–9), and showed 1000 U/mL in a culture flask scaled up to 5 L.

Also Wang et al. (2017) evaluated genetic shuffling between *A. oryzae* strains, recombining the fungi strains three times until they got a mutant fusion producer of fructosyltransferase. With optimal conditions, the fungi strains can produce fructosyltransferase activity of 180 U/g, and after the genetic shuffling between strains, the fructosyltransferase activity doubles its performance, obtaining 353 U/g. This genetic shuffle can be used in other strains and maybe FOS production can be multiplied.

There are a number of ways to produce FOS by enzymes. Most of the fungi strains used to produce them are *Aspergillus* sp. This fungi family is genetically prepared to produce enzymes able to consume high amount of sugars with fructose in their structure and produce short-chain FOSs.

18.7 AGROINDUSTRIAL MEDIA FOR ENZYME PRODUCTION (SUBSTRATES) IN SOLID AND SUBMERGED STATE FERMENTATION

Today, different culture sources have been explored for fructosyltransferases enzyme production through solid and submerged systems. Fructosyltransferases (FTases, EC 2.4.1.9), also called levansucrases, exhibit transfructosylating activity (Ganaie et al., 2013) when high concentrations of sucrose are used in the culture medium. This is in contrast to when low amounts of sucrose are used in a hydrolytic activity, resulting in glucose and fructose as by-products (Mussatto et al., 2009). FTases belong to the family 68 of glycoside hydrolases (GH68) and catalyze the synthesis of FOSs and the production of β-(2–6) levan (Vasileva and Bivolarski, 2012).

The production of FTases has been by submerged state fermentation (SmF) and fewer studies have been focused on solid-state fermentation (SSF) (Ganaie et al., 2017). In both fermentation systems, the fructosyltransferase production is significantly influenced by the carbon source used in the culture medium (Maiorano et al., 2008). In SmF, starch, maltose, glucose, fructose, galactose, and sorbitol have been used. However, the synthesis of FTases is poor in contrast to when sucrose is used as the carbon source (Dhake and Patil, 2007; Maiorano et al., 2008). Hidaka et al. (1988) evaluated the production of FOSs and FTases enzymes using different concentrations of sucrose as the carbon source. A treatment of 50% of sucrose allowed obtaining higher transfructosylating activity. It is known that sucrose acts as an acceptor of fructose molecules in the mechanism of reaction and therefore plays a key role in the synthesis of FTases to the FOS production of short chains, especially kestose (GF2), nystose (GF3), and fructofuranosyl nystose (GF4).

Liquid agroindustrial substrates for the FTases synthesis have been little used because in submerged fermentation processes (SmF), a sucrose solution enriched with other nutrients has been widely used. Prata et al. (2010) showed the FOS production and β-fructofuranosidase activity by *Penicillium expansum* in Erlenmeyer flasks containing a solution of sucrose (20%) in addition to other constituents, such as yeast extract, $NaNO_3$, K_2PO_4, $MgSO_4 \times 7\ H_2O$, and KCl. The maximum yield of FOS was 117.7 g/L with almost a total biotransformation of sucrose. Sangeetha evaluated alternate substrates of sucrose such as jaggery and sugar cane juice for the FOS production by FTase from *A. oryzae*. Muñiz-Márquez et al. (2016) used the aguamiel

of maguey as the culture medium for the FTase biosynthesis. They mention that the aguamiel is a substrate that represents an economical alternative for the FOS and enzyme FTase production.

On the other hand, in SSF, various agroindustrial materials have been used as the support substrate for the FTases production. Hang et al. (1995) studied the biosynthesis of kestose by extracellular fructosyl transferase from *A. foetidus* NRRL 337. In this work, apple pomace was used as part of the solid culture and made possible the formation of 50% kestose.

For the production of FOS in non-conventional substrates, Sangeetha et al. (2004) evaluated different by-products such as wheat bran, corn wastes, sugar cane bagasse, bagasse of cassava and byproducts of coffee and tea, enriched with sucrose processing and various sources of nitrogen as urea, extract of yeast and ammonium sulfate. Here, the major substrates for the synthesis of FTases by *A. oryzae* CFR 202 were rice bran and wheat bran. Mussatto et al. (2009) used vegetal fiber as a substrate for the immobilization of cells from *A. japonicus* for the FOS formation, with a yield of 69% and elevated activity of β-fructofuranosidase (42 U/mL). In addition, other synthetic materials were used in this study such as polyurethane foam, zeolites, pumice stones, foam glass, and stainless steel sponges, which were supplemented with sucrose. Later, Mussatto and Teixeira (2010) studied the FOS production with *A. japonicus* using corncobs, coffee silverskin, and cork oak under solid-state fermentation systems. The results obtained in this study showed that coffee silverskin supplemented or not supplemented with other nutrients gave the most interesting results for FOS production and β-fructofuranosidase activity (71 U/mL). However, 200 g/L of sucrose were added to the residues used. Sathish and Prakasham (2013) investigated the fructosyltransferases production by *Aspergillus awamori* GHRTS in solid-state fermentation. Between the organic substrates tested, corncobs were the best for their production. On the other hand, wheat bran and rice bran also showed satisfactory results in the enzymatic activity. Oat bran, sugar cane bagasse, redgram husk, green gram husk, bengal gram husk, black gram husk, ground nut oil cake, spent coffee, spent tea, pineapple waste, palm seed fiber, and coconut oil cake also were used in this study. Muñiz-Márquez et al. (2016) reported the production of FTase and FOS with aguamiel of maguey as the substrate in SSF using an inert support (polyurethane foam). More recently, Ganaie et al. (2017) optimized the process of FTase production with agriculture residues under solid-state fermentation. Here, organic wastes including wheat bran, corn straw, sugar cane bagasse, cassava peels, apple pomace, banana peels, beet root peels, orange peels, guava peels, guava seed powder, pine apple peels, papaya peels, mango peels, passion fruit peels, jabuticaba peels, and cashew peels were evaluated. But sugar bagasse cane proved to be the best substrate to produce fructosyltransferase in 96 h of fermentation with *A. flavus* NFCCI 2364.

18.8 PURIFICATION AND CHARACTERIZATION OF FTASE AND FFASE

The β-fructofuranosidase from mold, yeast, and bacteria has been characterized. In the case of the yeast *Schwanniomyces occidentalis*, Piedrabuena et al. (2016) characterized the FFase produced, finding that it can fructosylate 17 hydroxylated compounds as fructosyl-acceptors

alternative to sucrose. They included monosaccharides (galactose, glucose), disaccharides (gaxylose, lactose, lactulose, melibiose, sophorose, threalose), oligosaccharides (1-kestose, nystose, raffinose), alditols (erythritol, glucitol, lactitol, mannitol), and glycosides (acarbose, M-glucoside). The effect of sucrose concentration on FFase activity was also evaluated, and maximum FOS titers (78.3 g/L) were reached with 650 g/L sucrose and 6 h. On the other hand, Chu et al. (2014) purified, characterized, cloned, and overexpressed an organic β-fructofuranosidase from *Arthrobacter arilaitensis* NJEM01 in *Escherichia coli.* The enzyme had an estimated molecular mass of 55 kDa and was very stable in a wide pH range (5.0–9.5) and below 45°C. The main reaction product was 6-kestose and up to 476 g/L was synthesized. In the case of fungi, FFases from *A. pullulans* and *A. niger* have been typically used to produce FOS but also, these fungi form several kinds of FFases. For example, *A. pullulans* produces five FFases during different stages of culture (Yoshikawa et al., 2007). At an early stage of sucrose culture, the FFase I was formed and produced large amounts of FOS and glucose. This enzyme had the highest transfructosylating activity among the five enzymes. According to results, it was not repressed by glucose as other fungal FFases were (Jung et al., 1987), had a molecular weight of 430 kDa, and optimum pH of 5.0 but stability from 6.0 to 10.0. In contrast, the FFase of *A. niger* ATCC 20611 weighed 340 kDa, has an optimum temperature of 55 °C, a pH of 5.5, and is inhibited by Hg^{2+} (Hirayama et al., 1989).

In the case of FTase, high extracellular fructooligosaccharide-producing enzyme activity was reported in *A. aculeatus* M105. Evaluation of FOS production using immobilized and extracellular enzymes reached maximum yields of 65.4% and 67.54%, respectively. This enzyme has optimal pH and temperature of 5.0–6.0 and 65 °C. Relative activities declined significantly below pH 4.0 and above pH 7.0. Additionally, the encoding gene was characterized. Showing 99.4% identity with an FTase from *A. japonicus* CB05 and an FFase from *A. niger* as well as 96.5% with a FTase from *A. aculeatus* ATCC 16872 (Huang et al., 2016).

In the case of inulinases, in a study carried out by Kango (2008), a dandelion substrate allowed reaching levels with up to four times more enzymatic activity (52.3 U/mL) than with the medium containing pure inulin (12.3 U/mL), as well as higher activity than with extracts of onion bulbs (19.45 U/mL) and garlic (13.2 U/mL). *A. niger* NK-126 also showed higher growth in the medium composed of 40% dandelion extract with 2% yeast extract. It was thus that it reached an activity of 55 U/mL, incubating at 30°C for 96 h with a shaking of 150 rpm.

Exogenous intracellular inulinases have already been described for yeasts such as *Saccharomyces fragilis*, *Kluyveromyces fragilis*, and *Candida kefyr* as well as for the filamentous fungus *Penicillium* sp. Strain 1. Therefore, an in-depth study of the properties of intracellular inulinases is needed to better understand their secretion in a fungal system. In a study from Nakamura et al. (2001), the intracellular *exo*- and *endo*-inulinases of *A. niger* strain 12 were purified to compare their properties with those of an extracellular type. These were extracted from the mycelium using quartz sand and purified by chromatography. The intracellular enzymes were homogeneous according to the observed in the electrophoresis gel. *Exo*-inulinase showed activity of 6.6 U/mg on inulin and 22 U/mg on sucrose while *endo*-inulinase only showed activity on inulin (108 U/mg). Both enzymes were activated by Mn^{2+} and inactivated by Ag^{+} and Hg^{2+}. Inulinase P-II showed an apparent Km of 5.8 mM while P-III of 0.80 mM. In this way, it was concluded that the optimum conditions for said enzymatic activity were PH 5.0 and 55 °C for P-II, and pH 5.3 and 45 °C for P-III.

18.9 ENZYME IMMOBILIZATION FOR FOS PRODUCTION

Enzyme immobilization provides an advantages on the biocatalytic process. Immobilization provides a better product recovery and the separation of the biocatalytic process after the reaction (Plou et al., 2014). To enhance FOS production by enzymatic processes, the application of enzyme immobilization allows increasing the performance of the enzyme.

Smaali et al. (2012) immobilized a β-D-fructofuranosidase produced from *A. awamori* NBRC 4033 on a chitosan binding through glutaraldehyde linkages. This work provides a better view trying to scale-up an enzymatic process to produce FOS with a 53% sucrose conversion.

De Oliveira Kuhn et al. (2013) immobilized a commercial inulinase produced from *A. niger* in montmorillonite pretreated on pressurized propane and liquefied petroleum gas at 30 bar for 6 h. They found that this treatment produced a low rate of yield using sucrose as the substrate to the enzyme. Also, Aguiar-Oliveira and Maugeri (2013) added salts to immobilize a fructosyltransferase from *Rhodotorula* sp. LEB-V10.·They reported yields from 9% to 12% applying 5 and 10 mM $CuSO_4$, but when they used 10 mM $CuSO_4$, non-FOS was produced. Also, the production of FOS is affected using $MnCL_2$ and NaCl at the same concentration of 10 mM.

Immobilization of the enzymes can be made with chitosan, but when it is combined with another material. Ganaie et al. (2014) compared chitosan beads and alginate beads to immobilize a mycelial fructosyltranferase. The results obtained after this work show the efficiency of the alginate over the chitosan beads. Alginate shows 67% FOS formation in comparison with chitosan beads with 43% FOS formation. Lorenzoni et al. (2014) immobilized a Viscozyme L β-fructofuranosidase on glutaraldehyde-activated chitosan particles and the yield obtained was 55% of the conversion from sucrose to FOS. Also Chen et al. (2014) used chitosan-coated magnetic Fe_3O_4 nanoparticles and after 10 batches applying the same immobilized enzyme, this enzyme still had 55% of its enzymatic activity. Lorenzoni et al. (2015) used chitosan spheres with glutaraldehyde as a coupling agent. However, in this work, the catalytic process was evaluated in two packed bed reactors and compared these with two fluidized bed reactors. The yields of FOS produced from the sucrose substrate were 59% obtained from the packed bed reactors and 54% obtained from fluidized bed reactors. Mouelhi et al. (2016) evaluated a β-fructofuranosidase from *Sclerotinia sclerotiorum*, immobilizing the enzyme on chitosan and alginate and producing 72% yield of FOS in 12 h of the catalytic process.

Other supports to immobilize β-D-fructofuranosidase can be cellulosic compounds, as Gonçalves et al. (2015) reported the production of FOS applying sugarcane bagasse, string, and filter paper derivatives.

18.10 FUTURE PERSPECTIVES

Biotechnological processes have been used for FOS and enzyme production, including SmF and SSF. Particularly, solid-state fermentation is a bioprocess based on the utilization of agroindustrial wastes and by-products. Therefore, it needs to be further strengthened in order to improve the economics in the synthesis of prebiotics. Also, the use of cells and immobilized enzymes could improve FOS production because this process is considered an effective and economic method that could be used on a large scale. On the other hand, modern

biotechnology is focused in the use of recombinant FTases for the synthesis of FOS. Also, advances in analytical methods of the detection and purification of FOS are necessary. Other properties of FOS could be tested through assessments in vivo, including anticarcinogenic activity and control of diabetes. Finally, the encapsulation of prebiotics and their application for the food formulations could be strengthened. The progress in these aspects could be contributed to future developments in FOS production.

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CHAPTER

19

Antibiofilm Enzymes as an Emerging Technology for Food Quality and Safety

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19.1 INTRODUCTION

Biofilms are polymeric mixtures (polysaccharides, lipids, and nucleic acids) produced by microorganisms, which are supported on biotic and abiotic surfaces (Phillips, 2016). There are several favorable conditions for the formation of biofilms, such as the presence of moisture, nutrients, and inocula of microorganisms from the raw material. These conditions provide a persistent source of contamination, causing the deterioration of food as well as creating an important route for cross contamination during production processes (Laskar et al., 2017). Fig. 19.1 shows a scanning electron microscopy (SEM) image of biofilm formation on polystyrene coupons by *Aeromonas hydrophila* (Srey et al., 2013) and Fig. 19.2 shows atomic force microscopy (AFM) images of biofilms that have formed on knives used for poultry processing (Bata-Vidács et al., 2013). Microorganisms use biofilm formation as a "senile state strategy" to protect themselves against environmental stresses such as dehydration and starvation as well as against antimicrobial agents, namely antibiotics and biocides (Mah and O'Toole, 2001). In some scenarios, biofilm formation can play an important and beneficial role, for example, plant growth promotion, the biodegradation of environmental pollutants, and controlling the microbial balance in living organisms. However, in others, such as clinical settings and some industrial processes, it can cause significant problems (Bridier et al., 2015). Indeed, persistent human infections such as lung infections in cystic fibrosis patients and dental decay are associated with the development of biofilms (Simón-Soro and Mira, 2015), product contamination, the dissemination of pathogens, the corrosion and obstruction of metallic pipes, an increase in fluid frictional resistance, a decrease in heat

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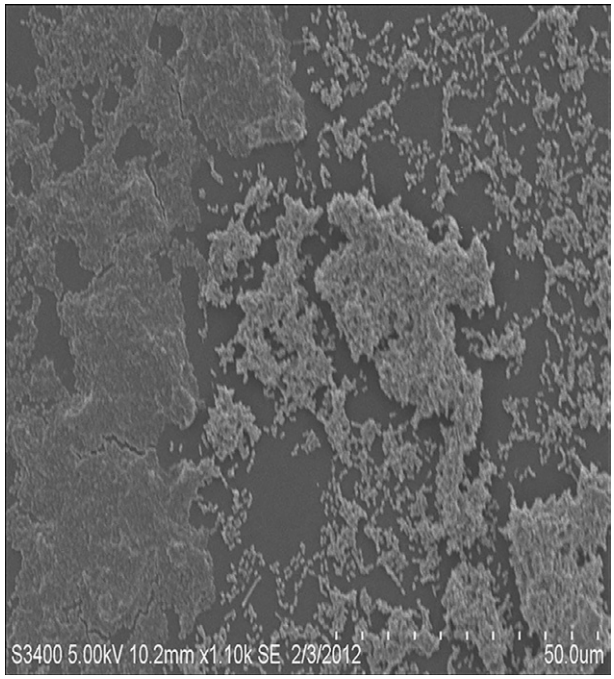


FIG. 19.1 Scanning electron microscopy (SEM) image of biofilm formation on polystyrene coupons in *A. hydrophila* (Srey et al., 2013). *Reproduced with permission from Srey, S., Jahid, I.K., Ha, S.-D., 2013. Biofilm formation in food industries: a food safety concern. Food Control 31, 572–585.*

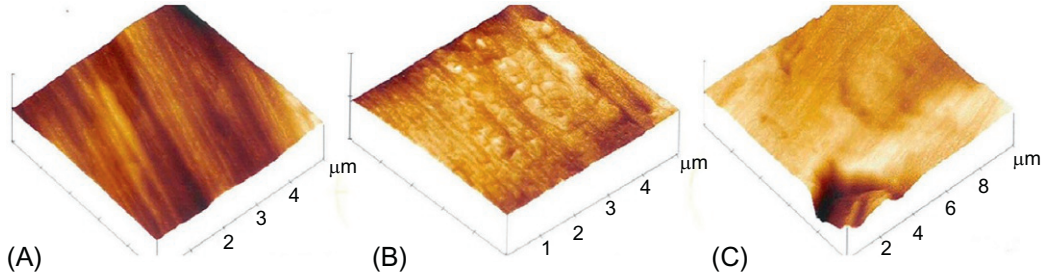


FIG. 19.2 Atomic force microscopy (AFM) images of poultry opening knives. (A) new, (B) at the end of the shift before cleaning, and (C) at the end of the shift after cleaning and disinfection (Bryers and Ratner, 2004).

transfer efficiency, and other equipment damage, all of which bring considerable economic and public health problems (Gilbert et al., 2003; Shi and Zhu, 2009). The failure of conventional methods to eradicate microorganisms contained in biofilms is due to: (1) the extracellular polymeric matrix, which generates a physicochemical diffusion barrier, (2) a reduced growth rate (an altered microbial metabolic state), in part because of nutrient/oxygen limitations, (3) the expression of specific resistance genes, and (4) the differentiation of cells into

phenotypic changes that are more resistant to treatments (e.g., the presence of persister cells) (Anderson and O'Toole, 2008; Gilbert et al., 2003).

Within the food industry, the adverse effects of biofilms are countered by the use of aggressive and corrosive chemical detergents (NaOH or NaClO), along with other environmental sanitation techniques. However, these products can damage machinery, endanger workers, and adversely affect the environment (Gilbert et al., 2003). In addition, these methods are not always successful for the removal of biofilms, especially as regards the deactivation of the inner cell layers and their surface elimination. The most recent developments for biofilm initiation and removal are those that focus on intercellular communication (quorum detection, QS), the production of extracellular polymer substances (EPS), cell-to-cell aggregation, and intrinsic cellular processes such as motility that are involved in the establishment and maturation of biofilms (Landini et al., 2010). One strategy that has proved effective for the removal of biofilms from industrial systems has been the use of enzymes, for example the treatment of biofilms formed in food areas (Akbas, 2015; Anand et al., 2014; Lequette et al., 2010).

The use of enzyme-based detergents or biocleaners, also known as "green chemicals," can serve as a viable option to overcome the problems incurred by biofilms in the food industry. Due to the heterogeneity of microorganism EPS, a mixture of enzymes may be necessary for adequate biofilm degradation. The potential application of enzymatic cleaning products against biofilms formed by microorganisms usually found in dairy products (*Lactobacillus bulgaricus*, *L. lactis*, *Streptococcus thermophilus*) has been demonstrated by Augustin et al. (2004). Nevertheless, their performance was significantly reduced in the presence of milk, particularly proteolytic enzymes. Oulahal-Lagsir et al. (2003) obtained interesting results after synergistically applying ultrasonic waves and proteolytic and glycolytic enzymes against stainless steel-attached *Escherichia coli* biofilms developed with milk. A 10 s treatment resulted in the removal of between 61% and 96% of the total amount of biofilm. Enzymes and detergents have also been used as synergists to improve disinfectant efficacy (Jacquelin et al., 1994; Johansen et al., 1997; Parkar et al., 2004). The combination of surfactants/proteolytic enzymes increased the wettability of biofilms formed by a thermophilic *Bacillus* species, thus enhancing cleaning efficiency (Parkar et al., 2004). Jacquelin et al. (1994) also reported the synergistic action of phenolic antimicrobials and surfactants in combination with enzymes. The specificity of the mode of action of each enzyme makes this technique complex. It also means that it is extremely difficult to identify enzymes that are effective against all the different types of biofilms. The formulation of mixtures containing several different enzymes thus seems to be fundamental for a successful biofilm control strategy. Generally, proteases and polysaccharide hydrolyzing enzymes have been found to be useful in this context (Meyer, 2003). However, the use of enzymes in biofilm control is still limited due to their relatively high cost compared to the chemicals currently used for this purpose (Oloketuyi and Khan, 2017; Sun et al., 2013). In addition, the technology and manufacture of these enzymes and the detergents derived from them are often protected by patents, and the resulting poor commercial accessibility limits their current use (Johansen et al., 1997). Nonetheless, several enzyme-based products are already marketed for their use in the food industry, and advances in synthetic biology, enzyme engineering, and DNA sequencing technologies show great potential for facilitating the development of more effective antimicrobial and antibiofilm enzymes. Finally, the control of biofilms with enzymes can be considered as an environmentally friendly strategy due to their nontoxic characteristics and biodegradability.

19.2 BIOFILM FORMATION

Different mechanisms are used by microorganisms to come into closer contact with a surface, attach firmly to it, promote cell-cell interactions, and grow as complex structures (Bryers and Ratner, 2004). Biofilm formation occurs in a sequence of steps (Bryers and Ratner, 2004). Since the mechanisms of biofilm formation are discussed only briefly, the reader is directed to several exhaustive reviews of this field (Bryers and Ratner, 2004; Verstraeten et al., 2008). Currently, the processes identified as governing biofilm formation include: (1) the preconditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface, (2) the transport of planktonic cells from the bulk liquid to the surface, (3) the adsorption of cells on the surface, (4) the desorption of reversibly adsorbed cells, (5) the irreversible adsorption of bacterial cells onto a surface, (6) the production of cell-cell signaling molecules, (7) the transport of substrates to and within the biofilm, (8) substrate metabolism by the biofilm-bound cells and the transport of products out of the biofilm accompanied by cell growth, replication, and EPS production, and (9) biofilm removal by detachment or sloughing (Fig. 19.3) (Bryers and Ratner, 2004).

Complex and varied processes intervene in the attachment of microorganisms to surfaces and their subsequent development (Table 19.1). Usually, attachment will occur most readily on surfaces that are more hydrophobic, rougher, and coated by surface conditioning films (Patel et al., 2007; Simões et al., 2008). Properties of the cell surface, specifically the presence of extracellular appendages; interactions involved in cell-cell communication; and EPS manufacture are all key to biofilm formation and development (Allison, 2003; Parsek and Greenberg, 2005). An increase in flow velocity or nutrient concentrations may also lead to increased attachment if these factors do not exceed critical levels (Simões et al., 2007). Biological aspects regulating biofilm formation are listed in Table 19.1.

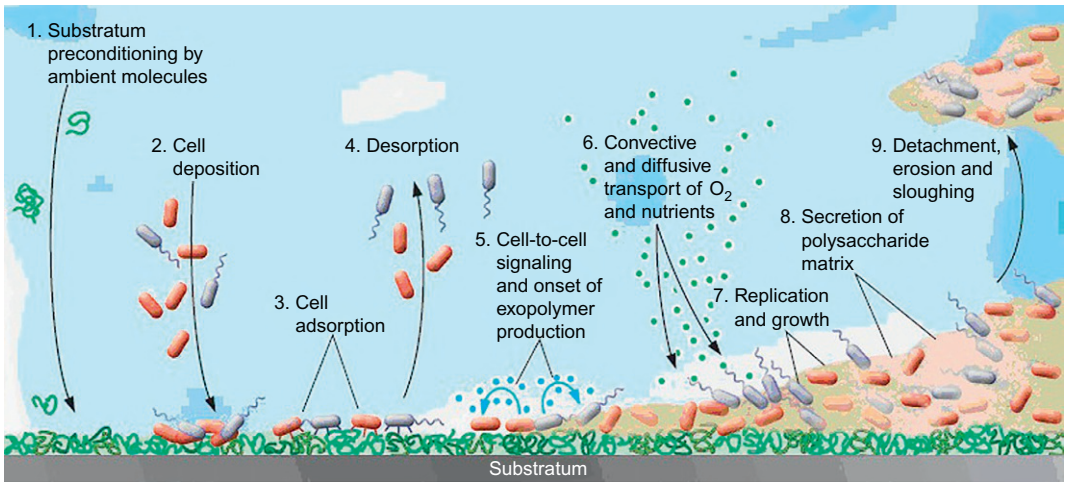


FIG. 19.3 Processes governing biofilm formation (Simões et al., 2010).

TABLE 19.1 Variables Important for Cell Attachment and Biofilm Formation and Development (Simões et al., 2010)

Adhesion Surface	Bulk Fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric substances
Charge	Cations	Signaling molecules
Conditioning film	Presence of antimicrobial products Nutrient availability	

Reproduced with permission from Simões, M., Simões, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. LWT—Food Sci. Technol. 43, 573–583.

19.3 ANTIBIOFILM ENZYMES

Enzymes are stereospecific proteins that act as natural biocatalysts and increase the rate of chemical reactions without being consumed (Shanmugam et al., 2012). It is well known that biochemical processes in cells depend largely on these macromolecules and even small changes to them can cause substantial metabolic consequences that affect the complex system of biochemical reactions (Cabral et al., 2003). Different parameters can alter the stereospecificity and activity of enzymes, namely pH, temperature, cofactors, the presence and/or lack of activators, and inhibitors or the substrates (Cabral et al., 2003). These biological macromolecules have many applications in the food and beverage, detergent, drug, textile, animal feed, paper, and pulp industries (Kirk et al., 2002). Table 19.2 shows the main

TABLE 19.2 Classification of the Main Enzymes

Enzyme Class	Example of Enzyme	Function
Transferases	Transaldolase, lipid kinase, phosphomutase, acyl-, methyl-, glucosyl-, phosphoryl-, transferase, etc.	It allows the transfer of an atom or group of atoms from one molecule to another
Oxidoreductases	Glucose oxidase, alcohol dehydrogenase, catalase, heme oxygenase, dihydrofolate reductase, phenylalanine hydroxylase, etc.	Catalyze redox reactions and transfer oxygen or hydrogen atoms
Isomerases	Isomerase, epimerase and racemase	Catalyze rearrangement reactions in a molecule
Hydrolases	Serine protease, pectinesterase, aminopeptidase, pyrophosphatase, glycosylase, oligoribonuclease, etc.	Catalyze hydrolytic reactions
Lyases	Pyruvate decarboxylase, synthase, hydratase, aldolase, etc.	Catalyze reactions by removing an atom or group of atoms
Ligases or synthetases	Carboxylase and synthetase	It can join two molecules together with a covalent bond.

classes of enzymes and their function as well as some examples of each class of enzymes (Cabral et al., 2003; Shen and Chou, 2007).

Today the application of enzymatic products has increased due to their success as biofilm removers from food contact surfaces (Taraszkiewicz et al., 2013; Thallinger et al., 2013). These processes have been approved by several regulatory agencies and there is no evidence that suggests that they affect food quality (Schmidt, 1997). In fact, food adulteration and contamination as well as any possible risk of an enzyme becoming an additional illegal additive is not feasible as long as the surfaces are well rinsed (Troller, 1993). These methods were developed in an effort to replace harmful and inefficient biocides and reduce the problems associated with the presence of biofilms in a more environmentally friendly way (Table 19.3) (Fig. 19.4) (Srey et al., 2013).

19.4 MODES OF ACTION

The EPS matrix surrounding the cells is usually the site of action of biofilm-degrading enzymes (Lequette et al., 2010). Nevertheless, their modes of action can vary as follows: (1) directly degrading the EPS matrix constituents, (2) promising cellular lysis, (3) interfering with the QS system, or (4) even catalyzing the production of antimicrobials (Augustin et al., 2004; Simões et al., 2010; Thallinger et al., 2013). The enzymatic action is closely linked to a reduction in biofilm structural integrity, and involves the disruption of the EPS matrix in monomers or molecules of lower molecular weight that can be carried and metabolized through the cell (Molobela et al., 2010). Before any enzymatic application, the chemical constituents of the EPS matrix must ideally be well characterized as the enzymes act on the biofilm (Molobela et al., 2010). These components are generally polysaccharides, carbohydrates, proteins (usually demonstrating amyloid-like properties), lipids, phospholipids, glycolipids, glycoproteins, and nucleic acids (Hobley et al., 2015). Nonetheless, matrix structure and composition is dependent on several extrinsic factors such as gas levels and nutrients, fluctuations in fluid shear (Simões et al., 2010), and a range of complex enzymatic and regulatory activities that take place within it (Allison, 2003).

The application of enzymes can either replace or at least significantly reduce the concentrations of in-use biocides, because the action of enzymes favors the biofilm disruption, thus allowing the easy entry of biocides within the microorganisms (Lequette et al., 2010; Srey et al., 2013). Because of the high chemical heterogeneity of biofilms, several classes of enzymes are needed to combat biofilms. Alternatively (and more commonly), enzyme mixtures combined with complementary treatments may be used (Augustin et al., 2004; Thallinger et al., 2013). Special attention has had four types of enzymes to biofilm control such as oxidative, anti-QS (Thallinger et al., 2013), proteolytic, and polysaccharide-degrading enzymes (Johansen et al., 1997; Thallinger et al., 2013). The types of enzymes previously identified correspond to three of the main classes of enzymes: oxidoreductases, lyases, and hydrolases (Fig. 19.5).

TABLE 19.3 Antibiofilm Applications of enzymes, Their Classification and Targets

Enzyme class	Action	Enzyme applied	Target biofilm producer	Surface material	Effect	Reference
Hydrolase	Anti-QS enzymes	Lactonase	*P. aeruginosa*	Polystyrene	66%–77% biofilm removal	Kiran et al. (2011)
		Acylase	Bacteria in a reverse osmosis membrane	Reverse osmosis membrane (material not specified)	9.0% biofilm removal	Kim et al. (2013)
		Lactonase (expressed by an Engineered T7 bacteriophage)	*P. aeruginosa* and *E. coli*	Polyvinyl chloride	Biofilm formation inhibition	Pei and Lamas-Samanamud (2014)
	Oxidative enzymes	DNase	*E. faecalis*	Polystyrene	Biofilm removal	Thomas et al. (2008)
		DNase	*L. monocytogenes*	Polystyrene	50% biofilm removal	Nguyen and Burrows (2014)
	Polysaccharide-degrading enzymes	Dispersin B	*S. epidermidis*	Glass	40% biofilm removal	Brindle et al. (2011)
		α-amylase	*S. aureus* and *S. epidermidis*	Polystyrene	79% *S. aureus* biofilm removal; no biofilm removal for *S. epidermidis*	Craigen et al. (2011)
	Proteolytic enzymes	Pandion, resinase, spezyme and paradigm used individually	*P. aeruginosa*	Polystyrene	4 log CFU/mL biofilm removal	Augustin et al. (2004)
		Bacteriophage enzyme	*E. coli* O157:H7	Stainless steel	Removal of 2.8 log CFU per stainless steel coupon	Sharma et al. (2005)
		Bacteriophage enzyme	*E. coli*	Plastic pegs	99.997% removal	Lu and Collins (2007)
		Pronase	*P. fluorescens*	Borosilicate glass	30% biofilm removal	Orgaz et al. (2007)
		Savinase	*Pseudoalteromonas* sp.	Polystyrene	Complete biofilm removal	Leroy et al. (2008)
		Savinase	*P. fluorescens*	Glass wool	80% biofilm removal	Molobela et al. (2010)
		Endolysin (LysHS)	*S. aureus*	Polystyrene	1–3 log biofilm removal	Gutiérrez et al. (2014)

Continued

TABLE 19.3 Antibiofilm Applications of enzymes, Their Classification and Targets–cont'd

Enzyme class	Action	Enzyme applied	Target biofilm producer	Surface material	Effect	Reference
	Anti QS + proteolytic enzimes	Acylase I + proteinase K	Bacteria in a reverse osmosis membrane	Reverse osmosis membrane (material not specified)	34% biofilm removal	Kim et al. (2013)
	Proteolytic + polysaccharide-degrading enzymes	Cellulose + pronase	*P. fluorescens*	Borosilicate glass	94% biofilm removal	Orgaz et al. (2007)
	Proteolytic enzyme + shear stress	Savinase + shear stress	*P. aeruginosa*	Polystyrene	90% biofilm removal	Pechaud et al. (2012)
	Proteolytic enzyme + ultrasounds	Amyloglucosidase + US	*E. coli*	Stainless steel	96% biofilm removal	Oulahal-Lagsir et al. (2003)
	Polysaccharide-degrading enzymes + chemical treatment	α-amylase + buffer with an anionic surfactant	*B. mycoides*	Stainless steel	2.98 log CFU/cm^2 biofilm removal	Lequette et al. (2010)
	Nucleolytic enzymes from marine cold-adapted bacteria	The recombinant alkaline phosphatase of *Cobetia amphilecti* KMM 296 (CmAP) and for gene cloning and expression of the full-length mature EEP-like enzyme (CmEEP)	*P. aeruginosa*, *B. subtilis*, *Salmonella enterica* and *S. aureus*.	Meat product (pork sausages) (see Fig. 13.4)	1.1 mg/mL, 2.5 u/mL led to a significant inhibition of microbial growth including yeast and mold ones within five days of the cold storage	Balabanova et al. (2017)

Oxidoreductase + hidrolase	Oxidative + polysaccharide-degrading enzymes	Glucose oxidase + lactoperoxidase	*S. aureus*, *S. epidermidis*, *P. aeruginosa* and *P. fluorescens*	Stainless steel	1–2 log CFU/disc biofilm removal of *Staphylococcus*; 3 log CFU/disc biofilm removal of *Pseudomonas* spp.	Johansen et al. (1997)
Lyase	Polysaccharide-degrading enzymes + antibiotic	Alginate lyase + gentamycin	*P. aeruginosa*	Cellulose fibers	Complete biofilm removal	Alkawash et al. (2006)

Modified and reproduced with permission from Meireles, A., Borges, A., Giaouris, E., Simões, M., 2016. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res. Int. 86, 140–146.

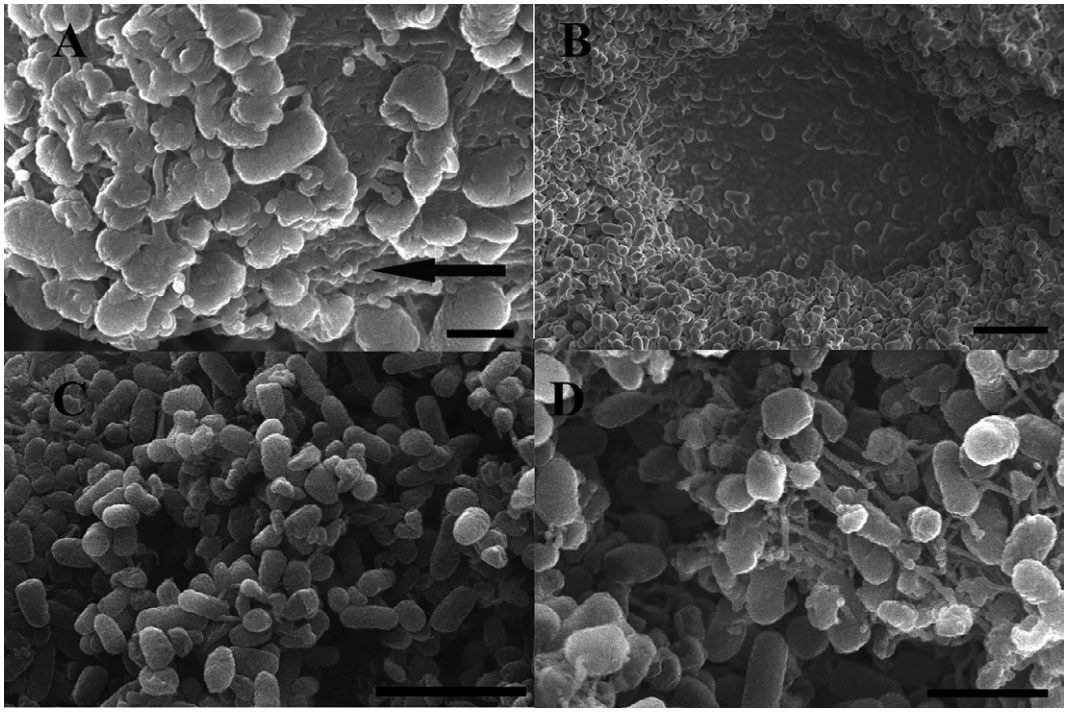


FIG. 19.4 10-Day old multilayer *P. aeruginosa* biofilm isolated from ready-to-cook meats: (A) Intact biofilm (0.5 μm). Black cursor shows dense extracellular matrix. (B) Zone of the biofilm treated by the recombinant alkaline phosphatase of *Cobetia amphilecti* KMM 296 (CmAP) (4 μm). (C) Zone of the biofilm treated by CmAP (2 μm): decreasing extracellular matrix compounds and intercellular connections (pilus). (D) Zone of the biofilm treated by the cloned EPP enzyme (CmEEP) (2 μm) where the genes contain conserved domains of a deoxyribonuclease (DNase)-like enzyme: loosening of the extracellular matrix without destruction of intercellular connections (pilus) (Balabanova et al., 2017). *Reproduced with permission from Balabanova, L., Podvolotskaya, A., Slepchenko, L., Eliseikina, M., Noskova, Y., Nedashkovskaya, O., Son, O., Tekutyeva, L., Rasskazov, V., 2017. Nucleolytic enzymes from the marine bacterium Cobetia amphilecti KMM 296 with antibiofilm activity and biopreservative effect on meat products. Food Control 78, 270–278.*

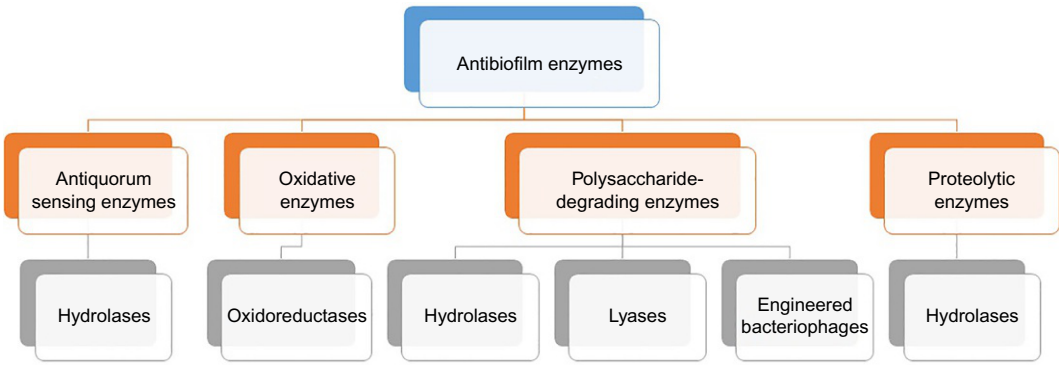


FIG. 19.5 Classification of enzymes relevant for biofilm control.

19.5 CLASSIFICATION OF THE MAIN ENZYMES FOR BIOFILM PREVENTION AND REMOVAL

19.5.1 Antiquorum Sensing Enzymes

Biofilm matrices provide optimal conditions for QS due to the spatiochemical conditions and close proximity of cells within them, which allow bacterial coexistence (Giaouris et al., 2015; Li and Tian, 2012). QS is an intercellular communication mechanism frequently used by several bacterial species as a result of an increase in the density of microorganisms. This complex system of genetic regulation is based on the production, release, and detection of small signaling molecules known as autoinducers (AIs) (LaSarre and Federle, 2013). Due to that, the use of anti-QS enzymes will be briefly discussed, and the reader is directed to some outstanding work on this subject (Grandclément et al., 2016; Kalia, 2013; LaSarre and Federle, 2013; Lade et al., 2014).

Different classes of signaling molecules derived from microorganisms have been identified, being the most frequently evaluated: (1) acyl homoserine lactones (AHLs), (2) autoinducing peptides (AIPs), and (3) autoinducer-2 (AI-2) (Miller and Bassler, 2001). Three components make up QS systems: (1) the AI, (2) the gene coding for the AI synthase protein, and (3) the gene coding for the response regulator protein. Whatever the specific methods used to inhibit the QS system, however, all the alternatives used are based on the disruption of some of these mechanisms (Kalia, 2013). The development and maintenance of biofilms is typically associated with QS, thus anti-QS enzymes can be successfully used for their removal (Lazar, 2011). Some of these enzymes are acylases and N-acyl homoserine lactonases. The lactones can hydrolyze the bonds in the homoserine ring, inhibiting the binding of AHLs to transcriptional regulators (Thallinger et al., 2013). AHL degradation enzymes have been found in many symbiotic bacteria and fungi (Dong et al., 2007). A significant number of α, β, γ proteobacteria and Gram-positive bacteria, particularly *Bacillus* spp., can secrete anti-QS or quorum-quenching enzymes (AHL lactonase and AHL acylase) that degrade AHL molecules (Czajkowski and Jafra, 2009). In general, the mechanism of action of these enzymes (AHL lactonases, AHL acylases, and oxidoreductases) is known: four potential cleavage sites in the AHLs are likely cut off after the catabolic digestion of carbon and nitrogen sources (Sadekuzzaman et al., 2015). Kiran et al. (2011) found that treatment with one unit of lactonase significantly reduced biofilm formation (from 69% to 77%) by four *Pseudomonas aeruginosa* strains. In addition, lactonase treatment at 0.3 U/mL disrupted biofilm structure and increased sensitivity to antibiotics (ciprofloxacin and gentamycin), thus causing a reduction in the production of virulence factors such as pyocyanin (from 85% to 93%), protease activity (from 86% to 96%), elastase activity (from 69% to 91%), and the secretion of pyochelin (from 40% to 90%). According to Kim et al. (2013) acylase I is able to cleave QS molecules. However, acylase I at 100 μg/mL was only able to degrade 9.0% of the EPS matrix found in a reverse osmosis membrane (Kim et al., 2013). In another work, an engineered T7 bacteriophage expressing a lactonase with broad-range activity for QS inhibition was developed by Pei and Lamas-Samanamud (2014). The inhibition of the development of biofilm in polyvinyl chloride microtiter plates was achieved by adding the engineered phage to mixed-species biofilms containing *P. aeruginosa* and *E. coli*. Alternatively, specifically in cell-to-cell communication in *Listeria monocytogenes*, biofilm development may occur mostly through AI-2, a mediator of QS similar to *Vibrio harveyi*, which is expressed by the lmo1288 gene encoding for the LuxS protein (Challan Belval et al.,

2006). LuxS is an enzyme found in many bacterial species and is involved in the production of AI-2, an interspecies signaling molecule synthesized by both Gram-positive and Gram-negative bacteria responsible for pathogenesis, motility, and biofilm formation (Oloketuyi and Khan, 2017). Gene encoding for two enzymes—*pfs* (*lmo1494*) and *luxS* (*lmo1288*)—are present in the genome of L. *monocytogenes* and plays a key role in cell-to-cell communication. These enzymes are formed by rearrangement into various cyclic compounds—AI-2 through the catalysis of the conversion of Sadenosyl homocysteine (SAH) into homocysteine and 4,5-dihydroxy-2,3-pentanedione (Sun et al., 2004). The peptide-mediated accessory gene regulatory system (*agr*) also plays an essential role in cell-cell communication through the production and regulation of auto-inducing peptides, gene clusters containing *agrBDCA*, and aids adherence in Gram-positive bacteria (Oloketuyi and Khan, 2017; Rieu et al., 2008). A detailed understanding of the mechanisms responsible for biofilm formation is thus key for determining the type of enzyme required to prevent the formation of biofilms.

19.5.2 Oxidative Enzymes

Enzyme-based products can also be targeted to extracellular DNA (eDNA) found in the biofilm matrix (Hall-Stoodley et al., 2008; Okshevsky and Meyer, 2015; Thomas et al., 2008). The enzymatic degradation of eDNA can prevent, disperse, or sensitize biofilms to antimicrobials (Okshevsky et al., 2015). The effects of deoxyribonuclease (DNase) on the biofilm of Gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative (*Acinetobacter baumannii*, *Haemophilus influenza*, *Klebsiella pneumoniae*, *E. coli*, and *P. aeruginosa*) bacteria have been extensively studied (Tetz et al., 2009). These authors demonstrated that DNase at a concentration of 5.0 μg/mL, reduced the active biofilm biomass by approximately 40% among all the tested organisms in 24 h. They also reported that the synergistic effects of DNase (5 μg/mL) and antibiotics (azithromycin, rifampin, levofloxacin, ampicillin, and cefotaxime) significantly reduced biofilm biomass (Tetz et al., 2009). Thomas et al. (2008) demonstrated that DNase applications diminished biofilm deposition by *Enterococcus faecalis*. The latter treatment was also applied to control *Streptococcus pneumonia* biofilms (Hall-Stoodley et al., 2008). Hall-Stoodley et al. (2008) observed that biofilm thickness decreased by more than 85%. Nguyen and Burrows (2014) evaluated the binding of *L. monocytogenes* to polystyrene-based plastic surfaces and found that DNase added at the start of biofilm formation diminished *L. monocytogenes* attachment by 50%. According to Whitchurch et al. (2002) and Nguyen and Burrows (2014), DNase possesses the ability to reduce adherence of *L. monocytogenes* to contact surfaces, inducing biofilm dispersal, by cleaving eDNA in the EPS matrix and hydrolyzing the phosphodiester bonds of the phosphate backbone, thus causing degradation. Eckhart et al. (2007) found that biofilm formation by *S. aureus* and *P. aeruginosa* was effectively reduced by DNase treatment. Furthermore, bovine DNase was reported to suppress biofilm formation by *P. aeruginosa*, *Streptococcus intermedius*, and *S. mutans* (Petersen et al., 2005; Whitchurch et al., 2002).

19.5.3 Polysaccharide-Degrading Enzymes

Among the degrading-polysaccharide antibiofilm enzymes, alginate lyase, cellulose, α-amylase, and lysozyme may be mentioned (Lequette et al., 2010; LewisOscar et al., 2016; Thallinger et al., 2013). The coadministration of a lyase with an antibiotic was tested to inhibit

and eradicate biofilms (Alkawash et al., 2006). These authors assessed the effects of a combined treatment with alginate lyase (20 μg/mL) and gentamycin (64 μg/mL) on a biofilm formed by two mucoid *P. aeruginosa* strains. Their results revealed that the treatment caused the liquefaction of the biofilm matrix as well as the complete eradication of the structure and all living bacteria within 96 h. The combined therapy also reduced viable counts of both strains by 2–3 log10 units (Alkawash et al., 2006).

Loiselle and Anderson (2003) concluded that cellulase inhibits *P. aeruginosa* biofilm formation. Cellulase was demonstrated to break down EPS by the decrease in its apparent molecular weight coupled with an increase in the production of reducing sugars (Loiselle and Anderson, 2003). The efficacy of enzymatic action is, however, an influence on the target bacteria. Craigen et al. (2011) found that α-amylase was able to reduce *S. aureus* biofilms by 79%, but was not efficient in removing *Staphylococcus epidermidis* biofilms. Specifically, α-amylases doses of 10, 20, and 100 μg/mL reduced *S. aureus* biofilms by 72%, 89%, and 90%, respectively while matrix formation was also inhibited by 82%. In a time course experiment, *S. aureus* biofilms were reduced by 79% and 89% within 5 and 30 min, respectively (Craigen et al., 2011). In addition, the data demonstrated that α-amylase compounds can disperse as well as inhibit *S. aureus* biofilms. These results led Craigen et al. (2011) to suggest that α-amylase compounds could be used in the near future to control *S. aureus* biofilm infections. Brindle et al. (2011) used the glycoside hydrolase, dispersin B (DspB), to hydrolyze poly-*N*-acetylglucosamine (PNAG), a polysaccharide responsible for the formation of biofilm structure excreted by *S. epidermidis*. DspB was applied at a concentration of 40 ppm to *S. epidermidis* biofilms on glass surfaces, resulting in 40% biofilm removal (Brindle et al., 2011). Similarly, Pavlukhina et al. (2012) showed that incorporating DspB into a layer-by-layer hydrogel also inhibited biofilm formation. A naturally occurring enzyme that can effectively invade biofilms is lysostaphin (LS) (Belyansky et al., 2011a, 2011b). Walencka et al. (2005) found that LS effectively eradicated biofilms formed by clinical and reference *S. aureus* and *S. epidermidis* strains. In addition, their study revealed that the combination of oxacillin and LS increased the sensitivity of the biofilm-growing bacteria to an antibiotic. Kokai-Kun et al. (2009) observed that treatment with LS (15 mg/kg) in combination with nafcillin (50 mg/kg) eradicated established *S. aureus* biofilms, including methicillin-resistant *S. aureus* (MRSA) biofilms. They also found that a single treatment with LS (10 mg/kg) totally protected surfaces from new biofilm infections. Aguinaga et al. (2011) found that the synergistic effect of doxycycline (an antibiotic) and LS against *S. aureus* biofilms was effective against both MRSA and methicillin-sensitive *S. aureus* (MSSA) biofilms.

19.5.4 Proteolytic Enzymes

Proteases are a class of proteolytic enzymes, and have been shown to hydrolyze protein macromolecules adhered to the surfaces of food processing facilities that are difficult to access such as pipes (Augustin et al., 2004). According to Lequette et al. (2010), serine proteases effectively reduce *Bacillus* biofilms whereas polysaccharides remove *Pseudomonas fluorescens* more efficiently than serine proteases. Molobela et al. (2010) essayed different enzymes and indicated Savinase as one of the most effective enzymatic formulations for eradicating *P. fluorescens* biofilms from glass wool. These researchers also reported that biofilm chemical constituents should be considered when choosing the correct enzyme. For example, proteases are more effective than α-amylases for degrading *P. fluorescens* biofilms (Molobela et al.,

2010). Similar results were also reported by Huang et al. (2014), i.e., proteolytic enzymes are more effective in removing biofilms than α-amylase enzymes. Franciosa et al. (2009) reported that protease K, a serine protease, inactivates *L. monocytogenes* biofilm by degrading proteinaceous adhesins, an important factor in attachment and matrix components. Leroy et al. (2008) used Savinase to inhibit *Pseudoalteromonas* sp. beginning and development of the biofilm, resulting in total elimination of the biofilm. Augustin et al. (2004) applied different products based on enzymes (Spezyme, Paradigm, Pandion and Resinase) separately for 30 min, achieving a 4-log reduction in a *P. aeruginosa* population. Orgaz et al. (2007) found that pronase applied to *P. fluorescens* biofilms formed on borosilicate glass surfaces removed 30% of the biofilm. More recently, Nguyen and Burrows (2014) showed that DNase and proteinase K are effective against *L. monocytogenes* biofilms, with DNase treatment over 24 h reducing the attachment of 72 h established biofilms, leading to incomplete biofilm dispersal. Moreover, the addition of proteinase K completely inhibited biofilm formation and 72 h biofilms were totally dispersed. "Generally recognized as safe" (GRAS) proteases such as bromelain and papain were less effective than proteinase K. The authors concluded that the biofilm matrix can be targeted for the effective prevention and removal of biofilms. Bacteria can be infected by phage (or bacteriophages), which in essence are viruses that can provoke cell lysis (Donlan, 2009; Fischetti, 2005). Phages can penetrate the EPS matrix via diffusion or with the help of phage-derived enzymes such as polysaccharide depolymerase. These enzymes can destroy biofilm architecture so that the phages can easily attach to lipopolysaccharides, outer membrane proteins, or other receptors necessary for their replication (Hughes et al., 1998a, b), and can thus also be applied as an antibiofilm alternative (Simões et al., 2010; Sillankorva and Azeredo, 2014). Phages produce lysins that cause cell lysis (Fischetti, 2005) as well as polysaccharide depolymerases that are capable of disrupting the EPS matrix, thus mounting a double attack on the biofilm (Donlan, 2009; Hughes et al., 1998a; Phillips, 2016). Sharma et al. (2005) used bacteriophages to reduce *E. coli* O157:H7 biofilms from 2.8 log colony forming units (CFU) per stainless steel specimen. Gutiérrez et al. (2014) were able to shrink *S. aureus* (obtained from food processing machinery) biofilms by 1–3 log units per polystyrene microtiter plate, applying LysH5 (an endolysin) to induce cell lysis (Borysowski et al., 2006). Rather than using phages per se, isolated bacteriophage enzymes may also be applied (Phillips, 2016). In this regard, several authors have sought to increase bacteriophage action by creating genetically engineered phages (synthetic biology) that produce biofilm-degrading enzymes during infection (Fischetti, 2005; Lu and Collins, 2007; Sadekuzzaman et al., 2015).

These genetically modified phages can act simultaneously and, therefore, are more efficient, provoking both EPS matrix disruption and cell lysis (Lu and Collins, 2007). For example, Lu and Collins (2007) found that an engineered enzymatic T7 bacteriophage (specific for *E. coli*) reduced cell counts in the *E. coli* biofilm by 4.5 log (99.997%) more than the nonenzymatic phage (noncloned phage) they tested. Specifically, the T7 bacteriophage was able to reduce the biofilm by about two orders of magnitude more than the nonenzymatic phage. The DspB enzyme was expressed intracellularly during infection so that it was liberated on host cell lysis. Other examples worthy of mention are a depolymerase enzyme extracted from a *Klebsiella*-specific bacteriophage against a biofilm-forming *Klebsiella* isolate from a food processing environment (Chai et al., 2014); peptidase $CHAP_K$ against *S. aureus* (Fenton et al., 2013); and PlyC (an endolysin) against *Streptococcus* (Shen et al., 2013).

19.6 COMBINATIONS OF ENZYMES

The application of enzyme mixtures has been amply used as an efficient antibiofilm alternative (Johansen et al., 1997; Orgaz et al., 2007). Because enzymes are specific in their matrix-removing activities, a mixture of enzymes is more likely to be effective than a single one (Simões et al., 2010; Srey et al., 2013). Nonetheless, because the chemical composition of biofilm matrices can vary, the enzyme mixtures may not be equally effective against all of them. Chaignon et al. (2007) confirmed this for staphylococcal biofilms, where the enzymatic detachment of the biofilm depended on the constituents of the matrix, which varied between different isolates. Johansen et al. (1997) evaluated the efficacy of commercially available enzymes against a range of bacterial biofilms, including *P. aeruginosa* and *S. aureus*, and concluded that glucose oxidase combined with lactoperoxidase was bactericidal but did not remove the biofilm whereas a combination of oxidoreductases and polysaccharide-hydrolyzing enzymes was both bactericidal and did remove the biofilm. Orgaz et al. (2007) eradicated 94% of a *P. fluorescens* biofilm, which was formed on borosilicate glass surfaces by applying cellulase followed by pronase. Kim et al. (2013) reported that the proteinase K (5 μg/mL)/acylase I (100 μg/mL) mixture removed 33.7% of the bacteria found on a reverse osmosis membrane device. Singh et al. (2015) recently demonstrated that *Aspergillus clavatus* (a natural source of fungi) produces a mixture of amylases, proteases, and pectinases that reduced *P. aeruginosa* and *Bacillus subtilis* biofilms by 82% and 75%, respectively. On the other hand, recombinant enzymes can be even more effective than natural enzymes as antibiofilm agents. For example, a recombinant DspB enzyme has been shown to have antibiofilm activity against *S. epidermidis* (Dobrynina et al., 2015) and *S. aureus* (Chaignon et al., 2007). In the latter case, the DspB enzyme followed by treatment with a proteinase treatment was more effective than dispersin alone.

19.7 COMBINING ENZYMES WITH CHEMICAL/PHYSICAL TREATMENTS

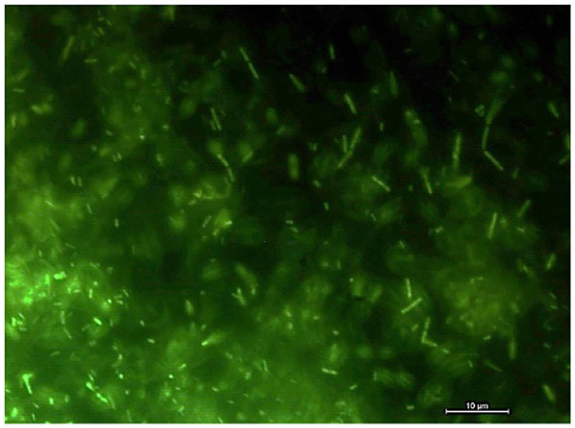
Due to variations in enzymatic activity and biofilm matrix composition, it has been suggested that a combination of enzymes directed at specific matrix constituents with surfactants, dispersing agents, and chelants can provide an effective alternative to chemical cleaning regimes (Lequette et al., 2010; Phillips, 2016). In addition, the synergistic effect of antimicrobial constituents or methods, namely biocides or ultrasounds, respectively, combined with enzymes has been shown to be more effective compared to individual methods. Oulahal et al. (2007) evaluated different enzymes (lysozyme, papain, amyloglucosidase, trypsin, and protease) combined with ultrasound (40 kHz for 10 s) and ethylene-diamine tetraacetic acid (EDTA) (a chelating agent) to degrade biofilms from stainless steel surfaces. This treatment resulted in the removal of 75% and 100% of *E. coli* and *S. aureus* biofilms, respectively (Oulahal et al., 2007). Alkawash et al. (2006) obtained the total eradication of *P. aeruginosa* biofilms on cellulose fibers after 96 h by applying gentamycin and alginate lyase. Pechaud et al. (2012) reported that the application of Savinase combined with a shear stress of 2.5 Pa led to a 90% removal of *P. aeruginosa* biofilms while a minor reduction (20%) was observed using NaClO (50 ppm). Oulahal-Lagsir et al. (2003) used proteolytic enzymes and ultrasound

(40 kHz for 10 s) to remove *E. coli* biofilms from stainless steel surfaces. The synergistic action of amyloglucosidase (50 U mL^{-1}) and ultrasound resulted in biofilm degradation by 96% (Oulahal-Lagsir et al., 2003). Lequette et al. (2010) mixed α-amylase with a buffer with an anionic surfactant and achieved the reduction of *Bacillus mycoides* biofilms on stainless steel from 2.98 log CFU/cm^2. The biofilm degradation action obtained from a surfactant (Biorem A1)/enzymes (Biorem 10) mixture can be observed in Fig. 19.6A and B. Fig. 19.6C shows the total biofilm eradication after using the above mixture, followed by application of sodium hypochlorite.

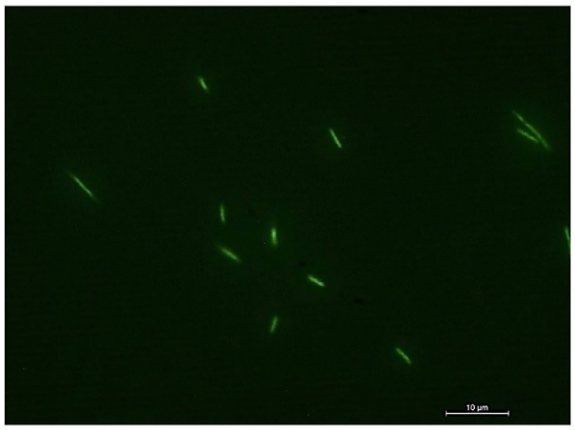
Enzymes and detergents have also been combined to increase disinfectant potency in the food industry. The synergistic effect of proteolytic enzymes with surfactants increases the wettability of biofilms, thus enhancing cleaning efficacy. Formulations containing several enzymes seem to be an effective novel biofilm control strategy. Proteases and polysaccharide-hydrolyzing enzymes are the most commonly used for this purpose (Shi and Zhu, 2009). Nevertheless, the difficulty of identifying enzymes that are effective against all types of biofilms coupled with their specific modes of action make this strategy very complicated and therefore impractical for biofilm control on a commercial scale. However, a mixture of several enzymes with disinfectants/antimicrobials has proved to be a promising and highly effective mechanism for the removal of biofilms (Sadekuzzaman et al., 2015). For example, the study by Chai et al. (2014) showed that the combination of pretreatment by a depolymerase enzyme followed by 30 min treatment with chlorine dioxide reduced the bacterial biofilm cells by approximately 92%, compared to the 80% and 75% reduction achieved after separate applications of either the phage enzyme or a chlorine-based disinfectant, respectively.

19.8 CONCLUSIONS

Currently, great efforts are being made to avoid the indiscriminate use of antibiotics and antimicrobial agents, due to the fact that many of these chemicals have given rise to genetically modified and highly resistant bacteria. The study of the mechanisms related to bacterial growth and their survival strategies when placed under stressful conditions, of which biofilm formation is one, has opened a technological window for microbial control, particularly against pathogenic and deteriorative bacteria that put food quality and safety at risk. The use of enzymes as an emerging technology for biofilm control has proved to be a very successful method of inhibiting and reducing microbiological growth in foods. These so-called antibiofilm enzymes not only render biofilms useless as a defense mechanism of bacteria, but also interrupt communication between them, resulting in their destruction and inactivation. Further advantages of these enzymes are that they have "green chemical" or "environmentally friendly" status, and do not impose selective pressures on bacteria. It is worth noting, however, that due to the complex polymeric matrices of the biofilms and their stereospecific characteristics, the use of enzyme mixtures, especially those combined with other alternative treatments, is highly recommended. Finally, future perspectives for the application of the enzymes in this field are currently oriented toward the use of enzymes engineered using synthetic biology techniques, and/or the simultaneous application of enzymes with other physical and/or chemical methods used as antimicrobials. Such perspectives could possibly reduce the high production costs of enzyme-based products, thus increasing the potential for their use at an industrial scale.



(A)



(B)

(C)

FIG. 19.6 (A) Microscopic visualization of *E. coli* biofilms (5 days) on stainless steel, (B) after application of Biorem A1 0.25% + Biorem 10 0.05% (Realco, Belgium) for 1 h at 25°C, and (C) combination of the enzymatic treatment with 50 ppm sodium hypochlorite for 20 min at 25°C. Magnification ×1000 and scale bar of 10 µm. Cells were stained with 0.1 µg/mL acridine orange (Sigma, Portugal). After 20 min of incubation in the dark, the slides were mounted with nonfluorescent immersion oil on glass microscope slides. The slides were examined using an epifluorescence microscope (LEICA DMLB2) (Meireles et al., 2016). *Reproduced with permission from Meireles, A., Borges, A., Giaouris, E., Simões, M., 2016. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res. Int. 86, 140–146.*

Acknowledgments

The author would like to thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Postdoctoral fellowship internal PDTS-Resolution 2417), Universidad Nacional de Mar del Plata (UNMdP) for financial support, and Dr. Mirian Carmona-Rodríguez.

Conflicts of Interest

The author declares no conflict of interest.

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CHAPTER

20

Enzyme and Bioactive Peptides—A Strategy for Discovery and Identification of Antihypertensive Peptides

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ABBREVIATIONS

ACE	angiotensin-converting enzyme
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BSA	bovine serum albumin
Cys	cysteine
EAE	enzyme-assisted extraction
ECE	endothelin-converting enzyme
ET	endothelin
Glu	glutamic acid
Gly	glycine
His	histidine
HPLC	high performance liquid chromatography
IC_{50}	the concentration of an inhibitor where the response (or binding) is reduced by half
Ile	isoleucine
Leu	leucine

https://doi.org/10.1016/B978-0-12-813280-7.00020-7

Lys	lysine
MALDI-TOF/TOF MS	matrix-assisted laser desorption ionization—time of flight/time of flight mass spectrometry
Met	methionine
Phe	phenylalanine
Pro	proline
RAS	renin-angiotensin system
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

20.1 INTRODUCTION—THE IMPORTANCE OF BIOACTIVE PEPTIDES

Bioactive peptides are specific protein-derived biomolecules that impart physiological effects and provide a positive impact to human body functions as well as health conditions (Danquah and Agyei, 2012). According to Dave et al. (2016), these peptides could be dietary or gut endogenous protein-derived bioactive peptides. These peptides are, however, inactive within the parental protein. They can only act as regulatory components with hormone-like activities once they are liberated via proteolytic reaction (Korhonen and Pihlanto, 2003; Sharma et al., 2011). The amino acid sequence in a particular peptide encodes the information that provides a diversity in properties. It was noted that a single peptide sequence could also exhibit different types of biological properties (Agyei et al., 2016). These physiological roles have made peptides a suitable candidate for the development of therapeutic agents.

Due to the side effects in using pharmaceutical drugs, the search for the pharmacological value of peptides has been conducted for many years. It was proven scientifically that they can be used as an alternative preventive or curative approach for different metabolic diseases due to their advantages, such as having a broad spectrum of action and being less allergenic as well as high in biospecificity, structural diversity, delivery, and activity (Agyei et al., 2016; Danquah and Agyei, 2012). Many studies reported the potential of bioactive peptides attributed to different bioactivities, such as antimicrobial (Holaskova et al., 2014), antioxidant (Zhang et al., 2010), antithrombotic (Wang and Ng, 1999), antihypertension (Zhao et al., 2007), and immunomodulatory activities (Gauthier et al., 2006). Table 20.1 shows the examples of bioactive peptides that are extracted from different sources and their potential biological activities.

The objective of this chapter is to assess the enzymatic-assisted approaches in the discovery of bioactive peptides. Although a number of approaches have been applied, there is limited knowledge on this aspect. In this chapter, we will elaborate on the challenges, discovery, and identification of bioactive peptide-using enzymes, followed by the advantages of enzymatic-assisted approaches as well as the current and future trends of peptide discovery. At the end of the chapter, we will discuss a case study that involved an overview of antihypertensive peptide discovery.

TABLE 20.1 Peptides Derived From Various Food Proteins and Their Biological Activities

Sources	Identified Peptide Sequences	Biological Activity	References
Rice	Phe-Arg-Asp-Glu-His-Lys-Lys Lys-His-Asn-Arg-Gly-Asp-Glu-Phe	Antioxidative	Zhang et al. (2010)
Casein	Tyr-Phe-Tyr-Pro-Glu-Leu	Antioxidative	Suetsuna et al. (2000)
Albumin	Lys-Leu-Pro-Gly-Phe	Antidiabetic	Yu et al. (2012)
Hoki skin gelatin	His-Gly-Pro-Leu-Gly-Pro-Leu	Antioxidative	Mendis et al. (2005)
Basil seeds	Ala-Cys-Gly-Asn-Leu-Pro-Arg-Met-Cys Ala-Cys-Asn-Leu-Pro-Arg-Met-Cys Ala-Gly-Cys-Gly-Cys-Glu-Ala-Met-Phe-Ala-Gly-Ala	Antioxidative and antidiabetic	Nurul Hidayatul Afifah and Gan (2016)
Soybean	Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr	Anticancer	Kim et al. (2000)
	Glu-Ile-Thr-Pro-Glu-Lys-Asn-Pro-Gln-Leu-Arg	Antiobesity	Martinez-Villaluenga et al. (2010)
	Lys-Asn-Pro-Glu-Leu-Arg	Antiobesity	Martinez-Villaluenga et al. (2010)
	Arg-Lys-Gln-Glu-Glu-Asp-Glu-Asp-Glu-Glu-Gln-Gln-Arg-Glu	Antiobesity	Martinez-Villaluenga et al. (2010)
Pinto bean	Pro-Pro-His-Met-Leu-Pro Pro-Leu-Pro-Trp-Gly-Ala-Gly-Phe Pro-Pro-His-Met-Gly-Gly-Pro Pro-Leu-Pro-Leu-His-Met-Leu-Pro Leu-Ser-Ser-Leu-Glu-Met-Gly-Ser-Leu-Gly-Ala-Leu-Phe-Val-Cys-Met	Antidiabetic	Ngoh et al. (2016)
Cumin seeds	Phe-Phe-Arg-Ser-Lys-Leu-Leu-Ser-Asp-Gly-Ala-Ala-Ala-Ala-Lys-Gly-Ala-Leu-Leu-Pro-Gln-Tyr-Trp	Antidiabetic	Siow and Gan (2016)
Oyster (*Crassostrea gigas*)	Leu-Leu-Glu-Tyr-Ser-Ile Leu-Leu-Glu-Tyr-Ser-Leu	HIV-1 protease inhibitor	Lee and Maruyama (1998)
Blood clam (*Tegillarca granosa*) muscle	Trp-Pro-Pro Gln-Pro	Antioxidant and anticancer	Chi et al. (2015)
Tuna	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Glu-Glu-Leu-Ser	Antioxidative	Je et al. (2007)
Grass carp muscle	Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val	Antioxidative	Ren et al. (2008)
Musca domestica	Lys-Ser-Ser-Ser-Pro-Pro-Met-Asn-His	Antitumor	Tang et al. (2009)
Squid	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	Antioxidative	Mendis et al. (2005)
Spanish dry-cured ham	Ala-Ala-Ala-Thr-Phe	Antidiabetic	Gallego et al. (2014)
Alaska Pollack	Leu-Pro-His-Ser-Gly-Tyr	Antioxidative	Je et al. (2005)
Wheat	Leu-Gln-Pro-Gly-Pyl-Gly-Gln-Gln-Gly Ala-Gln-Ile-Pro-Gln-Gln	Antioxidative	Suetsuna and Chen (2002)
Cowpea bean	Thr-Thr-Ala-Gly-Leu-Leu-Glu	Antidiabetic	De Souza Rocha et al. (2014)

20.2 CHALLENGES IN PEPTIDE DISCOVERY

The properties in the peptides, such as molecular weight, hydrophobicity or hydrophilicity, charge distribution, composition, and the sequence of amino acids, are essentially important to their biological activities. It was reported that, due to the primary structure changes, the binding mechanism or mode of action of bioactive peptides could be affected (Agyei et al., 2016). Therefore, the extraction process condition has to be well controlled. Apart from that, in order to achieve the targeted peptide as a commercial product, the developer is required to scale up the process to obtain a high production yield and less deterioration of their structure-activity property. This requirement is costly, time-consuming, and involves a series of tedious processes because the extraction process produces heterogeneous peptide sequences.

In the general workflow for peptide discovery, there were several approaches involved, such as selective precipitation, membrane filtration, chromatography methods, phage display techniques, etc. However, these approaches have their limitations (Agyei et al., 2016; Saxena et al., 2010). For example, if strong precipitation agents are used, an undesired reaction such as sulfydryl oxidation, might occur and affect the properties of the peptides. Subsequently, a purification procedure is required to remove the precipitation agents in order to obtain a pure peptide of interest. Apart from that, the recovery rate of highly concentrated peptides is low in the membrane filtration technique due to its low mass transfer rate and often, membrane fouling occurs. In addition, this approach has difficulty in separating peptides with a similar molecular mass. As for the chromatographic techniques, it is costly for the scale-up of production. The binding capacity is low and viscous protein hydrolysates can cause clogging of the column and reduce the flow rates (Agyei et al., 2016). Other than that, the binding affinity of the peptides using the phage display technique is too low to support their therapeutic use (Meiring et al., 2002). It has therefore presented scientific and technological challenges in the discovery and identification of peptides of interest (Udenigwe, 2014).

20.3 EXTRACTION OF BIOACTIVE PEPTIDES USING ENZYMES

The emerging trend of discovering natural food-derived bioactive peptides signifies the need for the most appropriate and applicable method to extract these nutritional protein fragments. Extraction is the key step in the discovery of bioactive peptides. Therefore, the selection of a proper extraction method is crucial in ensuring the smooth progress of subsequent phases (i.e., fractionation, identification, and characterization). Enzyme-assisted extraction (EAE) is the main approach in the extraction of bioactive peptides. The principle of EAE is the incorporation of enzymes in extracting targeted bioactive peptides from proteins. Enzymes are globular proteins composed of one polypeptide chain that catalyzes the conversion of substrates into products without being altered or consumed in the reaction (Sowbhagya and Chitra, 2010). They cleave the peptide bonds in the proteins to release the encrypted peptides of various sizes and free amino acids (Wouters et al., 2016). It is therefore essential to understand enzymes and their modes of action for an effective and successful EAE.

Enzymes can be derived from sources such as plants (i.e., papain, bromelain, keratinases), animals (i.e., trypsin, chymotrypsin, pepsin, and rennin), and microorganisms (i.e., bacterial, fungal, viruses). The production of enzymes from plants and animal sources has

limitations, such as availability of land for cultivation, suitability of climatic conditions for growth, availability of livestock for slaughter, and the time-consuming nature of production, which lead to increased usage of microbial enzymes. Microbial enzymes are hence preferable compared to plant and animal enzymes due to their broad biochemical diversity, susceptibility to genetic manipulation, and desirable characteristics for biotechnological applications. In addition, enzymes from microbial sources, such as Alcalase and Flavourzyme, are reported to have a higher catalytic activity compared to enzymes from gastric and intestinal origins (Segura-Campos et al., 2013).

The mechanism that underlies an EAE involves the binding of the substrate to an enzyme followed by enzyme catalysis upon substrate binding. Every enzyme has its own specific active site (Sowbhagya and Chitra, 2010). This region consists of residues that form temporary bonds with the substrate (binding site) and residues that catalyze a reaction of that substrate (catalytic site). In the binding site, substrates are bound to the enzymes through hydrogen bonds, hydrophobic bonds, or Van der Waals interactions to form an enzyme-substrate complex. The enzyme-substrate complex has to be in a specific conformation to ensure the occurrence of hydrolysis reaction and also increase the efficiency of the reaction. There are two models proposed for the fitting of a specific substrate into an enzyme: (1) the lock and key model and (2) the induced fit model (Koshland, 1994). Emil Fischer's lock and key model hypothesized that the substrate fits perfectly into the active site of the enzyme without any modifications. Meanwhile, Daniel Koshland's induced fit model hypothesized that the enzyme alters the shape of its active site until the substrate is perfectly bound into it, which resulted in shape modifications.

Catalysis occurs once the substrate binds to the active site of the enzyme. The catalytic residues of the enzyme will cleave the peptide bonds of the proteins to release the encrypted peptides, which are dependent on the specificity of the enzyme. This specificity can be distinguished, depending on the site of the substrate that is cleaved. Enzymes can be categorized into endopeptidases, which cleave internal peptide bonds in the substrates, and exopeptidases, which cleave terminal peptide bonds. Exopeptidases are further subdivided into aminopeptidases and carboxypeptidases, depending on the peptide bond cleavage at the N-terminal or C-terminal peptide bond, respectively (Nigel, 2002). In addition, enzymes can be specified into aspartic proteases, cysteine proteases, serine proteases, or metalloproteases, depending on their catalytic mechanism (i.e., the nature of the amino acids at the active site) (Mótyán et al., 2013). According to Rao et al. (1998), aspartic proteases are characterized by aspartic residues for their catalytic activity. They exhibit maximal activity at low pH (pH 3–4) with isoelectric points ranging from pH 3–4.5. Meanwhile, cysteine proteases depend on a catalytic dyad comprising cysteine and histidine in their active site. Serine proteases are identified by the presence of a serine group in their active site and contain broad specificities, including esterolytic and amidase activity. Metalloproteases are characterized by the requirement for a divalent metal ion for their catalytic activity. The eponymous residue in the active sites of serine and cysteine proteases is usually paired with a proton-withdrawing group to promote nycleophilic attacks on the peptide bond. Meanwhile, aspartic proteases and metalloproteases react by activating a water molecule to serve as the nucleophile instead of using a functional group of the enzyme itself (Erez et al., 2009). By understanding the nature of these enzymes, it could bring benefits to EAE in the discovery of peptides.

The advantages of EAE include its environmentally friendly concept (i.e., reduced amount of synthetic and organic chemical usage), shorter operating time, higher yield, and more quality extracts (Azmir et al., 2013). Furthermore, EAE enables the extraction of targeted or desired peptide sequences accredited to the high specificity of the enzyme. EAE can readily be tested on the laboratory scale at any time and only requires a sufficient amount of the raw material and enzyme. It does not require any additional provisions such as pressure, carbon dioxide, or electric pulses. The practice of immobilizing enzymes would also make EAE more economical because it facilitates the separation of the enzyme from the product and allows it to be reused (Michalak et al., 2017). Puri et al. (2012) reviewed different kinds of extraction methods and recommended EAE as the preferred extraction method over chemical (i.e., solvents used such as acetone, hexane, toluene, benzene, and ethanol) and physical (i.e., supercritical carbon dioxide, pressurized hot water, microwave, and ultrasound) extraction methods. Both chemical and physical methods showed limitations such as pretreatment of raw material prior to extraction, a single usage of chemicals and solvents, expensive due to bulk consumption of solvents, lower quality and yield, and hazardous due to the production of hazardous waste (Puri et al., 2012).

EAE of food proteins has produced many therapeutically valuable peptides that are applicable to various industries. In the pharmaceutics and health industries, bioactive peptides can be made into therapeutic products depending on their activities. The beneficial health effects exerted by peptides can be categorized into four categories: the cardiovascular system (i.e., antihypertensive, antiinflammatory, antioxidant, and hypocholesterlaemic); intestinal health (i.e., modulatory peptides of mineral absorption, antiinflammatory, satiety-induced, and antidiabetic); body defenses (i.e., antimicrobial, immunomodulatory, and antiproliferative); and the nervous system (i.e., relaxing and antinociceptive) (Hernández-Ledesma et al., 2013). These peptides can be applied as drugs in combating human diseases. Besides that, they can be consumed as supplements to prevent the occurrence of diseases. In the food industry, bioactive peptides can be applied as food preservatives or food emulsifiers due to their desired functional properties such as solubility, water- and fat-holding capacities, gelation, foaming, and emulsification (Wouters et al., 2016). Besides that, they can be marketed as food based on health claims. For example, some of the commercialized food products containing bioactive peptides include Calpis, Peptide Soup, Casein DP Pepto Drink, CholesteBlock, Evolus, and ProDiet F200 (Kannan et al., 2012). Interestingly, EAE-derived bioactive peptides are applicable in the cosmetics industry as well (Agyei et al., 2016). They can be incorporated as natural preservatives in cosmetic products, accredited to their antimicrobial property. In addition, they can improve skin health and functions such as combating wrinkles and skin aging as well as provide antiinflammatory effects.

20.4 IDENTIFICATION OF BIOACTIVE PEPTIDES

The identification of bioactive peptides is a crucial issue among researchers nowadays. Generally, the bioactive peptide content from enzyme hydrolysate crude is less and owing to that factor, the process of identification and purification is considered challenging. There are a few classical approaches in identifying and purifying the bioactive peptide, such as reverse phase high performance chromatography (Lee et al., 2009), high-speed counter current

chromatography (Knight et al., 1995), and ion exchange chromatography. However, there are limiting factors in those methods. As for high-speed counter chromatography, the method cannot be implemented on unprotected peptides, owing to the nature of the amphoteric, even though it gives high selectivity in purifying organic compounds (Knight et al., 1995). Although reverse phase high performance chromatography is widely used in purifying the peptide, the application is restricted as the separation of the compound is based on molecular ion exchange or weight. Hence, the purified sample still contains a trace amount of nonbioactive compounds (Lan et al., 2014). In overcoming the issues, enzyme-assisted identification was created as an alternative way of purifying and identifying the bioactive peptide.

Enzyme-assisted identification is best explained as a technique that implemented the principle of affinity selection. Based on the theory, affinity selection is one of the techniques in purification. As reported by Lowe (1996), it is estimated that 60% of the purification techniques implied the fundamental of affinity purification and became a quintessential principle for investigating the interaction in biochemistry. Historically, the pioneering concept of affinity purification was discovered by pharmacologist Emil Starkenstein in the 20th century through his studies on the enzymatic activity of liver α-amylase with the influence of chloride. This research, which demonstrated biospecific adsorption of the enzyme onto the substrate, has opened the opportunity to other researchers to further improve the experimental design (Roque and Lowe, 2008). In such a case, Cuatrecasas et al. (1968) implemented the principle in the purification of staphylococcal nuclease, carboxypeptidase A, and α-chymotrypsin through immobilizing the substrate and inhibitor. The affinity purification can still be developed and further used in application, especially in investigating the mechanism of enzyme and molecular structure elucidation. Furthermore, the affinity purification has been enacted in the "omics" technologies, especially in proteomics technology. Numerous fusion tag partners or proteins that are prominent for bioprocess applications have been adopted in the affinity technique in proteomics, as it's a way to enhance development. Among them are maltose binding protein, glutathione-S-transferase, and polyArg.

Upon further development of affinity purification, affinity selection was developed for identification of small biocompounds from biomolecule targets (Annis et al., 2007). In establishing the study, Freije and Bischoff (2003) discovered the use of peptides as biomolecule targets in identifying the metalloprotease based on the activity. Conversely, another adaptation of the affinity separation principle that has been incrementally famous in compound identification is known as ligand fish; it was introduced by Van Breemen et al. (1998). This technique is considered s an adequate and favorable technique, especially in compound isolation through the fish out of the ligand from complex mixtures (Zhuo et al., 2016). The fundamental of these experiments is that the compound with an affinity will remain attached to the immobilized target while the remains will be eluted out. Magnetic beads are commonly used in enhancing the isolation technique upon its expeditious capability in retrieval (Zhuo et al., 2016). Essentially, magnetic beads were used in immobilizing the target (protein or enzyme) through covalent binding. The immobilized target was then introduced into the mixture, which allows the ligand (biomolecule) to have the binding interaction with the target through the receptor. Subsequently, the magnetic field was acquainted to the mixture in order to retain the beads. At the same time, the unbound compound will be discarded while the potent ligand will be eluted out for identification by using mass spectrometry analysis.

Over the years, diverse studies have stated the implication of the enzyme in identifying the natural compound as an inhibitor through the affinity separation principle technique. As established by Tao et al. (2013), they managed to discover a natural compound inhibitor from *Morus alba* by coating α-glucosidases with magnetic beads. Despite that, Li et al. (2014) managed to find a technique using magnetic nanoparticle beads with HPLC integration in identifying bi-flavonoid, which acts as an α-amylase inhibitor from *Garcinia xanthocymus*. Among the bi-flavonoids are GB2a glucoside, GB2a, and fukugetin. In addition, the flavonoid group was isolated from *Tang-Zhi-Qin* extract, a Chinese medicinal herb, through the immobilization of lipase with magnetic beads based on the affinity selection technique.

Enzyme-assisted identification is a technique that implied all the principles as mentioned hereinafter. Conversely, in our context of research, this technique was used in identifying the bioactive peptide through the enzyme and peptide interaction. Based on the fundamental theory, the reactive group of the bioactive peptide interacted with the active site-probe of specific enzymes via electrostatic, hydrogen bonding, hydrophilic, and hydrophobic interaction. Through the interaction study of the angiotensin-converting enzyme (ACE) and its three-dimensional structure, there are 19 amino acid residues and zinc ion. These play roles as an ACE active side in the catalytic mechanism, and are partially involved in the binding interaction with the inhibitor. Among the 19 amino acids are His353, Ala354, Ser355, Ala356, His383, Glu384, His387, Phe391, Pro407, His410, Glu411, Phe512, His513, Ser516, Ser517, Val518, Pro519, Arg522, and Tyr523 (Corradi et al., 2007; Pan et al., 2011). To establish the study of interaction, Pan et al. (2011) elucidated the molecular interaction and metal zinc ion coordination of milk-derived peptides (i.e., Ile-Pro-Ala, Phe-Pr,o and Gly-Lys-Pro) and ACE through molecular docking and X-ray crystallography structure. They discovered that the peptides interacted with four residue amino acids (i.e., Ala354, Ala356, Arg522, and Tyr523) through hydrogen bonding and electrostatic interaction. Moreover, they also stated that a tetrahedral geometry was formed via the coordination of zinc ions of the enzyme and carbonyl group of the peptide.

Additionally, Ashok and Aparna (2017) stated in their manuscript that bioactive peptides (i.e., Ile-Gln-, Lys-Val-, Ala-Gly-, and Thr-Trp) isolated from *Bubalus bubalis* mimicked the interaction of lisinopril (ACE synthetic drug inhibitor) with ACE through the molecular docking studies. Throughout their study, they discovered that tryptophan terminal and carboxyl oxygen of the C terminal of the peptide formed metal coordination with a zinc ion and the interaction of the electrostatic and hydrogen bond with Glu384, Ala356, and Tyr523 of ACE. These interaction studies indicated that the enzyme can be a potential protein target in isolating the peptide through the affinity separation technique. A previous study managed to identify a synthetic peptide through immobilized α-amylase with amine-nanoparticle magnetic beads (Siow et al., 2017). In an established study by Lan et al. (2014), they managed and isolated antihypertensive inhibitory peptides from the fraction of the protein hydrosylate (<5 kDa) through immobilized ACE with magnetic agarose beads; MALDI-TOF/TOF MS was employed in sequencing the bioactive peptide. Despite that, De Almeida et al. (2017) identified the peptide isolated from tryptic-digested BSA by using ligand fishing with the magnetic bead technique.

20.5 ADVANTAGES OF ENZYMATIC-ASSISTED APPROACHES

To date, enzymatic extraction is attracting great attention due to its possibility of recovering high content and quality of the end products. Enzymes can assist in the extraction of bioactive peptides in several ways. The use of carbohydrases in attacking the cell wall components may increase the peptide yield by releasing more peptide from the matrix source (Rosenthal et al., 2001). A combination of cell wall-degrading enzymes (e.g., protease, cellulase, and viscozyme L) has been reported to be effective in cleaving linkages within the polysaccharide matrix, which increases the cell wall permeability and releases more intracellular peptides from the protein source (Guan and Yao, 2008; Rosenthal et al., 2001). In recent years, different proteases, alone or in combinations, have been employed to liberate encrypted bioactive peptides from the native protein sequence via enzymatic hydrolysis (Korhonen and Pihlanto, 2003). Such proteolytic action causes change in the molecular conformation of native proteins, which, in turn, improves the functional and nutritional properties of the extracted peptides (Adebiyi et al., 2008). A study from Alemán et al. (2011) demonstrated that enzymatic hydrolysis could increase the antioxidative action of the resulting hydrolysate via the enhancement of the radical scavenging activity. In fact, the enzymatic actions potentially influence the molecular size, hydrophobicity, and polar groups of the hydrolysate (Kristinasson and Rasco, 2000). These alterations of the hydrolysate characteristics have a direct effect on their physicochemical and functional properties (Kristinasson and Rasco, 2000). Various bioactive peptides, including antihypertensive, antidiabetic, antioxidative, and antiobesity peptides, have been released from plant materials and marine organisms through enzymatic hydrolysis (Chai et al., 2017; Ngoh and Gan, 2016; Siow and Gan, 2013, 2016).

The enzymes have been reported for their exquisite specificity and regioselectivity in catalyzing reactions (Gardossi et al., 2009). This makes them an ideal catalyst in assisting extraction, modification, or synthesis of complex bioactives of natural origin. Most enzymes work in mild operating conditions, which require simple and widely available equipment. Typically, enzymatic reactions are carried out at low temperatures (15–45°C), which is particularly useful for the extractions of thermolabile compounds (e.g., protein and peptides). In addition, enzymes can accelerate a reaction by factors of at least a million more than that of the rate of the same reaction in the absence of the enzyme (Cooper, 2000). Therefore, enzyme-assisted methods can overcome the limitations of nonenzymatic methods that are generally complicated, time-consuming, and lacking specificity.

Nevertheless, all the aforementioned desirable attributes of enzymes and their large-scale applications are often hindered by their lack of shelf life, reusability, and long-term operational stability as well as by their tedious recovery process (Homaei et al., 2013). These limitations related to the use of free enzymes (i.e., in solution) can be generally overcome by the immobilization of enzymes. Compared to their free form, immobilized enzymes appear to be more stable, robust, and resistant to environmental changes as well as easier to handle. In addition, the immobilized techniques can largely prevent the contamination of either the bulk product or the prepared enzyme solution (Homaei et al., 2013). As in the case of a protease, the rate of its autolysis process can be decreased due to the improved stability and repetitive use upon immobilization (Massolini and Calleri, 2005). An added advantage of this method is that a rapid turnover rate can be obtained due to the increasing availability of the enzyme

to the substrate, enabling the reduction of the redundant downstream and purification processes (Datta et al., 2013). In short, enzyme immobilization provides excellent systems for an easy recovery of both enzymes and products with greater purity, automation, multiple reuse of enzymes, continuous economic operation, high investment/capacity ratio, rapid termination of reactions, and greater variety of bioreactor designs (Homaei et al., 2013). The use of immobilized enzymes in assisting extraction and identification of bioactive peptides could be a reliable method for fundamental research as well as an economically competent and efficient biotechnology for a vast number of industrial applications.

Due to its exceptional sensitivity and specificity, enzyme immobilization is also well suited for the screening of enzyme inhibitors. In other words, it offers a direct detection system on the basis of the enzyme-inhibitor interaction that could exploit the chemical specificity of the inhibitor and provide qualitative and quantitative measurement of the target analytes (Acker and Auld, 2014; Luque de Castro and Herrera, 2003). This method also provides a very sensitive platform for detecting a very low concentration of target analytes while, at the same time, reducing nonspecific background signals (Acker and Auld, 2014). Another merit of this approach is that it provides insights into identifying key modes of inhibitor binding (e.g., competitive, noncompetitive, and mix-mode inhibition) by assessing the strength and pattern of binding between an enzyme and its inhibitor (Acker and Auld, 2014). Collectively, these advantages of enzyme inhibition-based determination may enhance its use as a biochemical tool for screening and identifying small-molecule inhibitors (e.g., peptides) in a fast and reliable manner.

20.6 CURRENT TRENDS OF STUDY

The catalytic and hydrolytic functions of enzymes have contributed to most of their applications in various fields. Using an enzyme to screen/identify its potent ligand based on binding interactions has led to the establishment of novel methods of identification for completing new applications where enzymes were rare or not previously used. Enzymes can exhibit direct physical interactions with other molecules, including proteins, peptides, and substrates as well as small molecule ligands such as metabolites and drugs. Molecular recognition and specific binding of enzymes have provided the basis for the sensitive and efficient development of enzyme-assisted methods of screening and discovery of potential bioactive compounds. However, the downstream separation and purification of the end products as well as enormous variations in enzymatic activity remain the most challenging aspects of using enzymes as an identification-aided tool. Therefore, the use of molecular docking techniques (e.g., AutoDock, Pepsite2, and CABS-DOCK) to simulate the bindings and interactions between peptides and enzymes for evaluating the physiological effects of the peptides (Siow et al., 2017; Velarde-Salcedo et al., 2013; Zenezini Chiozzi et al., 2016) has become popular. It was expected that the integration of these bioinformative-driven tools to enzyme-assisted strategies for discovering bioactive peptides will continue to grow in importance.

In recent years, enzyme immobilization has been used for enhancing the enzyme activity and stability while reducing product inhibition, nonspecific adsorption, and microbial contamination (Singh, 2009). Recent developments involve the immobilization of enzymes onto magnetic nanoparticles (e.g., beads) via affinity interactions (Sassolas et al., 2013).

These magnetic beads provide a number of binding sites for biochemical reactions, leading to a fast and efficient method for quantitative measurement of the bindings. Immobilization of enzymes onto these beads (the bait) can be used to "pull down" a large quantity of enzyme-binding partners (the prey), offering exciting possibilities to be developed as important diagnostic and research tools. A growing trend in recent years has been the use of pull-down assays as purification methods to enrich the population for a particular protein, as confirmation tools to verify the existence of a previously predicted protein–protein interaction, and as research aids to identify unknown protein-protein interactions (Arifuzzaman et al., 2006; Jäger et al., 2011; Yadav et al., 2014). However, based on the literature, the degree of exploitation for their uses as discovery tools in assisting the identification process of bioactive peptides is still very low. One recent example of a useful application of the pull-down assay in a biopeptides study is by Siow et al. (2017). The authors used α-amylase-coated magnetic beads for pulling down the binding peptides with the purpose of confirming the existence of binding interactions as previously predicted by the phage-display method. Such binding characteristics were postulated as important evidence for their inhibitory actions against α-amylase, which is useful for the management of type-II diabetics (Siow et al., 2017). Two novel peptides (i.e., Arg-Cys-Met-Ala-Phe-Leu-Leu-Ser-Asp-Gly-Ala-Ala-Ala-Ala-Gln-Gln-Leu-Leu-Pro-Gln-Tyr-Trp and Asp-Pro-Ala-Gln-Pro-Asn-Tyr-Pro-Trp-Thr-Ala-Val-Leu-Val-Phe-Arg-His) that were identified by the pull-down assay have been experimentally verified for their α-amylase inhibitory potencies, indicating the accuracy, reliability, and specificity of the pull-down assay, which is well suited for biopeptide discovery. In addition, the physiological effects of bioactive peptides depend on their ability to reach the target; in most cases, this is associated with their binding characteristics. The pull-down technique provides a "bait-prey" model, which could be useful for the development of high-throughput screening via the detection of direct bindings between enzyme and peptide. The screening criteria can be established based on either high affinity (strong) or low affinity (weak) binding of peptides toward the targeted enzyme. Therefore, the pull-down technique could be the next wave of search strategies, particularly in the discovery of a promising peptide-based activator/inhibitor that has enzyme specificity.

20.7 CASE STUDY: AN OVERVIEW OF ANTIHYPERTENSIVE PEPTIDE DISCOVERY

20.7.1 Hypertension and the Related Enzymes

High blood pressure or hypertension is defined medically as repeatedly elevated blood pressure exceeding 140/90 mmHg (i.e., systolic pressure above 140 mmHg or diastolic pressure above 90 mmHg) (Achelrod et al., 2015). This cardiovascular disease is one of the leading causes of global mortality. According to the World Health Organization (2014), about 40% of the adults >25 years old suffer from this chronic disease. Approximately 9.4 million deaths were reported in 2010, and about 45% and 51% of these were due to heart disease and stroke, respectively.

In the aspect of physiology, the renin-angiotensin system (RAS) is responsible for regulating the blood pressure and fluid balance (Daien et al., 2012). Two major enzymes, namely

renin and ACE, play crucial roles in this system. In the kidney, renin, an aspartyl protease, is formed via the conversion of prorenin zymogen. It initiates the pathway by cleaving the 10 amino acids from the N-terminus of angiotensinogen and subsequently forms angiotensin I. The level of renin in the blood changes with the alteration of blood pressure and fluid balance. Therefore, the level of renin determines the overall activity of RAS (Sparks et al., 2014). Similarly, ACE, a dicarboxypeptidase, is ubiquitous in the bloodstream. The angiotensin I generated by renin is subsequently converted by ACE to vasoactive peptide angiotensin II via cleaving its dipeptide residue from the C-terminus. The presence of angiotensin II will then trigger the secretion of aldosterone from the adrenal glands, which results in an increase of salt and water resorption as well as blood pressure by constricting the artery (Chen et al., 2009). Apart from that, ACE is able to degrade bradykinin, which exhibits a vasodilator property, into an inactive peptide (Margolius, 1996).

It should also be noted that another enzyme—namely the endothelin-converting enzyme (ECE), which is a specific phosphoramidon-sensitive metalloprotease—plays an essential role in regulating blood pressure. It cleaves big endothelins (i.e., 37- to 41-amino-acid peptide) and forms endothelin-1 (ET1, a 21-amino-acid peptide with a hydrophobic C terminus and two cysteine bridges at the N terminus), endothelin-2 (ET2, a 2-amino-acid peptide), or endothelin-3 (ET3, a 6-amino-acid peptide). Similar to ACE, ECE is also able to hydrolyze bradkynin. Therefore, this endothelial system induces different physiological effects. For example, it plays a vasoconstricting role in the blood vessels and causes the development of hypertension. It also monitors the tone of airways and blood vessels in the lungs and thus develops pulmonary hypertension (D'Orleans-Juste et al., 2003).

20.7.2 Inhibition of Enzyme Targets as the Treatment for Hypertension

20.7.2.1 ACE Inhibition

According to Udenigwe and Mohan (2014), ACE inhibition may not be fully accountable for reducing the blood pressure because angiotensin II could be generated in a chymase-catalyzed reaction, which is an ACE-independent pathway. However, a huge amount of antihypertensive peptides were investigated for their ACE inhibitory activity. For example, two purified ACE inhibitory peptides (i.e., Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro and Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg) were extracted from the skate skin with the IC_{50} values of 95 μM and 148 μM (Lee et al., 2011).

Further study using docking analysis showed that Ala354, Glu384, and Tyr523 in region S1; Gln281, His353, Lys511, His513, and Tyr520 in region S2; and Glu162 in region S1′ were the active sites of ACE (Rohit et al., 2012). Lisinopril, a commercial ACE inhibitor, was reported to bind at similar active sites, such as His353, Ala354, His383, Glu384, His387, Glu411, Lys511, and Tyr520 (Wang et al., 2011). If these regions were bound by the bioactive peptide, the inhibition of ACE activity would occur (Wu et al., 2016). Therefore, these active sites were highlighted as the indicator for the ACE inhibitors in the design of antihypertensive drugs.

An In vivo study was also conducted to validate the efficacy of the ACE inhibitors as antihypertensive drugs. It was reported that the C-domain of ACE is the predominant site that is responsible for the cleavage of angiotensin I (Wei et al., 1991). Therefore, it was suggested that using the selective inhibitor could reduce the blood pressure in the in vivo study (Georgiadis et al., 2003). This theory was supported by the studies that showed that the administration of

these drugs could induce the hypotensive effect in rats. The researchers reported that the oral administration of a hexapeptide (Ac-Arg-Lys-Trp-His-Phe-Trp-NH_2) and two heptapeptides (Ac-Arg-Lys-Trp-His-Phe-Leu-Trp-NH_2 and Ac-Arg-Lys-Trp-Leu-Phe-His-Trp-NH_2) successfully induced a reduction in systolic blood pressure for 3 h, whereas intravenous injection of these peptides managed to induce acute transient reductions in the mean blood pressure of spontaneously hypertensive rats (Ruiz-Giménez et al., 2011).

20.7.2.2 Renin Inhibition

The advantage of using a renin inhibitor is that the enzyme has a unique specificity for angiotensinogen and hence, the inhibitor does not interfere with other renin-like enzymes, such as cathepsin D and tonins. Therefore, other metabolic pathways are not disrupted. The development of such a drug was started with peptide analogues as the first generation renin inhibitors, followed by peptide mimetics as the second generation inhibitors (Gradman and Kad, 2008). According to researchers, both drugs were tested for their mechanical and hemodynamic effects in both animals and humans. Examples of these inhibitors are CGP29287, CGP38560A, enalkiren, remikiren, and zankiren (Staessen et al., 2006). Recent developments have shown that food-derived peptides also exhibited the same function. Five peptides present in the pea protein hydrolysate were found to significantly inhibit renin activities. Leu-Thr-Phe-Pro-Gly showed a higher decrease in systolic blood pressure after 2 h of administration whereas Ile-Phe-Glu-Asn-Leu-Gln-Asn and Phe-Glu-Gly-Thr-Val-Phe-Glu-Asn-Gly gave maximum effects after 4 h (Aluko et al., 2015).

The active sites of human renin that interacted with inhibitors were identified through molecular modeling analysis. S1 and S3 pockets that form a large hydrophobic cavity enhanced the van der Waals interaction with inhibitors. The $S3^{sp}$ subpocket was also surprisingly found to contribute to a higher potency and affinity (Politi et al., 2010; Rahuel et al., 2000). In addition, the specific residues of Tyr14, Asp32, Gly34, Arg74, Thr77, Ser76, Asp215, Gly217, and Ser219 in renin were reported to be bound to the inhibitors via hydrogen bonding (Rahuel et al., 2000).

20.7.2.3 ECE Inhibition

The hyperactivation of the endothelin system has been reported to be responsible for hypertension (D'Orleans-Juste et al., 2003). Considering the evidence that a systematic administration of ECE inhibitors could block the biologically active endothelin actions, there is a growing interest in developing ECE inhibitors. Fernández-Musoles et al. (2010) reported that eight lactoferricin B-derived peptides exhibited an ECE inhibitory effect using big endothelin as the substrate. They also reported that there was a positive correlation with the inhibitory effect on the ECE-dependent vasoconstriction.

20.7.3 Enzyme-Assisted Extraction of Antihypertensive Peptides

Bioactive peptides are required to be released from the parent protein in order to exhibit their biological activities. Among the processes, enzymatic hydrolysis is the most well-accepted approach due to its selectivity and specificity (Korhonen and Pihlanto, 2006). There were a number of peptides derived using different enzymes and protein sources that are known to exhibit the hypotensive effect. From Table 20.2, it could be observed that alcalase is one of the most commonly used enzymes for the production of ACE-inhibitor peptides.

TABLE 20.2 Antihypertensive Peptides Extracted Using Different Enzymes and Sources

Enzyme	Source	Peptide Sequence	Reference
Alcalase	Hemoglobin	Phe-Gln-Lys-Val-Val-Ala Phe-Gln-Lys-VaI-Val-Ala-Gly Phe-Gln-Lys-Val-Val-Ala-Lys Gly-Lys-Lys-Val-Leu-Gln	Mito et al. (1996)
	Peanut	Lys-Leu-Tyr-Met-Arg-Pro	Shi et al. (2014)
	Snakehead fish	Leu-Tyr-Pro-Pro-Pro Tyr-Ser-Met-Tyr-Pro-Pro	Ghassem et al. (2014)
	Corn	Ala-Tyr	Yang et al. (2007)
	Rice	Val-Tyr	Li et al. (2007)
	Soy Protein	Asp-Gly Asp-Leu-Pro	Li et al. (2004)
	Wheat Germ	Ile-Val-Tyr	Li et al. (2002)
	Sweet sorghum grain	Thr-Leu-Ser	Wu et al. (2016)
	Mung bean	Lys-Asp-Tyr-Arg-Leu Val-Thr-Pro-Ala-Leu-Arg Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe	Li et al. (2006)
	Green algae (*Enteromorpha* spp.)	Pro-Ala-Phe-Gly	Pan et al. (2016)
	Seaweed pipefish (*Syngnathus schlegeli*)	Thr-Phe-Pro-His-Gly-Pro His-Trp-Thr-Gln-Arg	Wijesekara et al. (2011)
Trypsin	Bovine β-lactoglobulin	Ala-Leu-Pro-Met-His-Ile-Arg	Vermeirssen et al. (2002)
	Bovine α_{S2}-casein	Phe-Pro-Gln-Tyr-Leu-Gln-Tyr Phe-Ala-Leu-Pro-Gln-Tyr Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys Thr-Val-Tyr	Tauzin et al. (2002)
	Rice bran protein	Tyr-Ser-Lys	Wang et al. (2017)
	Rice protein hydrolysate	Val-Asn-Pro Val-Trp-Pro	Chen et al. (2013)
	Yeast protein hydrolysate	Tyr-Gly-Lys-Pro-Val-Ala-Val-Pro-Ala-Arg	Mirzaei et al. (2015)
	Milk (whey)	Ala-Leu-Pro-Met-His-Ile-Arg	Ruiz et al. (2004)
	Cauliflower by-products	Ala-Ala-Gly-Gly-Phe-Gly-Gly-Leu-Arg Ala-Gly-Gln-Ala-Ala-Phe-Gly-Asn-Met-Cys-Arg Phe-Phe-Ala-Pro-Tyr-Ala-Pro-Asn-Phe-Pro-Phe-Lys Gly-Gly-Pro-Val-Pro-Ala-Pro-Cys-Cys-Ala-Gly-Val-Ser-Lys Ile-Leu-Tyr-Asp-Phe-Cys-Phe-Leu-Arg Met-Leu-Asp-Phe-Asp-Phe-Leu-Cys-Gly-Arg	Chiozzi et al. (2016)

TABLE 20.2 Antihypertensive Peptides Extracted Using Different Enzymes and Sources—cont'd

Enzyme	Source	Peptide Sequence	Reference
Pepsin	Porcine skeletal myosin B	Lys-Arg-Val-Ile-Gln-Try Val-Lys-Ala-Gly-Phe	Muguruma et al. (2009)
	Pacific cod skin gelatin	Gly-Ala-Ser-Ser-Gly-Met-Pro-Gly Leu-Ala-Tyr-Ala	Ngo et al. (2016)
	Porcine skeletal muscle troponin	Lys-Arg-Gln-Lys-Tyr-Asp-Ile	Katayama et al. (2008)
	Krill (*Euphausia superba*)	Ile-Pro-Ile-Lys	Park et al. (2016)
	Algae protein waste	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe	Sheih et al. (2009)
Alkaline Proteases	Milk (α-casein)	Tyr-Lys-Val-Pro-Gln-Leu Thr-Val-Tyr Phe-Phe-Val-Ala-Pro	Guan et al. (2009)
	Sea bream scales	Gly-Tyr, Val-Tyr Gly-Phe Val-Ile-Tyr	Fahmi et al. (2004)
	Sardine	Ala-Lys-Lys Gly-Trp-Ala-Pro Lys-Trp	Meisel and Walsh (2006)
Thermolysin	β-lactoglobulin	Leu-Asp-Ala Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp Leu-Gln-Lys-Trp	Herńandez-Ledesma et al. (2006)
	Bitter melon seed	Val-Ser-Gly-Ala-Gly-Arg-Tyr Val-Asp-Ser-Asp-Val-Val-Lys-Gly	Priyanto et al. (2015)
Subtilisin	Rapeseed protein	Ile-Tyr Arg-Ile-Tyr Val-Trp Val-Trp-Ile-Ser	Marczak et al. (2003)
	Cupuassu seed	Met-Val-Val-Asp-Lys-Leu-Phe Leu-Asp-Asn-Lys Phe-Leu-Glu-Lys Met-Glu-Lys-His-Ser Gly-Ser-Gly-Lys-His-Val-Ser-Pro	Cruz et al. (2016)
Neutrase	Corn gluten meal proteins	Not identified	Zhou et al. (2013)
Chymotrypsin	Sorghum grains	Not identified	Kamath et al. (2007)
Neutral protease AS1398	Grass carp fish scale	Not identified	Zhang et al. (2009)
Flavourzyme	Defibrinated bovine plasma	Gly-Tyr-Pro His-Pro-Tyr His-Pro-Gly-His Ser-Pro-Tyr Tyr-Pro-His	Wanasundara et al. (2002)

Continued

TABLE 20.2 Antihypertensive Peptides Extracted Using Different Enzymes and Sources—cont'd

Enzyme	Source	Peptide Sequence	Reference
Papain	Buffalo milk	Phe-Pro-Gly-Pro-Ile-Pro-Lys Ile-Val-Pro-Asn Ile-Pro-Pro-Lys Gln-Pro-Pro-Gln	Abdel-Hamid et al. (2017)
SK1–3-7 proteinases	Tilapia	Met-Ile-Leu-Leu-Leu-Phe-Arg Leu-Asn-Leu-Gln-Asp-Phe-Arg Leu-Asn-Leu-Gln-Asp-Phe	Toopcham et al. (2015)
Crude bacterial proteases	Goby (*Zosterisessor ophiocephalus*) muscle proteins	Ala-Arg-Ser Val-Val-Ala-Pro-Phe-Ala-His-Gly-Thr Arg-Ser-Thr-Ala Phe-Tyr-Pro-Pro Arg-Cys-Ser-Ala-Gly-Val	Nasri et al. (2013)
Alcalase and flavourzyme	Sunflower protein	Not identified	Megías et al. (2009)
Alcalase, pepsin, and Pancreatin	Defatted raw and roasted peanut flour	Not identified	Quist et al. (2009)
Alcalase, α-chymotrypsin, neutrase, pepsin, papain, and trypsin	Skate skin hydrolysate	Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg	Lee et al. (2011)
Trypsin, α-chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain, and pepsin	Porcine skeletal muscle proteins	Met-Asn-Pro-Pro-Lys Ile-Thr-Thr-Asn-Pro Met-Asn-Pro Asn-Pro-Pro Pro-Pro-Lys Ile-Thr-Thr Thr-Thr-Asn Thr-Asn-Pro	Arihara et al. (2001)
Acid proteinase from *Monascus purpureus*	Soybean proteins, β-conglycinin and glycinin	Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro Leu-Pro-His-Phe Ser-Pro-Tyr-Pro Trp-Leu	Kuba et al. (2005)
Pepsin and papain	Box jellyfish (*Chiropsalmus quadrigatus*) Haeckel venom hydrolysate	Ala-Cys-Pro-Gly-Pro-Asn-Pro-Gly-Arg-Pro	So et al. (2016)
Pepsin and pancreatin	Human milk and infant formula	His-Leu-Pro-Leu-Pro Trp-Ser-Val-Pro-Gln-Pro-Lys	Hernández-Ledesma et al. (2007)
Pepsin, papain, and alcalase	Sesame seed protein	Tyr-His-Pro-Ser-Pro-Arg Asn-Asn-Tyr-Asn-Pro-Arg Gly-Gly-Trp-Asn-Gly-Pro-Gly-Pro-Lys Leu-Val-His-Phe-Val-Leu-Lys-Lys Ile-Thr-Arg-Asn-Val-Pro-Val-Arg-Leu Lys-Met-Ser-Glu-His-Ile-Leu-Leu-Leu Val-Gly-Asn-Asn-Thr-Arg-Val-Met-Cys-Arg	Chatterjee et al. (2015)

TABLE 20.2 Antihypertensive Peptides Extracted Using Different Enzymes and Sources—cont'd

Enzyme	Source	Peptide Sequence	Reference
Pepsin and papain	Jellyfish (*Rhopilema esculentum*) protein hydrolysates	Gln-Pro-Gly-Pro-Thr Gly-Asp-Ile-Gly-Tyr	Liu et al. (2013)
Alcalase, neutrase, papain, trypsin, pepsin, and α-chymotrypsin	Pacific cod (*Gadus macrocephalus*) skin gelatin	Thr-Cys-Ser-Pro Thr-Gly-Gly-Gly-Asn-Val	Ngoa et al. (2011)
Alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin	Chum salmon (*Oncorhynchus keta*) skin	Gly-Leu-Pro-Leu-Asn-Leu-Pro	Lee et al. (2014)
Bromelain and alcalase	Sea cucumber (*Acaudina molpadioidea*)	Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp	Zhao et al. (2009)
Pepsin, trypsin and α-chymotrypsin	Silkworm pupa (*Bombyx mori*) protein	Ala-Ser-Leu	Wu et al. (2015)
Alcalase, thermolysin, pepsin, trypsin, and α-chymotrypsin	Larvae of the cotton leafworm (*Spodoptera littoralis*)	Not identified	Vercruysse et al. (2009)
Compound proteinase AQ	Jellyfish	Val-Gly-Pro-Tyr Phe-Thr-Tyr-Val-Pro-Gly Phe-Thr-Tyr-Val-Pro-Gly-Ala Phe-Gln-Ala-Val-Trp-Ala-Gly	Liu et al. (2015)
Alcalase and N120P	Peanut	Lys-Leu-Tyr-Met-Arg-Pro	Shi et al. (2014)
Pepsin and trypsin	Pistachio	Ala-Cys-Lys-Glu-Pro	Li et al. (2014)
Pepsin and Bromelain	Green seaweed (*Ulva rigida C. Agardh*)	Ile-Pro Ala-Phe-Leu	Paiva et al. (2016)
Pepsin and Pancreatin	Ostrich (*Struthio camelus*) egg white protein	Trp-Glu-Ser-Leu-Ser-Arg-Leu-Leu-Gly	Asoodeh et al. (2016)
Pepsin and Corolase	Manchego cheese	Val-Arg-Tyr-Leu	Ruiz et al. (2004)
Flavourzyme and Protamex	Kacang goat (*Capra aegagrus hircus*)	Phe-Gln-Pro-Ser	Mirdhayati et al. (2016)

For example, Wu et al. (2016) hydrolyzed sweet sorghum grain protein in different protein (10–30 g/L) and enzyme (0.4–1.0 g/L) concentrations using the same enzyme at the hydrolysis temperature of 50°C and pH 8.0. This hydrolysis process yielded a novel peptide, Thr-Leu-Ser, with an IC_{50} value of 102.1 μM of ACE inhibitory activity. Trypsin is another popular choice of enzyme. A recent publication reported that rice bran protein could be hydrolyzed under the conditions of hydrolysis temperature of 37°C, pH 8, and an enzyme-to-substrate ratio of 1500 U/mg in 2 h. This condition generated Tyr-Ser-Lys, which exhibited ACE inhibitory activity with an IC_{50} value of 75.95 μM (Wang et al., 2017). Apart from that, a mixture

of enzymes or crude enzymes could also be used. For example, crude proteinases extracted from *Virgibacillus halodenitrificans* were used to generate ACE-inhibitory peptides from tilapia muscle proteins (Toopcham et al., 2015). The hydrolysate was reported to generate peptides, in particular, Met-Ile-Leu-Leu-Leu-Phe-Arg, Leu-Asn-Leu-Gln-Asp-Phe-Arg, and Leu-Asn-Leu-Gln-Asp-Phe, which exhibited the highest inhibition with an IC_{50} value of 0.29 μM. Other common examples of enzymes are listed in Table 20.2.

The above-mentioned enzymatic hydrolysis was often conducted using the in-solution approach, where a free enzyme was used. However, this traditional approach was reported to have several drawbacks, such as sample contamination by the presence of enzymes, enzyme autolysis, and being unsuitable for a continuous process (Ruan et al., 2014). Enzyme immobilization was suggested in order to improve stability and recyclability (Agyei and He, 2015). One of the examples is the work of Rocha et al. (2011), which established a protocol to immobilize trypsin on spent grains via adsorption and covalent attachment. The researchers reported that immobilization via the multipoint covalent attachment showed a good storage and operational stability with a value of >90%, which allowed the use of the enzyme at a broader range of temperatures. In addition, no significant differences were found in the generated bioactive peptides between the free and the immobilized enzymes.

20.7.4 Identification of an Anti-ACE Peptide Using Enzymes

In general, the identification of bioactive peptides has to go through a series of procedures. Wang et al. (2017) demonstrated the fractionation using ultrafiltration with different MW cutoff values. From the reported results, the fraction (<4 kDa) that portrayed the highest activity (IC_{50} = 300 μg/mL) was chosen for further purification. Following the purification procedure using gel filtration chromatography, Fraction 2 exhibited the highest activity with an IC_{50} value of 32 μg/mL. Therefore, it was subjected for subsequent purification using reversed-phased high performance liquid chromatography, which gave the highest activity in Fraction 2A. This purified peptide was then subjected for amino acid sequencing using the mass spectrometry approach, where Tyr-Ser-Lys was identified. There was a concern in this conventional approach because of the low yield of the peptides after this extensive process; it was also very time consuming (Udenigwe, 2014). Therefore, an alternative approach was suggested.

As mentioned in Section 20.4, the ligand binding assay, where the interaction of the targeted enzyme (e.g., ACE) and the bioactive peptide took place, is a form of affinity purification. It is therefore proposed that bioactive peptides could be identified. In this approach, the bait protein will be the ACE, renin, or ECE, which will be immobilized/captured. The interacting peptides will then come into contact with the enzyme during incubation with the hydrolysate. From this point of view, those interacting peptides could be the potential inhibitors of the targeted enzyme. The elution of the peptides in the latter procedure will then allow us to screen and identify the peptides. De Almeida et al. (2017) used a similar approach with immobilized ACE as the bait protein onto magnetic beads in order to "fish out" the inhibitor, including peptide inhibitors from a pool of tryptic-digested bovine serum albumin. Due to the high specificity of the interaction, this approach can be used in detecting inhibitor peptides.

20.8 CONCLUSION

In the pharmaceutical or food sectors, a conventional approach in bioactive peptide discovery usually involves the screening and selection of peptides. This chapter has highlighted the prospect of enzymes as a useful medium for discovery and identification of bioactive peptides. The mechanism of disease curing or prevention could be predicted using an in silico study based on the interaction of the enzyme model and peptides. The enzyme could be used as a ligand that could assist in "fishing out" the resultant peptides. An in vitro study using an enzyme inhibition assay would confirm the potential biological activity. Bioavailability and safety evaluation should then be performed, followed by cell-line, animal, and human studies prior to regulatory approval. This would accelerate the process for the design of peptides with targeted biological activity.

Acknowledgments

We would like to acknowledge Universiti Sains Malaysia RUI Grant (1001/CAATS/814257) and MyBrain scholarship in supporting the related research activities.

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CHAPTER

21

Transglutaminase Cross-Linked Edible Films and Coatings for Food Applications

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21.1 INTRODUCTION

Transglutaminases (EC 2.3.2.13, TGase) are a family of intra- and extracellular enzymes that are able to catalyze the posttranslational modification of proteins by introducing isopeptide bonds, which are highly resistant toward enzymatic proteolysis and mechanical stress, between *endo*-protein Lys ε-amino (acyl acceptor substrate) and Gln γ-carboxamide (acyl donor substrate) groups. The resulting reaction products range from peptides and polypeptides containing either intramolecular cross-links (Fig. 21.1A) or intermolecular cross-links, the latter classified as linear (Fig. 21.1B) or branched homo- (Fig. 21.1C) and heteropolymers (Fig. 21.1D), respectively. Free ammonia derived from γ-carboxamide group is released during the reaction. Although protein cross-linking is the reaction that has always drawn the greatest attention, the significance of TGase-mediated structural modifications by primary amine or polyamine covalent incorporation into polypeptides is also well documented. In addition, TGase is able to catalyze Gln deamidation in the absence of free amines, with water acting as an acyl acceptor (Eckert et al., 2014; Kuraishi et al., 2001; Motoki and Seguro, 1998). Therefore, all the reactions catalyzed by this enzyme result in significant changes, not only of the structure but also of the physical and chemical characteristics of the proteins acting as substrates, such as viscosity, thermal stability, and elasticity (Kieliszek and Misiewicz, 2014).

https://doi.org/10.1016/B978-0-12-813280-7.00021-9

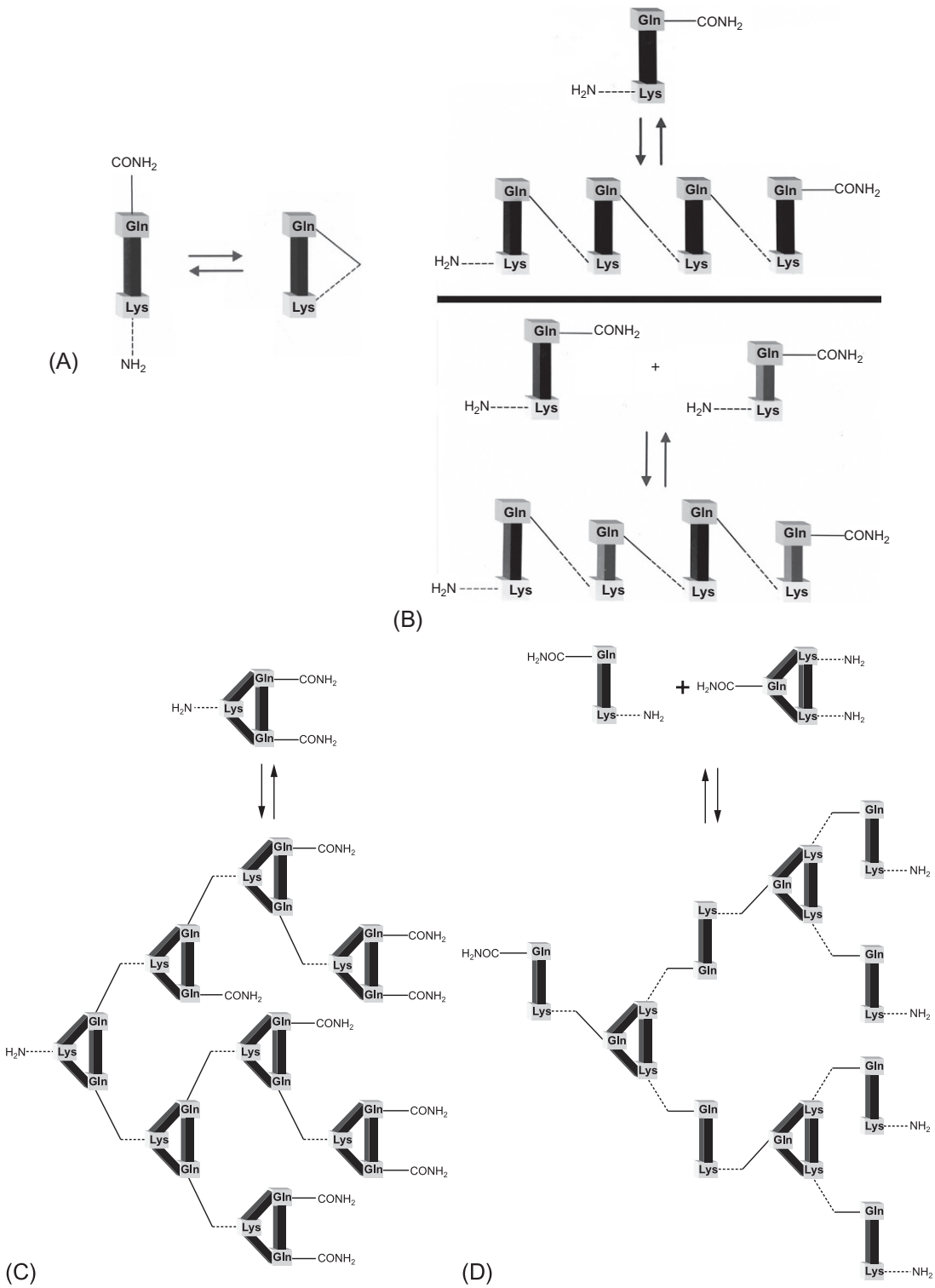

FIG. 21.1 (A) TGase-catalyzed intramolecular cross-link; (B) TGase-catalyzed formation of linear homo- (upper) and hetero- (under) polymers by intermolecular cross-links; TGase-catalyzed formation of branched homo- (C) and hetero- (D) polymers by intermolecular cross-links.

TGases are widespread in nature (Kashiwagi et al., 2002). Since 1959 discovery of a transamidating activity in guinea pig liver extracts due to the most widely distributed and extensively studied molecular form of the enzyme, afterwards designated as TGase2, several additional proteins exhibiting the same catalytic activity were identified in unicellular, invertebrate, and vertebrate organisms as well as in plants (Griffin et al., 2002; Yasueda et al., 1994; Yu et al., 2008). Nine TGase genes have been demonstrated in humans, eight of them able to give rise to catalytically active proteins (the best known is the blood coagulation Factor XIII responsible for fibrin clot stabilization), whereas that relative to the erythrocyte membrane protein band 4.2 leads to an enzymatically inactive product. The first TGase primary structure established was that of Factor XIII (Ichinose et al., 1990) and only a faint sequence homology was then observed among the different enzyme molecular forms. However, all the multiple forms of TGase share an identical sequence at the level of the active site, that is, a catalytic triad of cysteine, histidine, and aspartate (Cys-314/His-373/Asp-396 and Cys-276/His-334/Asp-358 in Factor XIII and TGase2, respectively), with a proceeding of the reaction through an intermediate product linked to the Cys–SH group and where hydrogen bonds between Asp and His residues contribute to maintain a catalytic orientation of the protein (Strop, 2014). Six mammalian TGases with a total mass ranging from 77 to 90 kDa have been well characterized. All of them show structures consisting of a β-sandwich core domain, which contains both active and regulatory sites, and two C- terminal β-barrels (Strop, 2014).

In higher organisms, TGases have been suggested or demonstrated to play different roles by modifying different proteins in different organs (Eckert et al., 2014; Lorand and Graham, 2003). Other than Factor XIII, involved in blood coagulation, TGase2 has been proposed to have a function in cellular differentiation, tissue stabilization, and apoptosis as well as to promote cell adhesion, whereas keratinocyte and epidermal enzymes cross-link proteins on the outer surface of the squamous epithelium. In addition, some molecular forms of the enzyme have also been suggested to be involved in additional specific human physiological as well as pathological conditions, including immunosuppression, neurodegeneration, and coeliac and neoplastic diseases, so as to be considered as potential therapeutic targets (Eckert et al., 2014; Paonessa et al., 1984; Peluso et al., 1994). Finally, the eukaryotic enzyme is regulated by guanosine-5′-triphosphate and requires calcium concentrations above physiological conditions to be catalytically active. Therefore, its reaction products are formed only under conditions disrupting cellular homeostasis, such as those occurring during blood coagulation and wound healing (Eckert et al., 2014). In contrast to the extensively studied mammalian TGases, there have been so far limited studies on the presence and role played by such enzyme in plants, where it was first detected in pea seedlings (Icekson and Apelbaum, 1987). It then was showed in further tissues, from soy to topinambour, from fodder beet to apple, and was immunodetected in the protein extracts of maize meristematic calli and their isolated chloroplasts (Campos et al., 2010; Falcone et al., 1993; Luciano and Arntfield, 2012; Sobieszczuk-Nowicka et al., 2008). As far as the occurrence of TGase in unicellular organisms, it was investigated by screening different microorganisms with the aim mainly to have available an inexpensive and stable source of the enzyme for possible biotechnological applications (Kieliszek and Misiewicz, 2014; Rachel and Pelletier, 2013; Strop, 2014). The enzyme, isolated from *Streptoverticillium* sp. and *Physarum polycephalum*, has also been detected in *Bacillus subtilis* spores and, as an extracellular molecular form, it was shown to be produced by *Streptoverticillium cinnamoneum* subsp., *Streptoverticillium griseocarneum*, *Streptoverticillium*

ladakanum, *Streptomyces netropsis*, and *Streptomyces lydicus* (Aidaroos et al., 2011; Duran et al., 1998; Færgemand and Qvist, 1997; Gerber et al., 1994; Ho et al., 2000; Yu et al., 2008). Although microbial TGase (mTGase) catalyzes the same reaction catalyzed by the other molecular forms of the enzyme, it does not share with them a sequence or structural homology, is not regulated by calcium or guanosine-5′-triphosphate, and has a broader substrate specificity and pH dependence (Kieliszek and Misiewicz, 2014). In fact, mTGase, the isoelectric point of which is 8.9, is active over a wide range of pH with an optimum value between 5 and 8. Moreover, the enzyme is active at high temperatures up to 70°C with an optimum at 55°C, although at this temperature it loses catalytic activity over time (Kieliszek and Misiewicz, 2014; Jaros et al., 2006; Yokoyama et al., 2004). mTGase (38 kDa, a single polypeptide chain of 331 amino acids) has a globular structure determined by 11 α-helices and eight β-strands, with the active site located in a central pocket composed of five acidic residues (Asp-3, -4, -255 and Glu-249, -300) and numerous aromatic residues (Tyr-62, -75, -278, -291, -302 and Trp-59, -69) (Kashiwagi et al., 2002; Yokoyama et al., 2004). A Cys protease-like mechanism of reaction, in which Asp-255 plays the role of His residue in Factor XIII and TGase2, has been hypothesized for mTGase, with the formation of a tetrahedral enzyme/substrate complex following the thioester binding between Cys-64 and the Gln γ-carboxamide carbonyl group of the protein acyl donor substrate. Then, a proton is donated by Asp-255 to the oxyanion hole and an enzyme-bound thioamide is formed with the release of ammonia. Finally, an acyl acceptor substrate (either an endoprotein Lys or a free primary amine) attacks the thioamide carbonyl group, facilitated by Asp-255 deprotonation, and the cross-linked or aminated protein is released while the enzyme is regenerated in its native form (Kashiwagi et al., 2002; Yokoyama et al., 2004). mTGase specificity toward the acyl donor substrates is determined by a combination of primary and secondary structure of the protein domain containing the reactive Gln residue, as well as by protein substrate flexibility around its reactive γ-carboxamide group (Strop, 2014). Conversely, and similarly to the other TGases, minor structural requirements have been demonstrated for the reactive sites of acyl acceptor substrates. However, mTGase molecular mass, which is about half with respect to the one of its counterparts, the single domain and the absence of sequence homology with the other enzyme molecular forms suggest that mTGase is probably a product of convergent evolution (Ando et al., 1989; Kanaji et al., 1993; Jaros et al., 2006). The only feature similar to that of all TGases is its obvious posttranslational activation because undesired protein cross-linking might be harmful to both unicellular and multicellular organisms. Finally, the biological function of mTGase still remains largely unknown, even though it has been hypothesized that in *Streptomyces mobaraensis* the enzyme might play the role to cross-link inhibitory proteins participating in the formation of an antibiotic shield against host proteases (Sarafeddinov et al., 2011).

Applied research carried out up to now with TGase involved almost exclusively two molecular forms of the enzyme, i.e., TGase2 and mTGase. These two forms have been extensively studied both in academia and industry but, while the interest in TGase2 started from a medical perspective to better understand the role of the enzyme in various diseases, the microbial molecular form of the enzyme encountered great attention in many different food applications (Góes-Favoni and Bueno, 2014; Kieliszek and Misiewicz, 2014; Kuraishi et al., 2001; Mariniello and Porta, 2005; Mariniello et al., 2008; Motoki and Seguro, 1998; Motoki and Kumazawa, 2000; Rachel and Pelletier, 2013). In fact, mTGase, considered to be "generally recognized as safe" (GRAS) for human intake, has been widely proposed as a processing aid

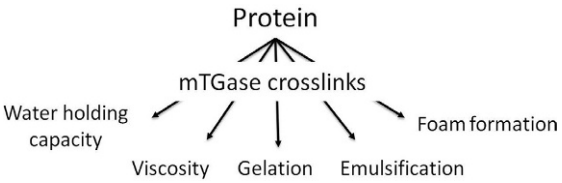

FIG. 21.2 Main functional properties of proteins of food interest influenced by mTGase.

for modifying the functional properties of many different food products. With proteins being one of the main classes of food components, their structural modification via chemical, physical, or enzymatic methods is an alternative available for the improvement and/or development of new functional properties of specific protein-based foods. In view of the considerable interest of the food industry in using enzymes due to their specificity, mild reaction conditions, and lower risk of formation of toxic products (Özrenk, 2006), gaining an understanding of their effects and mechanism of action in the modification of the functional properties of proteins is the first step in promoting their industrial use. On the other hand, according to the requirements of Article 6.4 (c) of Directive 2000/13/EC and Article 20 (b) of Regulation (EU) N. 1169/2011, food enzymes used as processing aids are not required to be declared in the list of ingredients on prepacked food. Therefore, many attempts were performed with the aim to improve the physical and biological properties of food proteins by mTGase (Fig. 21.2) because a variety of them have been shown to act as effective acyl donor and/or acceptor substrates for the enzyme (Bönisch et al., 2006; Giosafatto et al., 2012; Hong and Xiong, 2012; Mariniello and Porta, 2005, Mariniello et al., 2007, 1993, 2014; Porta et al., 2011a).

21.2 TRANSGLUTAMINASE AND FOOD EDIBLE COATINGS

The current scenario of plastic pollution shows that >700 thousand plastic shopping bags and 400 thousand plastic bottles are consumed worldwide every minute. Consequently, about 35 million tons of plastic waste are annually produced in the entire world while only 7% of that is recycled (Sabbah and Porta, 2017). One possible solution to reduce the pollution due to plastics of petrochemical origin is their increasing replacement with biodegradable materials derived from aliphatic polyesters (e.g., polylactates and polyhydroxyalkanoates) and/or natural biopolymers such as polysaccharides and proteins (Gómez-Estaca et al., 2016; Pathak et al., 2014; Song and Zheng, 2014). A great number of agroresources rich in proteins, such as soybeans (38%–44%), sunflower seeds (28%–42%), peas (22%–28%), cereal grains (8%–15%), and milk whey (1%) may be available and possibly recycled to produce biomaterials useful for food biodegradable and/or edible coatings and packaging (Rhim, 2007). The application of such "bioplastic" films in the food industry was proposed in the late 1960s with the aim to prolong the shelf life of different fresh and manufactured foods and to improve their quality (Krochta and De Mulder-Johnston, 1997). Then, the use of edible coatings increased with time, showing to be a simple and effective technology in preventing the textural deterioration of several food products (Porta et al., 2013; Zink et al., 2016). Coatings can be obtained in several ways, generally by dipping or spraying foods with specifically tailored film-forming solutions (FFSs) (Coltelli et al., 2016; Song and Zheng, 2014). However, films made of polysaccharides and/or proteins, while possessing good gas barrier features toward oxygen and carbon

dioxide, usually exhibit poor mechanical properties and, due to their hydrophilic nature, are too water sensitive to be applied to the majority of foods having high or intermediate moisture (Krochta and DeMulder-Johnston, 1997; Nisperos-Carriedo, 1994). These disadvantages can be resolved by the preparation of blended films, as protein-polysaccharide films and/or by adding lipids or other components such as nanoparticles able to reinforce the film network (Porta et al., 2011b; Rhim, 2007; Rostamzad et al., 2016). An additional strategy to improve the characteristics of protein-based films is to create a cross-linked structure of its network either chemically or enzymatically. Among the enzymes able to create protein cross-linkings, TGase is certainly the most efficient, being able to catalyze isopeptide bonds between reactive Gln and Lys residues existing in the polypeptide sequence. Mahmoud and Savello (1992, 1993) were the first to utilize TGase as a cross-linker to produce whey protein (WP) homo- and heteropolymers containing films. Afterwards, different authors proposed further proteins and methodologies to produce TGase cross-linked films with specific properties suitable for coating specific food products (Cui et al., 2017; Elango et al., 2017; Mariniello and Porta, 2003; Porta et al., 2011b, c; Rossi Marquez et al., 2014, 2017). This kind of food coating needs, of course, to be produced by using proteins able to act as acyl donors and/or acceptor substrates of the enzyme. Therefore, most of the studies were mainly focused on a few specific proteins able to easily form cross-linked polymers in the presence of mTGase. An overview of the main effects of mTGase-catalyzed reactions on the properties of different protein-based films and food coatings is given hereafter and summarized in Table 21.1.

TABLE 21.1 Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings

Protein Film[a]	Effect	References
White (milk) proteins		
Casein	Mechanical strength and flexibility increases, solubility decrease	Oh et al. (2004) and Al-Saadi et al. (2014)
α_{s1}-Casein	Tensile strength increase and water solubility decrease	Motoki et al. (1987)
Sodium caseinate/hydroxypropylmethylcellulose	Solubility decrease and stiffness increase	Perone et al. (2014)
Casein/type I collagen	Improvement of thermal stability	Wu et al. (2017)
Succinylated casein/egg-white protein	Improvement of mechanical properties, water resistance, and thermal stability	Peng et al. (2017)
Sodium caseinate/WP	Water solubility decrease and mechanical/water barrier properties increase	Qiao et al. (2014)
WP	Tensile strength and elongation at break increase. Delayed lipid oxidation	Rodriguez-Turienzo et al. (2013)
WP/chitosan	Lower permeability to water vapor	Di Pierro et al. (2006)
WP/carboxymethylated chitosan	Improvement of water vapor barrier and mechanical properties	Jiang et al. (2016)

TABLE 21.1 Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings—cont'd

Protein Film[a]	Effect	References
WP/pectin	Tensile strength, elongation at break, and gas barrier increase. Oil content decrease in coated fried foods. Prolonged shelf life of coated fruits and vegetables	Di Pierro et al. (2013) and Rossi Marquez et al. (2014, 2017)
Green (plant) proteins		
WG	Increased solubility and integrity. Improved mechanical properties and water vapor permeability	Larré et al. (2000) and Jinshui et al. (2005)
WG/α-polylysine	Improved mechanical properties and water stability, more compact network structures and higher hydrophobicity	Cui et al. (2017)
Zein hydrolysate/WPs	Increased integrity and flexibility	Oh et al. (2004)
Zein/oleic acid	Improved mechanical and water barrier properties	Masamba et al. (2016)
SPI	Increased tensile strength and surface hydrophobicity thermostability	Tang et al. (2005)
SPI/pectin soy flour proteins/pectin	Higher homogeneity, increased tensile strength, reduced flexibility, and lower gas exchange	Mariniello et al. (2003) and Di Pierro et al. (2005)
Soy flour proteins/ putrescine-pectin	Improved mechanical properties and decreased water vapor permeability	Di Pierro et al. (2010)
Skimmed soy proteins/WPI	High water-keeping capacity and strong elasticity	Su et al. (2007)
SPI/gelatin films	Improved thermal stability, water resistance, and tensile strength properties	Weng and Zheng (2015)
SPI/type I collagen	Improved thermal stability and mechanical properties	Wu et al. (2017)
Phaseolin/grapefruit albedo	Improved mechanical characteristics and barrier properties to carbon dioxide and water vapor	Mariniello et al. (2010)
Phaseolin/citrus pectin	Easy digestion by the human gut, lower CO_2 and O_2 permeability than LDPE and MATER-BI	Giosafatto et al. (2014a)
Phaseolin/pectin/trehalose	Smoother surface and antioxidant properties	Giosafatto et al. (2014b)
BVP	Decreased CO_2 and O_2 permeability and increased resistance and stiffness	Porta et al. (2015)
BVP/pectin	Decreased gas permeability and improved mechanical properties	Porta et al. (2016)
Red (animal) proteins		
Type I collagen/casein, keratin or SPI	Improved thermal stability and mechanical characteristics	Wu et al. (2017)

Continued

TABLE 21.1 Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings—cont'd

Protein Film[a]	Effect	References
Shark catfish skin collagen	Higher tensile strength, stiffness, water solubility, and stability against proteolysis	Elango et al. (2017)
Gelatin	Improved water vapor barrier, mechanical properties, water resistance, and thermal stability	Babin and Dickinson (2001), Carvalho and Grosso (2004), and Liu et al. (2016, 2017)
Fish-skin gelatin	Decreased tensile strength, elongation at break, and solubility	Piotrowska et al. (2008)
Catfish gelatin	Increased elongation at break and decreased tensile strength	Oh (2012)
Gelatin/calcium carbonate	Reduced water vapor permeability, increased mechanical properties, and facilitated film formation	Wang et al. (2015)
Gelatin/casein	Increased elongation at break and decreased water vapor permeability	Chambi and Grosso (2006)
Fish gelatin/nanoclay	Decreased tensile strength and elongation at break	Bae et al. (2009)
Fish gelatin/chitosan	Increased deformation and water vapor permeability, decreased tensile strength and solubility	Alvarado et al. (2015)
Fish gelatin/starch	Increased tensile strength and Young's modulus, decreased solubility and elongation at break	Al-Hassan and Norziah (2017)
Pork gelatin-coated paper	Reduced water vapor permeability	Battisti et al. (2017)
Fish myofibrillar protein	Increased tensile strength, barrier properties, and thermal stability. Decreased elongation at break and solubility	Kaewprachua et al. (2017)
Fish myofibrillar protein/nanoclay	Improved water gain, water vapor permeability, solubility, tensile strength, and elongation at break	Rostamzad et al. (2016)
Ovalbumin/chitosan	Improved mechanical properties	Di Pierro et al. (2007)

[a] *WPI = Whey protein isolate, WG = Wheat gluten, SPI = Soy protein isolate, BVP = Bitter vetch proteins.*

21.2.1 White (Milk) Proteins

21.2.1.1 Caseins

Caseins are the most abundant proteins occurring in milk, accounting for about 80% of the total milk protein content. Native caseins exist as large highly hydrated colloidal micelles that coagulate under the effect of specific proteolytic enzymes to give rise to many different dairy products. However, since caseins present in their structures many acyl acceptor (Lys) and donor (Gln) reactive sites, they could be easily polymerized by TGase (Schorsch et al., 2000).

The obtained casein-based films have a hydrophilic character and, consequently, they exhibit high water vapor permeability (Chick and Hernandez, 2002). Oh et al. (2004) tried to improve their properties by adding TGase to FFSs containing casein alone or in combination with other proteins, such as zein hydrolysates, to obtain films with higher mechanical strength and flexibility, whereas Al-Saadi et al. (2014) observed a lower solubility of the casein films prepared in the presence of the enzyme. Among the different composite films prepared with other proteins or polysaccharide derivatives (Perone et al., 2014; Wu et al., 2017), Qiao et al. (2014) observed that water solubility decreased while both mechanical and water barrier properties increased when sodium caseinate film was blended with WPs and cross-linked by TGase. These results are in agreement with the previous ones showing that the presence of the enzyme increased the mechanical properties and decreased the film water solubility of α_{s1}-casein-based films (Motoki et al., 1987). Furthermore, Peng et al. (2017) recently investigated the effect of TGase on succinylated casein films blended with egg white protein. They observed that the TGase-containing films were more homogeneous and smoother and possessed better water resistance and thermal stability compared to the ones prepared in the absence of the enzyme. In addition, the spatial conformation and degree of crystallinity of such composite films were also found to be positively affected by TGase.

21.2.1.2 Whey Proteins

Nevertheless, the main interest in milk-derived protein films and coatings has been focused on the protein soluble fraction occurring in the milk whey, that is, the liquid by-product of cheese manufacturing that is available in large amounts and whose annual production increases year over year (Khwaldia et al., 2004). There is great concern in the food industry to find out multiple and suitable methodologies for recovering this valuable by-product that, otherwise, may represent a dangerous polluting waste for its lactose and protein content (Di Pierro et al., 2006; Siso, 1996; Smithers, 2008). The milk WP fraction has been widely tested for the preparation of biodegradable films, due to its unique nutritional and functional properties (Ferreira et al., 2009; Schmid, 2013; Schmid et al., 2014). Depending on the WP manufacturing process, various proteins such as immunoglobulins, bovin serum albumin, and several low mol. wt. polypeptides may occur in different proportions in the WP preparations, either as isolates or concentrates, in addition to the two main components α-lactoalbumin and β-lactoglobulin (Andersson, 2008; Herrmann et al., 2004; Lent et al., 1998). Although WP-derived bioplastics, as the other protein-based films, have excellent gas barrier properties, they show low tensile strength and high water vapor permeability due to the high proportion of hydrophilic amino acids occurring in the polypeptide sequences of both α-lactoalbumin and β-lactoglobulin (Ghanbarzadeha and Oromiehi, 2008). WP films are generally formed in aqueous solutions in the presence of different plasticizers. Their properties have been shown to be significantly influenced by the presence of lipids, salts and TGase (Hamman and Schmid, 2014). In particular, the addition of mTGase to WP FFSs was demonstrated to induce an enhancement of film mechanical resistance and a reduction in its deformability. Even the barrier efficiency toward O_2 was found to be markedly improved in the cross-linked films, which also showed a lower permeability to water vapor (Di Pierro et al., 2006, 2013; Rossi Marquez et al., 2014). Studies were also carried out by blending WP with different polysaccharides, such as free (Di Pierro et al., 2006) or carboxymethylated (Jiang et al., 2016) chitosan and pectin (Di Pierro et al., 2013; Rossi Marquez et al., 2014,

2017), with the aim to improve film coating properties. In particular, Rossi Marquez et al. (2014) demonstrated a clear effect of the TGase-cross-linked WP/pectin films in decreasing moisture loss in both doughnuts and French fries when applied as coating before food frying. A concomitant decrease in oil content was also observed in the coated fried foods with no difference in their texture noted during the sensory evaluation tests recorded between uncoated and coated foods. The same coating methodology was effective at hindering moisture absorption by biscuits during their storage, being able to prevent the conversion of the food matrix from a glassy state to a rubbery state. Furthermore, Rodriguez-Turienzo et al. (2013) evaluated the effect of the enzyme on either heated or ultrasound-treated WP films. In particular, they investigated mTGase ability to improve the quality parameters of coated frozen Atlantic salmon (*Salmo salar*). The authors concluded that the enzyme addition to the heated WP coatings delayed lipid oxidation, even though it did not significantly modify the yields, drip losses, color, or chemical composition of the fish fillets. Finally, Jiang et al. (2016) reported that mTGase was effective at improving the water vapor barrier and mechanical properties of WP/carboxymethylated chitosan films, whereas Rossi Marquez et al. (2017) showed that WP/pectin films obtained in the presence of mTGase significantly increased the shelf life of fresh cut apples, potatoes, and carrots without affecting their sensory properties.

21.2.2 Green (Plant) Proteins

21.2.2.1 Wheat Gluten

Wheat gluten (WG) is a term generally used to define >50 different salt water-insoluble wheat flour proteins of different classes. It includes the glutelin and prolamine fractions of wheat flour proteins, typically referred to as glutenin and gliadin, respectively (Coltelli et al., 2016). WG is well known to form a unique viscoelastic network that gives integrity to wheat dough, which has been used worldwide for centuries for a myriad of bakery and other food products. WG films are typically prepared from aqueous/alcoholic FFSs. Because casting is the method widely used for gluten film formation, homogeneous FFSs are required, aided by mechanical mixing, heating, and adjusting to alkaline or acidic pH values (Zhang and Mittal, 2010). Larré et al. (2000) reported that TGase is effective in introducing isopeptide covalent bonds into films obtained from slightly deamidated gluten. Protein cross-linkings induce the formation of polymers of high molecular weight that are responsible not only for the greater insolubility of the treated films, but also for the reduced film surface hydrophobicity. The determination of film mechanical properties showed that the addition of the enzyme increased film integrity and resistance as well as its capacity to stretch. In addition, Jinshui et al. (2005) showed that the films prepared from TGase-modified gluten exhibited higher tensile strength but lower elongation at break and water vapor permeability, compared to the native gluten films. They also showed that, when the modified gluten films were immersed in water at 25°C, their water resistance result increased. Although gluten proteins can be easily manufactured into various types of biological materials, the lack of primary amino groups in their sequence limits the possibility of modification. Recently, Cui et al. (2017) investigated the effect of α-polylysine in gluten TGase-mediated cross-linking and the consequences on the properties of the derived films. The mechanical properties as well as the water stability of the obtained films were found to

be significantly improved in the presence of the enzyme. The films also exhibited rougher surfaces, more compact network structures, and higher hydrophobicity.

21.2.2.2 Zein

Zein, an alcohol-soluble protein enriched in the endosperm of corn, belongs to the class of prolamine proteins and is one of the best studied plant biomacromolecules (Lawton, 2002; Momany et al., 2005). In the absence of plasticizers, zein is known to form brittle films with poor flexibility (Lawton, 2002). Interestingly, Gln is an abundant amino acid at the zein surface, forming polyGln strands. Due to the existence of these polyGln turns and to the fact that some Gln are particularly exposed, it was first hypothesized and then demonstrated that zein is an effective acyl donor substrate of TGase (Cui et al., 2016). The addition of TGase and WPs to zein hydrolysate decreased the solubility of the derived films, which exhibited lower tensile strength and higher elongation at break values. Therefore, enzyme-catalyzed cross-linkage between zein hydrolysate and WPs markedly increased the integrity and flexibility of the protein films (Oh et al., 2004). Furthermore, the effect of different drying temperatures and pH values on the properties of TGase-cross-linked zeine/oleic acid composite films has been recently reported (Masamba et al., 2016). The results of these investigations showed that low pHs and high drying temperatures are ideal processing conditions in improving the mechanical and water barrier properties of TGase cross-linked films. Conversely, high pHs and low drying temperatures did not improve tensile strength, solubility, or water vapor permeability, instead only improving elongation at the break. The effects observed at low pHs were explained with protein aggregation as evidenced by the enzyme-catalyzed increase in zein molecular weight.

21.2.2.3 Soy Proteins

Soy protein isolate is a soybean oil by-product containing a high amount of proteins (≥90%). It is abundantly available, cheap, and easily modified by TGase (Guoa et al., 2015). Moreover, having a good film-forming ability, soy proteins have been extensively studied for food coating applications, leading to the formation of films with high thermostability as packaging material, owing to both hydrogen and disulfide bonds as well as to hydrophobic interactions (Li et al., 2008, 2016). Tang et al. (2005) demonstrated that mTGase was able to increase soy protein film tensile strength and surface hydrophobicity while decreasing film elongation at break, moisture content, and transparency. Microstructural analyses indicated that the cross-linked films had a rougher surface and a more homogeneous and compact cross-section compared to the controls. The mTGase-catalyzed cross-linking of soy protein films was also investigated in the presence of pectins, which were shown to facilitate the formation of high molecular weight protein polymers due to polysaccharide/protein electrostatic interactions (Di Pierro et al., 2005; Mariniello et al., 2003). Film homogeneity was improved and its tensile strength was increased whereas gas permeability was reduced. Furthermore, a putrescine/pectin conjugate was tested to prepare blended soy flour protein films in the presence of mTGase. The obtained cross-linked films were found to possess a decreased water vapor permeability as well as improved mechanical properties, with respect to the ones obtained with not aminated pectin (Di Pierro et al., 2010). Soy protein blended films were also prepared by adding to FFSs other proteins of different origin. Su et al. (2007) used skimmed soybean protein powder to investigate the formation of soy protein/WP blended films by a purified mTGase

produced from a new strain of *Streptomyces* sp. They found that the tensile strength values of cross-linked films were much greater than those of the control while the films had high elasticity and the prevention rates against the permeability of water vapor and oxygen were also upgraded by >70%. TGase-cross-linked soy protein films were also obtained in the presence of gelatin, and these blended films showed better thermal stability as well as improved water resistance properties with respect to the ones prepared in the presence of only mTGase. In addition, SEM morphological characterization revealed that the enzyme also produced a more compact network structure of the blended films (Weng and Zheng, 2015). Recently, also Wu et al. (2017) observed that the cross-linking not only improved the thermal stability of soy protein/collagen complex but enhanced the mechanical properties of the combined films in terms of both tensile strength and elongation at break.

21.2.2.4 Phaseolin

Phaseolin, a bean (*Phaseolus vulgaris* L.) protein having structural properties very similar to those of the globulins occurring in soybean and other legumes, has been shown to act as both an acyl donor and acceptor substrate of mTGase (Mariniello et al., 2007). Accordingly, albedo-phaseolin films prepared in the presence of the enzyme have been suggested as promising candidates to be used to obtain edible food wraps because of their suitable mechanical characteristics and barrier properties to carbon dioxide and water vapor (Mariniello et al., 2010). Afterwards, Giosafatto et al. (2014a, b) characterized the hydrocolloid films made by using *Citrus* pectin and TGase-cross-linked phaseolin. For the first time, trehalose, a nonreducing homodisaccharide into which two glucose units are linked together by a α-1,1-glycosidic linkage, was used as a component of hydrocolloid films constituted of both proteins and carbohydrates. These investigations demonstrated that the obtained films acted as very effective barriers to gases and exhibited a high antioxidant capability.

21.2.2.5 Bitter Vetch Proteins

Bitter vetch (BV, *Vicia ervilia*) is an annual *Vicia* genus cultivated for forage and seed yield. In particular, BV seeds, with up to 25% protein, are an abundant, inexpensive, and renewable source of both protein and energy (Sadeghi et al., 2009). Thus, BV proteins might represent an affordable alternative protein source to produce edible films for both pharmaceutical and food applications. In particular, Arabestani et al. (2013, 2016), Porta et al. (2017), Sabbah et al. (2017), and Fernandez-Bats et al. (2018) recently described edible films obtained from BV protein concentrate (BVPC), showing promising barrier and mechanical properties that are useful to give rise to nanocomposite or bilayered films, as well as biodegradable containers. Porta et al. (2015) determined some properties of BVPC films reinforced by TGase cross-linking. The surface of films prepared in the presence of the enzyme appeared more compact and smoother and the film cross-sections showed the disappearance of the discontinuous zones observed in the control films and, on the contrary, a very homogeneous structure. TGase cross-linked films also exhibited a markedly decreased oxygen (700-fold) and carbon dioxide (50-fold) permeability compared to the controls, as well as significantly different mechanical properties being increased their resistance and stiffness. Moreover, BVPC/pectin blended films have been shown to exhibit a tensile strength double the one observed by using films containing only BV proteins, with an increase of about 3-fold observed in the presence of TGase (Porta et al., 2016). Also, the elongation at break resulted higher in the films containing

the enzyme, leading to the conclusion that films were more extensible mostly when both pectins and TGase occurred in the FFSs. A direct correlation between the improved film mechanical properties and the negative increase of the zeta-potential of the originating FFSs was recorded. Conversely, gas permeability of BVPC/pectin films markedly decreased and TGase addition determined a further enhancement of their barrier properties. Finally, cross-sectional SEM analysis of the same samples showed that BVPC/pectin films cross-linked by TGase possessed a more compact and homogeneous microstructure in comparison with the reticular structure observed in the control samples.

21.2.3 Red (Animal) Proteins

21.2.3.1 Collagen

Collagen is the main protein component in animal connective tissues such as skin, cartilage, and bone, appearing in a fibrous triple helical structure consisting of three protein molecules (Coltelli et al., 2016; Sahithi et al., 2013). Type I collagen is the most common among 28 different types of collagen and it has been widely used in biomedical materials, pharmaceuticals, and foods (Shoulders and Raines, 2009; Sinthusamran et al., 2013). Few studies have been made on the formation of collagen films cross-linked by TGase. Only recently, Wu et al. (2017) investigated the thermal stability and mechanical properties of type I collagen enzymatically cross-linked with casein, keratin, and soy proteins. The obtained results indicated that the high molecular weight particles produced could be effective for collagen film application as food packaging material. On the other hand, Elango et al. (2017), by studying the effects of TGase on mechanical and functional properties of shark catfish skin collagen films, concluded that the enzyme containing films showed improved tensile strength and stiffness. In addition, the cross-linked collagen films exhibited a high stability against in vitro biodegradation by proteolytic enzymes. Gelatin is a water-soluble protein obtained from collagen by acid or alkaline hydrolysis. Babin and Dickinson (2001) showed that the gelatin treatment with TGase determined either negative or positive effects on the protein film stiffness, depending on the order in which the enzyme-catalyzed cross-linkages were formed, and also if they appeared before or after the formation of junction zones induced by the cooling of the solution. Afterwards, Carvalho and Grosso (2004) reported an improvement of the gelatin film water vapor barrier and mechanical properties by TGase treatment, whereas Piotrowska et al. (2008) showed that enzymatic cross-linking of fish skin gelatin significantly decreased film tensile strength and elongation at break as well as film solubility in an aqueous medium without improving water vapor permeability. Conversely, Oh (2012) observed that the mechanical properties of catfish (*Ictalurus punctatus*) gelatin film were significantly affected by TGase cross-linking, with the tensile strength significantly decreasing and elongation at break increasing while no significant difference in film water vapor permeability was detected. More recently, Wang et al. (2015) successfully utilized the enzyme in the preparation of gelatin-calcium carbonate composite films, showing that TGase improved both mechanical and barrier properties of gelatin films as well as the protein thermal stability. Finally, Liu et al. (2016, 2017) determined the effect of drying temperature and glycerol on the properties of gelatin films modified with TGase. They demonstrated that drying temperature might be used to tailor the physical properties of enzyme-modified gelatin films for specific applications and that an improvement of the mechanical properties was associated with film glycerol content. As far as the gelatin composite films are concerned, Chambi

and Grosso (2006) reported that enzymatic cross-linking induced a substantial increase in the high molecular weight protein components of gelatin/casein FFSs. The derived films (casein/gelatin, 3:1) showed a significant increase in the elongation at break values and a significant decrease in water vapor permeability as compared to films made from gelatin or casein alone and from gelatin/casein blended films untreated with TGase, while no difference was detected in the tensile strength values. A gelatin/nanoclay (unmodified Na-montmorillonite) composite film was also produced, and its mechanical and barrier properties were investigated after treatment with mTGase (Bae et al., 2009). The viscosity of the originating FFS was shown to increase in the presence of enzyme because of protein cross-linking, whereas both tensile strength and elongation at break of the derived films decreased; no significant effect on either oxygen or water vapor permeability was observed. Furthermore, Alvarado et al. (2015) reported that films made from fish gelatin and chitosan, in the presence of glycerol and mTGase, showed adequate properties as packaging material. In fact, by using a 3:1 (chitosan:gelatin) ratio, a decrease in tensile strength and an increase in deformation and water vapor permeability were observed. Conversely, the addition of TGase to fish gelatin/starch blended films was recently shown to significantly increase both tensile strength and Young's modulus and to reduce elongation at break as a result of gelatin enzymatic polymerization (Al-Hassan and Norziah, 2017). Finally, one recent application of gelatin film prepared in the presence of mTGase was developed by Battisti et al. (2017). These authors produced a paper sheet coated with a polymeric film based on enzyme cross-linked gelatin containing citric acid. The coated papers, endowed with antimicrobial and antioxidant properties, might be used as active packaging of fresh beef because the obtained material was shown to be able to reduce water vapor permeability.

21.2.3.2 Myofibrillar Proteins

Myofibrillar proteins of fish muscle are generally used as a film-forming material. In fact, although these proteins are insoluble in water, they become soluble by adjusting the pH of the solution. The obtained films exhibit a homogeneous and smooth surface, similar to the commercial wrap films of polyvinyl chloride. However, as compared to the latter, myofibrillar protein films have relatively poor mechanical and barrier properties (Kaewprachua et al., 2016). Therefore, different attempts have been performed to improve these features to render such films comparable to the commercial ones through optimization of various parameters such as the addition of different amounts and types of plasticizer and/or nanoparticle as well as of cross-linking agent. To this aim, Kaewprachua et al. (2017) investigated mTGase effects on the properties of fish myofibrillar protein films. They observed an increased thickness, thermal stability, tensile strength, and gas barrier properties whereas film lightness, transparency, elongation at break, water vapor permeability, moisture content, solubility, and degree of swelling all decreased. Furthermore, investigations on the combination of montmorillonite nanoclay addition to myofibrillar protein FFS and TGase treatment showed a significant improvement of water gain and water vapor permeability of the obtained films, as well as of their solubility. Moreover, these nanocomposite films also exhibited an improved tensile strength and elongation at break (Rostamzad et al., 2016).

21.2.3.3 Ovalbumin

Egg white contains many globular functionally important proteins (main solutes present in egg white representing 10% of its weight) with high potential for industrial applications.

Ovalbumin, the most abundant of these (54% of the total egg white proteins), is widely used as a nutrient supplement (Abeyrathne et al., 2013). Flexible, smooth, transparent, and slightly yellowish films have been obtained by mixing ovalbumin with chitosan, but the addition of mTGase to the chitosan/ovalbumin blended FFS gave rise to biomaterials characterized by a lower solubility at a wide range of pH and better enzymatic hydrolysis by trypsin (Di Pierro et al., 2007). The degree of swelling was also reduced and the mechanical resistance of the cross-linked films was shown to be significantly improved, whereas the barrier efficiency toward water vapor was only slightly affected. The protein enzymatic cross-linking of such composite material confirms once again that the approach in modifying the protein film network by TGase described throughout the present chapter represents a very useful strategy in preparing edible films with tailored characteristics for specific food coatings.

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CHAPTER

22

Application of a Novel *Endo*-β-*N*-Acetylglucosaminidase to Isolate an Entirely New Class of Bioactive Compounds: *N*-Glycans

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22.1 INTRODUCTION

Mammalian milk has evolved for more than 200 million years. To date, scientists have yet to fully identify all milk biomolecules. It has been showed that breast-fed infants have a reduced risk of diarrhea and eczema compared to infants fed formula (Hamosh, 2001; Holman et al., 2006). One interesting characteristic of milk that is now being recognized is the extent of glycosylation of its proteins, which is common to bovine and human milk and considered to be active on beneficial effects. Glycosylation is a posttranslational modification where small carbohydrate residues are added during protein synthesis. Importantly, the milk glycans are indigestible to the host whereas they promote the growth of beneficial intestinal microbes (especially Bifidobacteria), thus further protecting the infant from pathogens and stimulating a healthy intestinal environment (Karav et al., 2016; Marcobal and Sonnenburg, 2012). Although the backbone of glycoproteins has been widely investigated through proteomics studies, the functions of glycosylation have not been elucidated. For example, it has been shown that lactoferrin stimulates growth in bacteria but the role of the glycans in its biological activity has been typically neglected. Similarly, the antimicrobial effect of lactoferrin has been known for decades, but the role of conjugated glycans to the biological function of lactoferrin is still not clear. It is obvious that glycosylation can also protect the peptide backbone by covering cleavage sites, making it more resistant to proteases during digestion. The common highly *N*- and/or *O*-glycosylated proteins found in nature are lactoferrins, immunoglobulins, caseins, mucin, and fetuin (Anumula, 2012; Melmer et al., 2011; van Veen et al., 2004). Glycan

https://doi.org/10.1016/B978-0-12-813280-7.00022-0

diversity of these proteins and glycosylation sites is widely investigated (Barboza et al., 2012; Huhn et al., 2009; Parry et al., 2006) and found to be unique for proteins. Some proteins such as lactoferrins show different *N*-glycan diversity in different mammalians, but their functions are not well elucidated due to the limitations of large-scale production of these glycans (Le Parc et al., 2014; Royle et al., 2008). Various enzymes (glycosidases) and some chemical methods are extensively used for removing glycans from glycoproteins. However, they have many limitations such as low glycan release specificity, high cost, disruption glycans, and the remaining polypeptide structure that hinders the investigation of the biological roles of glycans. There is great interest in the biological functions of glycans (Bode et al., 2004; Morgan and Winick, 1980; Yolken et al., 1992). However, the investigations are limited because of their low concentration in glycoprotein substrates and the lack of the enzymatic deglycosylation techniques. Therefore, novel approaches should be considered to release these glycans from dairy streams. Here, we mention a new enzyme that removes the *N*,*N*′-diacetyl chitobiose moiety from the *N*-glycan core of various glycsoproteins.

22.2 BIOLOGICAL ROLES OF GLYCANS

Glycans can be found as free or attached to the other structures in nature such as proteins, lipids, or peptides. Free glycans (oligosaccharides) in milk contain a lactose core and are modified by *N*-acetylglucosamine, sialic acids, and fucoses. Glycans are also linked to proteins with *O*- or *N*-glycosidic binding. The appearance of each type of glycans in glycoproteins can be exclusive but both types can coexist on the same protein. The biological roles of free or attached glycans have not been fully understood. However, they can be divided into two main groups: the structural contributions and the modulatory characteristics of glycans (Varki et al., 2009).

22.2.1 Free Oligosaccharides

Human milk's free oligosaccharides can be found as branched or linear. These small-chain carbohydrates contain 3–14 different monosaccharide compositions (Wu et al., 2011). Human milk has hundreds of different free oligosaccharides that vary between individuals (Ruhaak and Lebrilla, 2012). The amount of oligosaccharides and their composition vary between mothers and the lactation period (Kunz et al., 2000). Human oligosaccharides mostly originate from a lactose core that is modified with *N*-acetylactos-amine blocks. Further elongation is achieved regardless of whether it is linear or branched with the addition of fucose and/or *N*-acetylneuraminic acid (Ruhaak and Lebrilla, 2012). Although human milk contains free glycans as the third most abundant compound, these oligosaccharides are not digestible by infants due to the lack of glycosidases present in the gastrointestinal tract in infants. However, LoCascio et al. (2009) showed that these oligosaccharides are preferentially consumed by various *Bifidobacteria* strains. Another important role of free oligosaccharides is antipathogenic activity including HIV, enteropathogenic *Escherichia coli*, *Listeria monocytogenes*, *Streptococcus pneumonia*, *Vibrio cholera*, *Salmonella*, and *Campilobacter jejuni* (Coppa et al., 2006; Hong et al., 2008; Ruiz-Palacios et al., 2003). As well as their recently found bifidogenic activity and antiadhesive antimicrobial activity, these small-chain carbohydrates play an important role in

brain development (Wang, 2009). A study performed in piglets suggests that dietary sialic acid enriched glycans improve memory and learning skills (Wang et al., 2007).

22.2.2 Conjugated Glycans

O-glycans are generally bound to the proteins through *N*-acetylgalactosamine to a hydroxyl group of a serine or threonine residue, and can be modified into various core classes (Varki et al., 2009). *O*-Glycans have a number of core structures, at least eight of which are known. *O*-Glycans are linked to either serine or threonine through an *N*-acetylgalactosamine with no known consensus sequence. One of the most common types of *O*-linked glycans contains a GalNAc residue attached to the polypeptide through Ser/Thr residues. This process depends on the primary, secondary, and tertiary structure of the glycoprotein (Steen et al., 1998). These types of glycans are mostly referred to as mucin-type glycans. Other *O*-linked glycans have different initial sugars such as glucosamine, xylose, galactose, fucose, or manose. The molecular mass of *O*-linked glycoproteins is generally larger than 200 kDa with biantennary and less branching than *N*-glycans. Free oligosaccharides in milk are more structurally similar to *O*-glycans than *N*-glycans. *N*-linked glycans (*N*-glycans) are attached to an asparagine residue of proteins through *N*-acetylglucosamines (HexNAc) in a specific amino acid sequence AsN-X-Ser/Thr. *N*-Glycans have a single core consisting of two *N*-acetylglucosamines (GlcNAc) followed by three mannoses (Fig. 22.1). Further glycosylation of the mannoses determines the types of *N*-glycans. The presence of only mannose residues yields high mannose-type oligosaccharides. Other types of glycans can add to the core mannoses such as *N*-acetylglucosamine, fucose, galactose, and sialic acid residues to produce hybrid and/or complex types.

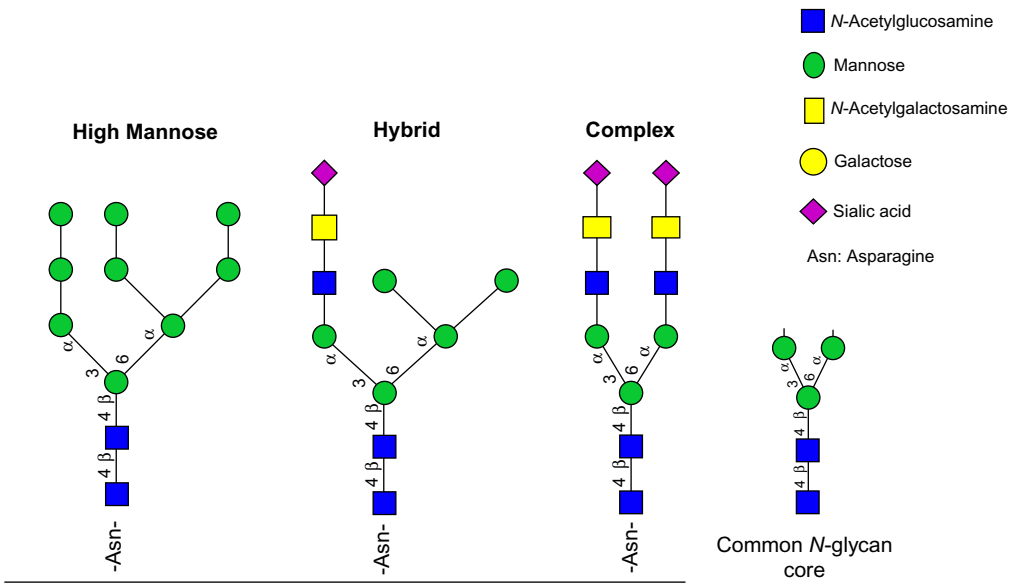

FIG. 22.1 Common *N*-glycan core and different *N*-glycan types: high mannose, hybrid, and complex.

Glycans play an important role in various cellular mechanisms that are associated with health and disease. Studies on the glycan role in cell adhesion and receptor activation (Barboza et al., 2012) suggest that the structure of glycoproteins provides the host with protection against various microbial and viral attacks. *N*-Glycans are also important for the recognition and connection of microbes with lectins found on the cell membrane (Nwosu et al., 2012). Moreover, glycans also contribute proteins with the help of behavioral and structural properties (Spik et al., 1994). One of the recent studies on lactoferrin glycans suggests that *N*-glycans inhibit *Pseudomonas aeruginosa*, which is responsible for epithelial cell invasion and bacterial keratitis (Kautto et al., 2016). In addition to the glycan contribution to the folding and conformation of proteins, they also influence the protein resistance against proteolysis, solubility, and antigenicity (Wormald et al., 2002).

22.3 MILK GLYCOPROTEINS

Proteins in milk can be classified as whey proteins and caseins. Caseins are highly *O*-glycosylated proteins whereas whey proteins are mostly *N*-glycosylated. Whey, a by-product of the cheese-making process, contains a high amount of glycoproteins. The content of whey is mostly water with 94% of total content proteins (0.6%) and lactose (4.5%). Globally, whey production is more than 200 million tons annually, which provides 1 million tons of protein for the industry (Mollea et al., 2013). The glycoprotein content of whey is mostly glycomacropeptide, immunoglobulins, lactoferrin, osteopontin α-lactalbumin, and transferrin (Horowitz, 2012; Walstra et al., 2005) (Table 22.1).

22.3.1 Immunoglobulins

Immunoglobulins are also known as antibodies, which are bound specifically to antigens found in the blood. They are highly abundant in human secretions from mouth to

TABLE 22.1 Common Glycosylated Proteins, Their Abundance in Milk, and Their Glycosylation Patterns

Glycoprotein	Molecular Weight (kDa)	Concentration (g/kg)	Glycosylation Type	Reference
GMP	7.0	1.5 g/L	*O*-linked	(Manso and Lopez-Fandino, 2004)
Lactoferrin	86	0.1	*N*-linked	(Recio et al., 2009)
Transferrin	76	0.01–0.03	*N*-linked	(Walstra et al., 2005)
IgG	150	1.8	*N*-linked	(O'Riordan et al., 2014)
IgA	385	0.4	*N*-linked	(Fuquay et al., 2011)
IgM	900	0.2	*N*-linked	(Fuquay et al., 2011)
Osteopontin	60	0.018 g/L	*O*-linked	(Schack et al., 2009)
α-Lactalbumin	14.2	1.2	*N*-linked	(Hart et al., 1979)

Adapted from Karav, S., Cohen, J.L., Barile, D., de Moura Bell, J.M.L., 2017. Recent advances in immobilization strategies for glycosidases. Biotechnol. Progress 33(1), 104–112.

genital organs (El-Loly, 2007). Moreover immunoglobulins are also found in the colostrum to transfer the mother's defense molecules into the neonate. There are different types of immunoglobulins, but the most abundant ones are IgA, IgM, and IgG, which are found in human and bovine milk (El-Loly, 2007). The variety of immunoglobulin types depends on the source of milk and varies from one organism to another. Age, breed, and the state of health of a species affect the amount and variety of immunoglobulins found in the milk (Korhonen et al., 2000). Immunoglobulins have two different chains, heavy and light. They are bound to each other with disulfide bridges (Spiegelberg, 1974; Steward, 1984). The light chain, also called VL, includes 100–110 amino acids and four λ chains, which have 23 kDA weight. On the other hand, the heavy chain is also known as VH and consists of 110 amino acids; the heavy chain also contains the CH region, which contains 300–440 amino acids. There are five different types of heavy chains that include 53–75 kDA weight (Spiegelberg, 1974). Immunoglobulins play a vital role for the defense mechanism against o all kind of microbes, pathogens, and toxins because they possess antimicrobial effects (El-Loly, 2007). The primary function of the immunoglobulins is binding to viruses, bacteria, or toxins. This antigen-antibody complexes result in Ag elimination and provide protection for the host. Immunoglobulins generally produced by the immune system are categorized into five classes that contain Ig (A, E, G, M, D), according to their biochemical structure and biological activities. The most abundant type of Ig in humans is IgA (Watanabe et al., 2005). It is found in serum as a monomeric, but it is found as a polymeric when it is secreted by human secretions such as tears or mucus (Royle et al., 2003). One type of immunoglobulin response to allergic reactions is IgE (Watanabe et al., 2005). IgE binds to pathogens or toxic material and destroys them in order to protect the body from the harmful adverse effects of these materials (Watanabe et al., 2005). One of the most abundant immunoglobulins is IgG, which is a heavy gamma chain. It is found on the B cell surfaces as a receptor for antigens (Pan and Hammarström, 2000). IgG is the only immunoglobulin that is able to pass through the placenta to the baby. Thus, the most abundant immunoglobulin found in the newborn is IgG. It binds to the receptors found on the surface of the pathogens and makes them more recognizable for the immune defense system so that they are recognized as soon as possible and destroyed quickly (Hashira et al., 2000; Janeway Jr et al., 2001). The biological roles of immunoglobulins are thought to be modulated by glycans, which are bound to the crystal part on the fragment. It has been reported that missing fucoses on *N*-glycan structure result in the stimulation of antibody-mediated cellular cytotoxicity and glycans with a high sialylation degree play an important role in the immunosuppressive properties (Huhn et al., 2009).

22.3.2 Lactoferrin

Lactoferrin is a multifunctional glycoprotein secreted from specific granules and exocrine glands (Iyer and Lonnerdal, 1993). Lactoferrin can be found widely in milk, salvia, mucus, and saliva (Levay and Viljoen, 1995; Steijns, 2001). Its concentration in the milk of different mammalians varies depending on the species, sometimes even with individuals (Hirai et al., 1990). Lactoferrin that contains 692–697 amino acids folded into two globular lobes attached by an α-helix is also known as lactotransferrin. Lactoferrin has an ability for iron binding and Fe^{3+} ion transferring because it is a member of the transferrin family (the amino acid sequence

similarity with serum transferrin is 60%) (Metz-Boutigue et al., 1984). Lactoferrin has similar amino acid compositions with other transferrins in addition to similar secondary and tertiary structures (disulfide linkages included). However, it possesses different biological functions and physicochemical characteristics (Bluard-Deconinck et al., 1974; Querinjean et al., 1971). The variance in glycan profiles of these proteins is believed to be the reason for the differences in biological function of lactoferrins. Lactoferrins have attracted many scientists because they have various biological functions. As well as its strong iron-binding capability, it has bifidogenic, antimicrobial (Qiu et al., 1998), immunomodulatory (Legrand et al., 2006), anti-inflammatory (Kane et al., 2003), and anticarcinogenic properties (Bezault et al., 1994; O'Riordan et al., 2014). Lactoferrin is also associated with the immune host defense by providing the proliferation, activated immune system cells, and differentiation (Adlerova et al., 2008).

The antimicrobial activity of lactoferrin is one of the most interesting ones, because it disables the growth of various microorganisms such as viruses, bacteria, fungi, and protozoa. It is considered that the antimicrobial effect of lactoferrin is caused by the depletion of iron (an essential nutrient for all microorganisms). Barboza et al. (2012) has demonstrated that human milk glycans protect the mucosal layer of the intestines from pathogens. Moreover, Antonini et al. have shown that direct interaction of lactoferrin with pathogens causes cell lysis, and glycans are thought to be the signaling agents in this mechanism (Antonini et al., 2015). Surprisingly, lactoferrin stimulates the growth of beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium* while also acting as an antimicrobial by killing pathogens. Lactoferrins from different mammalian milks show different growth-promoting characteristics on beneficial microbes. Petschow et al. (1999) demonstrated that bovine milk lactoferrin specifically promotes the growth of *Bifidobacterium breve* and *B. infantis*, whereas human milk lactoferrin stimulates greater growth of *B. infantis*. Karav et al. have also demonstrated that *N*-glycans formed by five monosaccharides-hexose (Hex), *N*-acetylglucosamines (GlcNAc), fucose (Fuc), sialic acid (NeuAC), and *N*-glycolylneuraminic acid (NeuGc)-isolated from concentrated bovine whey by a novel *endo*-β-*N*-acetlyglucosaminidase selectively increased the growth of *B. infantis* (Karav et al., 2016). Especially the lactoferrin glycan compositions 4Hex-3HexNAc-1Fuc, 3Hex-5HexNAc, 5Hex-2HexNAc-1NeuAc, 5Hex-4HexNAc-1NeuAc, and 5Hex-3HexNAc-1NeuAc were preferentially utilized as substrates by *B. infantis* while *B. lactis* did not use these glycans as a carbon source.

Lactoferrins from different mammalian milks have different glycosylation patterns, although they have a high amino acid homology (65%–100%). The *N*-glycosylation sites potentially found in human lactoferrin are asparagine (Asn) 138, 479, and 624; in caprine, bovine, and ovine are Asn233, 281, 368, 476, and 545; and murine lactoferrin contains one potential *N*-glycosylation site, Asn476 (Baker and Baker, 2009). Considering the potential sites, only two sites are commonly glycosylated in humans: Asn138 and 479 (Haridas et al., 1995), and Asn233, Asn368, Asn476, and Asn545 are glycosylated in bLF (Moore et al., 1997). Moreover, the glycans that attach to the protein are mostly unique for the species. Le Parc et al. (2014) showed that human lactoferrin contains 16 unique glycans, whereas bovine and goat have 18 and six unique glycans, respectively. These lactoferrins also have mutual glycans (13 in total) such as 5Hex2HexNAc, 6Hex4HexNAc, 4Hex4HexNAc, and 5Hex4HexNAc1NeuAc. Additionally, the glycan monosaccharide content varies depending on the species. For example, NeuGc can be found in the glycan composition of ruminant species, such as bovine and

goat lactoferrin, but it is not present in human lactoferrin (Le Parc et al., 2014; O'Riordan et al., 2014). The fucose and sialic acid content of different mammalian lactoferrins is different. The fucosylation degree in human lactoferrin is greater than in goat and bovine lactoferrin. This variance might be the reason for the different biological functions of lactoferrins. Almond et al. (2013) demonstrated that different glycan patterns can change the immunogenicity and allergy characteristics of lactoferrin. Barboza et al. (2012) also showed that the glycosylation in the second week of lactation is decreased and then an increase is observed in total glycosylation. Moreover, Le Parc et al. (2014) demonstrated that human lactoferrin produced in transgenic cows shows a different glycan profile compared to the natural human lactoferrin. Although they share identical amino acid sequences, the profile of the human lactoferrin recombinantly expressed in cows was more similar in glycosylation to the bovine milk lactoferrin than to the human lactoferrin, sharing 16 *N*-glycan structures with bovine lactoferrin and nine structured with human lactoferrin, respectively. This variation indicates a strong organism-dependent influence on lactoferrin posttranslational modifications, and suggests that the proteins so produced might have different biological roles due to altered/unique glycan profiles. The effects of glycans on the biological functions and the structure of lactoferrin are not shown yet. Therefore, an efficient deglycosylation method should be developed to isolate these glycans without disrupting their structures for further investigation.

22.4 GLYCAN RELEASE STRATEGIES FROM GLYCOPROTEINS

Increasing interest in large-scale production of bioactive glycans requires efficient deglycosylation techniques to investigate their biological roles and commercialization. Currently, deglycosylation of glycoproteins is based on two approaches: chemical and enzymatic. These techniques have been created to isolate these oligosaccharides in high efficiency with wide diversity, low cost, and easy application. Producing the glycans without disrupting their structures is also an important criterion for a deglycosylation method. However, there is no single method that meets these characteristics fully yet. Therefore, a combination of two or more strategies is preferred to obtain the desired deglycosylation process.

22.4.1 Glycan Release by Using Chemicals

Chemical methods were widely used as deglycosylation agents of glycoproteins in early glycobiology science due to their easy and fast application, low cost, and high substrate specificity (Sojar and Bahl, 1987). The deficiency of commercial *O*-glycosidases has led to the further requirement for chemical deglycosylation (Roth et al., 2012; Turyan et al., 2014). Common chemical deglycosylation methods are β-elimination and hydrazination (Dwek et al., 1993). β-Elimination is based on the cleavage of glycans by exposing the glycoproteins to alkaline conditions. The released free glycans might be degraded by subsequent β-elimination reactions (peeling action). However, this can be avoided by the use of reducing agents such as sodium borohydride (Carlson, 1968; Roth et al., 2012; Turyan et al., 2014). The limitation of this method is that glycans have a single group for labeling that is converted to alditols by the reductive agent, which hinders the ability to bind a fluorophore or a chromophore. As a result, it becomes challenging to monitor glycan release (Roth et al., 2012; Turyan et al., 2014).

β-Elimination also might result in sample losses due to the cleaning step of high salt content (Turyan et al., 2014). Hydrazine treatment is based on the hydrolysis reaction started with the addition of anhydrous hydrazine to the glycoprotein. This method has been widely used because it allows manipulable isolation of *N*- and *O*-linked glycans by adjusting the reaction conditions such as temperature (Patel et al., 1993). The higher potential of hydrazine treatment has been demonstrated by Turyan et al. (2014), who showed that hydrazinolysis is more effective than ammonium-based *β*-elimination to release *O*-glycans with higher activity and wide selectivity. Interestingly, a combination of both these methods has also been demonstrated as an efficient technique for the isolation of pig gastric mucin (Kisiel et al., 2008). In addition to *β*-elimination and hydrazine treatment, anhydrous forms of hydrogen fluoride and trifluoroacetic acid treatment are also employed for chemical deglycosylation (Edge et al., 1981; Fryksdale et al., 2002; Sojar and Bahl, 1987).

22.4.2 Enzymatic Deglycosylation

The enzymatic release of glycans from glycoproteins is mostly performed by peptidyl-*N*-glycosidases (PNGases) (Altmann et al., 1995). A PNGase generally cleaves asparagine-linked glycans with the aim of hydrolyzing the amide side chain (Nuck et al., 1990; Takahashi, 1977). Commercially available PNGases release *N*-linked glycans, regardless of their charge and size (Morelle et al., 2009; O'Neill, 1996; Szabo et al., 2010), if there is a fucose linked to *N*-acetylglucosamine, these enzymes are not able cleave the glycans (Tretter et al., 1991). Another challenge for PNGases activity on *N*-glycans is the form of the glycoprotein. PNGase activity requires high heat and detergent use for the denaturation of glycoproteins to increase the enzyme accessibility to the glycans. However, these harsh conditions might result in the disruption of the released glycan and remaining polypeptide structures, which is directly linked to the biological activity of these compounds. Various endoglycosidases such as F1, F2, and F3 have more activity on the native form of proteins than PNGase F. However their activity is extremely low on multiple-antennary glycans (Trimble and Tarentino, 1991). *O*-Glycan release from glycosproteins enzymatically is very limited due to the complex *O*-glycan cores and insufficient commercial *O*-glycosidases that are active on these cores. A commonly used commercial *O*-glycosidase isolated from *Enterococcus faecalis* and then produced by *E. coli* show high activity on *O*-glycan cores one and three among the eight cores (Makadiya et al., 2016).

22.4.3 A Novel *Endo*-β-*N*-Acetylglucosaminidase

We isolated an *endo*-β-*N*-acetylglucosaminidase (EndoBI-1) from an infant gut microbe *Bifidobacterium longum* subsp. *Infantis* ATCC 15697. This enzyme is capable of cleaving the *N*,*N*′-diacetyl chitobiose moiety that is found on the *N*-glycan core, regardless of the *N*-glycan type (high mannose, hybrid, and complex *N*-glycans). Another advantage of this enzyme is that it is not affected by core fucosylation such as PNGase F (Garrido et al., 2012). The optimal working conditions of EndoBI-1 were determined by using a two-level factorial design (2^4) in a central composite design with eight axial points and four center points (Karav et al., 2015a). Various parameters such as reaction time (15–475 min), temperature (27.5–77.5°C), pH (4.45–8.45), and enzyme/protein ratio (1:3000–1:333) were tested on the isolation of glycans by EndoBI-1 from bovine colostrum. It was found that medium temperature values (~52°C),

longer reaction times, lower pH values, and higher enzyme/protein ratios showed high glycan release from milk glycoproteins. Under these optimal conditions, EndoBI-1 releases 20 mg *N*-glycan from 1 g of bovine colostrum. These results suggest that bovine whey, which is waste by-product of cheese making, can be used a glycan source. The parameters tested here are also suitable to be applied in dairy processes, enabling the production of a completely new class of compounds (*N*-glycans and *N*-glycan-free milk). The activity of EndoBI-1 was also compared with PNGase F (a commercially available enzyme), suggesting that EndoBI-1 shows higher activity on native proteins than PNGase F and can be efficiently utilized during pasteurization because it still maintains its activity at high temperature values. To facilitate EndoBI-1 commercially, we calculated the kinetic parameters for EndoBI-1 on bovine lactoferrin ribonuclease B, in addition to concentrated bovine colostrum whey (Karav et al., 2015b). K_m values were found to be 0.25, 0.43, and 0.90, whereas V_{max} values were from 3.5×10^{-3}, 7.75×10^{-3}, and 5.2×10^{-2} mg/mL×min for ribonuclease B, lactoferrin, and whey, respectively. Briefly, EndoBI-1 showed the strongest enzyme-substrate affinity for ribonuclease B, whereas it showed the maximum reaction rate for concentrated whey. Another study that we performed regarding the activity of EndoBI-1 showed that different types of glycans are being released under different reaction conditions (Parc et al., 2015). The various combinations of time, temperature, pH, and enzyme/protein ratio were determined (Combination 1: pH 6.15, 245 min, 52.5°C, enzyme/protein ratio 1/1000 Combination 2: pH 5.30, 360 min, 40°C, enzyme/protein ratio 1/500 Combination 3: pH 7, 130 min, 65°C, enzyme/protein ratio 1/500). We detected 41, 37, and 18 different *N*-glycan compositions in the combinations 1, 2, and 3, respectively. These data suggest that a wide diversity of the structures, including all types of glycans such as neutral and sialylated complex/hybrid *N*-glycans, could be isolated from bovine whey by the application of EndoBI-1. According to the combinations, the low pH values, long reaction times, and high enzyme use of combinations 2 and 3 mostly favored the enzyme activity, resulting in a higher number of total structures and sialylated glycans for combinations 2 and 3. These results showed that EndoBI-1 is able to cleave all compositions of *N*-glycans, whose production could be selectively manipulated by the application of various processing parameters. Many commercially available glycosidases require substrate denaturation by heating or adding detergents or reducing agents to increase the accessibility of the enzymes. However, this process might disrupt the glycan structure or the remaining polypeptide chain that hinders the further investigation of these compounds' biological characteristics. EndoBI-1 is compatible with food processing, where it is scalable on the release of *N*-glycans with a wide diversity while preserving the native form of protein. Free human milk oligosaccharides are shown to be selectively used by *Bifidobacteria*. The achievement, with release of *N*-glycans that are structurally similar to these free oligosaccharides (HMOs), will be an opportunity to investigate the importance of the structures to stimulating the growth of the gastrointestinal microbes, including *Bifidobacterium*.

22.5 IMMOBILIZATION

When considering any enzymatic process at an industrial scale, immobilization of the enzyme is an important step to achieve a stable, easier, and cheaper bioprocess. However, the application of the immobilized enzymes on large molecules (i.e., proteins) is limited

for industrial use. To immobilize glycosidases for their application in the food industries, various techniques such as adsorption, covalent binding, and entrapment as well as immunoaffinity binding have been considered. Moreover, various support materials have been applied, resulting in different enzyme stabilities and activities. These different techniques have advantages and disadvantages resulting in the requirement of optimization studies to determine the optimal approach for immobilization. While there are diffusional limitations of macromolecular substrates such as milk glycoproteins in certain immobilization methods, there is also a deficiency of research on suitable substitutes such as spacer arms. The application of spacer arms is shown to be an especially critical factor to improve the enzyme accessibility to the substrate, which is the main problem for the glycosidase-glycoprotein complex. Covalent binding of enzymes is one of the most common strategies. The first application of the immobilization of *N*-glycosidases (PNGASE G) was performed with cyanogen bromide (CNBr)-activated Sepharose 4B beads in 1987 (Damm et al., 1987). Similarly, the only applied immobilization technique to an *endo*-β-*N*-acetylglucosaminidase for glycoprotein deglycosylation was achieved with cellulose beads in 2012 (Garrido et al., 2012). When immobilized and free EndoBI-1 tested on RNAse B, there was no significance observed between the samples in terms of yield and the diversity of *N*-glycan profile (Kwan et al., 2005). Adsorption is another commonly used method for enzyme immobilization, based on the binding of enzymes on the surface of support with weak forces. These forces are hydrogen bonds, Van der Waals forces, hydrophobic interactions, and electrostatics (Jegannathan et al., 2008). This method is very simple because it generally requires few reagents and activation steps. Moreover, this method enables the enzyme to maintain high accessibility capability to the substrate because the native form of the enzyme is not changed during the immobilization process. However, the enzyme might leak from the carrier due to the wear force between enzyme carries. Therefore, immobilized enzymes via adsorption provide high enzyme activity but the reusability of the immobilized enzyme is limited.

22.6 GLYCAN ANALYSIS BY MASS SPECTROMETRY

Glycans are structurally diverse and very challenging to analyze. They have numerous monosaccharides attached to each other with various linkages and branching. The determination of these compounds analytically is quite challenging because of their isomeric and anomeric configurations (glycans with the same molecular formula but different branching) (An example is given in Fig. 22.2). However, with the recent advances in mass spectrometry, glycans can be analyzed qualitatively and quantitatively with the help of the ionization of these compounds (Ho et al., 2003; Huang et al., 2017). Combined high-pressure liquid chromatography coupled (HPLC) mass spectrometry (MS) systems and tandem MS (MS/MS) have been commonly used for the sensitive glycan analysis.

Mass spectrometry is an optimal approach for the analysis of glycans because it provides high mass accuracy for the identification of structures based on their mass-charge ratio. Mass spectrometry is capable of detecting more structures compared to the traditional approaches and enables low-abundant molecule detection. The application of desorption/ionization

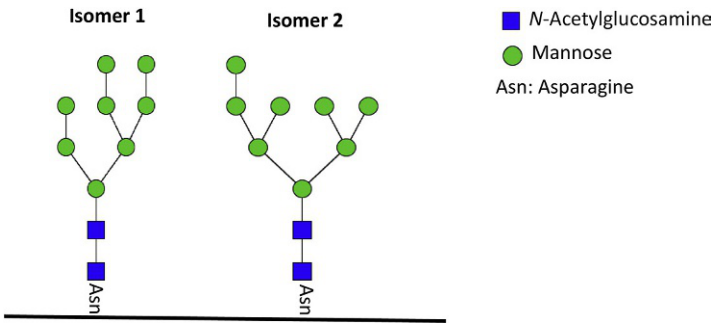

FIG. 22.2 Two isomers of 2HexNAc-8Hec (m/z: 2191. 0819).

approaches such as matrix-assisted laser desorption ionization (MALDI) as well as electrospray (ESI) has increased glycan research with MS. When obtaining the only mass of the intact molecule is not enough to understand the composition details, another technique called collision-induced dissociation (CID) is used to get information about glycan monosaccharide composition. In CID, all unknown ions are sequentially isolated and fragmented so that just by observing characteristic patterns of glycan fragmentation, one can identify the composition of unknown/novel glycans. MS also enables a sensitive method to help the identification of the composition of glycans. However, it is crucial that the samples should be prepared as pure to perform optimal ionization. Indeed, without separation, all types of molecules would be in a competition for ionization. Glycans are described as low ionization efficiency molecules. Therefore, it would be a risk to detect them because their signals would be masked by other molecules with high ionization efficiency (e.g., peptides or proteins present in the same sample). Novel instruments such as the nano liquid chromatography Chip Quadrupole Time of Flight MS have a built-in ability to separate structures regarding their affinity with the help of a chip. Separation of molecules enables the gradual entrance of these molecules to the mass spectrometry that results in the opportunity for the detection of more compounds. Carbon columns (graphitized) can separate glycans with high sensitivity (isomers level), therefore enabling the study of *N*-glycans, *O*-glycans, and free oligosaccharides. Because of the more reproducible retention times achieved with the nanoLC system, novel bioinformatics libraries based on accurate mass, tandem spectra, and retention time can be assembled to aid in the automated identification of known molecules in any chromatogram. These libraries, when integrated with software for data analysis, can also provide each compound's abundance, significantly speeding up data analysis time. To analyze complex *N*-glycan samples, accurate mass data is not enough to distinguish the glycans from each other. In this case, MS/MS system should be used, where initial ions are fragmented to produce ions that are used by QTOF. For example, *N*-glycans have common fragments such as protonated Hexose (163.06 m/z), *N*-acetylglucosamine (204.09 m/z), and Hexose+ *N*-acetylglucosamine (366.14 m/z) can be seen as a separate fragment (Fig. 22.3.) The identified glycans are entered into the libraries that contain previously characterized glycans' monosaccharide profile, retention time, and mass.

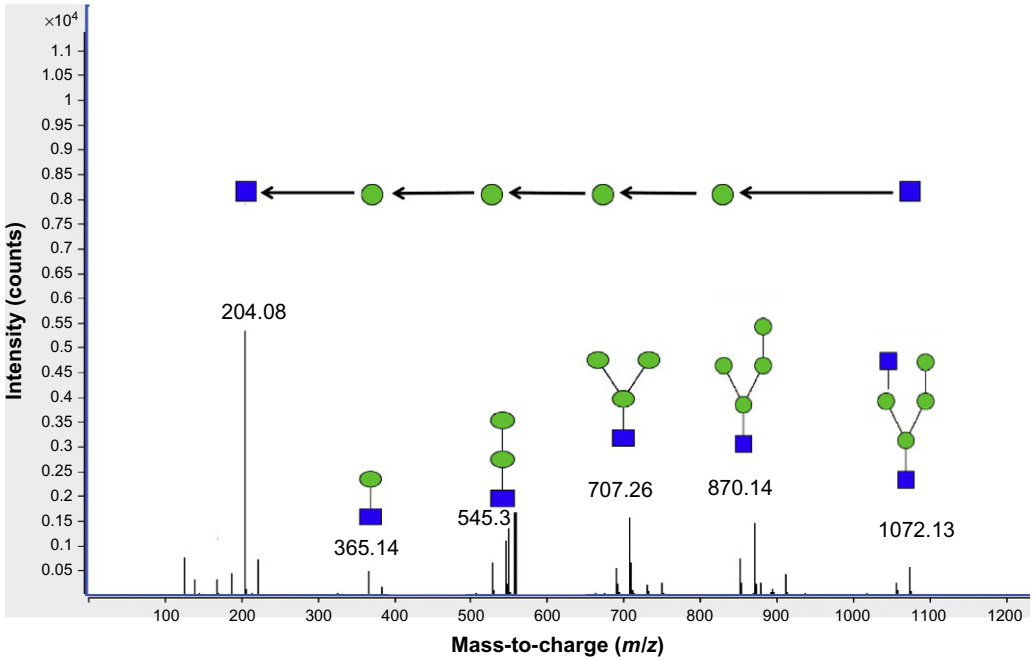

FIG. 22.3 MS/MS spectra of a neutral. (A) Deconvoluted tandem spectrum of the neutral *N*-glycan 4Hex-2HexNAc from bovine milk glycoproteins. This glycan corresponded to 1072.13 m/z with $z=+1$. *Circles* and *squares* represent mannose and HexNAc residues, respectively.

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CHAPTER

23

Enzymatic Production of Steviol Glucosides Using β-Glucosidase and Their Applications

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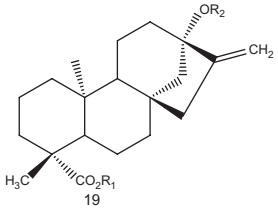
23.1 INTRODUCTION

Natural sweeteners have received much interest due to increasing health concerns over the consumption of sugar as well as problems related to the safety of some nonnutritive artificial sweeteners. Steviol glucosides (stevioside, rebaudioside) are extracted from the leaves of the plant *Stevia rebaudiana* Bertoni, a rhizomatous perennial shrub in the family of Asteraceae that originated in Paraguay and Brazil. For the first time, they were approved for consumption as a natural sweetener in the United States, the European Union, Australia, and New Zealand (Risso et al., 2014). The sweet taste of leaves of *S. rebaudiana* is due to the ent-kaurane type diterpenoid glycosides commonly containing aglycone and steviol that differ from each other only in the position (C13 and/or C19) of glycosidic constituent (Ko et al., 2012; Nguyen et al., 2014). More than 30 steviol glycosides at various concentrations are accumulated in *S. rebaudiana* (Wolwer-Rieck, 2012). Glycosides found in *S. rebaudiana* include stevioside (Ste, 5%–10%), rebaudiosides (Reb)A (2%–5%), RebC (1%), dulcoside (0.5%), other Rebs (RebD, RebE, and RebF, ≤0.2%), and rubusoside (≤0.2%) (Table 23.1) (Yadav and Guleria, 2012; Chatsudthipong and Muanprasat, 2009). Steviol glucosides have gained great attention due to their usage as a

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00023-2

TABLE 23.1 Major Biochemical Components of *Stevia rebaudiana*

Components	Structure		MW	Value (g/100 g dry leaf weight)	Plant Source	Sweetness*	Reference
	R1 (C-19/ Carboxylic Acid)	R2 (C-13/ Hydroxyl)					
Steviol			318.2	Trace <0.01%	*S. rebaudiana*		
	H	H					
Stevioside	β-Glc	β-Glc-β-Glc(2→1)	804.9	5%–10%		300	Yadav and Guleria, 2012; Chatsudthipong and Muanprasat, 2009
RebA	β-Glc	β-Glc-β-Glc(2→1)-β-Glc(3→1)	967.0	2%–5%		250–450	Chatsudthipong and Muanprasat, 2009
RebC	β-Glc	β-Glc-α-Rha(2→1)-β-Glc(3→1)	951.0	1%		50–120	
Dulcoside A	β-Glc	β-Glc-α-Rha(2→1)	788.9	0.5%		50–120	
RebD	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)-β-Glc(3→1)	1129.2	0.2%		250–450	
RebE	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)	967.0	0.2%		150–300	
RebF	β-Glc	β-Glc-β-Xyl(2→1)-β-Glc(3→1)	937.0	0.2%			
Steviolbioside	H	β-Glc-β-Glc(2→1)	642.7	0.1%		100–125	
Rubusoside	β-Glc	β-Glc	642.73	Trace		115	Ko et al., 2012
				5.3%	*R. suavissimus* S. Lee		Tanaka et al., 1981

Reb, rebaudioside; Glc, glucosyl; Rha, rhamnosyl; Xyl, xylosyl; Sweetness*, times sweeter than sucrose at concentration of 0.025%.

low-cost natural sweetener in food and beverages. They are intensively sweet, low in calories, nonnutritive, highly stable, and have the therapeutic properties of being antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, antidiarrheal, and antidiuretic while also having immunomodulatory effects (Clos et al., 2008; Wolwer-Rieck et al., 2010; Goyal et al., 2010). Although stevia occupies only 1% of the global artificial sweeteners market, its market is growing at a rate of 4% per annum with an estimated value of around $1.3 billion. Japan solely invests 40% in the international sweetener market. The estimate of the Japanese total market value of stevia sweetener was approximately $25–35 million per year (Megeji et al., 2005). China accounts for 75% of global *Stevia* plant cultivation, which is 80,000 acres of land. The Chinese stevia industry experienced a significant increase in yield, from 2073 tons in 2007 to 3096 tons in 2009, and 80% of the yield was exported. It has been estimated that worldwide stevia sweetener capacity grew from 5000 tons per year in 2007 to 11,789 tons per year in 2009 (Yadav and Guleria, 2012; Chatsudthipong and Muanprasat, 2009). Steviol, steviolbioside, and Ru are rare in nature, unlike Ste. *S. rebaudiana* only contains trace amounts of them. Ru can be extracted from *Rubus suavissimus* S. Lee (Rosaceae). However, the yearly yield of Ru is various depending on regional climates (Wan et al., 2012). Thus, mass purification of Ru was considered to be complicated and economically impractical (Ko et al., 2012; Sugimoto et al., 2002). Hydrolysis of Ste has been suggested as a method because Ste contains three glucosyl groups at the C13 and C19 positions, which can yield steviolbioside, steviol, isosteviol, steviol mono-glucosyl ester, Ru, or steviol mono-glucoside after full or partial cleavage. β-Glucosidase is an enzyme produced by all life domains. It can hydrolyze the β-D-glucosidic bonds of various compounds comprised of alkyl-β-D-glucosides, aryl-β-D-glucosides, cyanogenic glucosides, disaccharides, and short-chain oligosaccharides, liberating glucose from a nonreducing end. Therefore, researchers have tried to hydrolyze Ste by using β-glucosidases to produce specific products for industrial applications with high yields. This review will summarize recent advances in the enzymatic production of steviol glycosides from Ste by using β-glycosidases and the characterization of products with a particular focus on their potential industrial applications. In steviol glucosides, the number of carbohydrate groups at the C13 and C19 sites will determine the degree of sweetness of steviol (Adari et al., 2016; Gerwig et al., 2016). It has been reported that Ste is 300 times sweeter than sucrose (Yadav and Guleria, 2012; Chatsudthipong and Muanprasat, 2009). Although the concentration of Ste in the *S. rebaudiana* leaves is higher than that of RebA, the bitter aftertaste of Ste and its low solubility in water limit its use for human consumption. That also limits its application in food and pharmaceutical products (Adari et al., 2016; Ko et al., 2016). Compared to Ste, RebA has an extra glucosyl group at the C13 site. It imparts the greatest potency of sweetness with a less bitter aftertaste (Kohda et al., 1976; Adari et al., 2016).

23.2 ENZYMATIC MODIFICATION OF STEVIOL GLUCOSIDES

23.2.1 Production of Steviol

Ste contains three β-glycosidic bonds (β-linked sophomore, β-1,2-D-glucopyranosyl on C13, and an ester β-glucosidic linkage on the C19 carboxyl group). Ru can be produced by selective cleavage of β-1,2-glucosidic linkage of the sophorosyl moiety at site C13, whereas hydrolysis of different positioning glycosides with different numbers of Ste will produce

steviol, isosteviol, steviolmonoside, steviolbioside, and a mixture of these compounds (Okamoto et al., 2000). Unlike Ste, steviol is rare in nature. Therefore, few studies have reported its synthetic methods (Ogawa et al., 1980; Ko et al., 2013; Nguyen et al., 2016; Milagre et al., 2009; Chen et al., 2014). Steviol is an aglycone of steviol glucosides. It has been pharmaceutically used to improve cognitive functions including memory, alertness, learning, and psychotic stability. In addition, it has been used as a plant-growth factor (Ko et al., 2013; de Oliveira et al., 2008). It might also have potential antihyperglycemic effects on stimulating pancreatic beta cells to secrete insulin (Jeppesen et al., 2000). Wang and Lu have separated 4 μg of steviol/g dry weight of *R. suavissimus* (Wang and Lu, 2007) or 5.9 ± 0.8 μg of steviol/g dry weight of *S. rebaudiana* by high-performance liquid chromatography (Minne et al., 2004). To produce steviol, a chemical method involving the hydrolysis of Ste under extremely acidic conditions has been used. However, steviol produced in that way is rearranged into isosteviol automatically (Kohda et al., 1976). Briefly, to produce steviol, 1 g Ste and 1.5 g sodium periodate in 75 mL water are stirred for 16 h. Then 7.5 g potassium hydroxide is added and refluxed for 1 h. The mixture is then carefully acidified with acetic acid and extracted using ether. The organic layer is then washed with water, dried using magnesium sulfate, and concentrated in vacuo to give crystalline residue (Ogawa et al., 1980). Extraction and crystallization can afford steviol with a yield of 75%. This process requires a highly diluted system and a large amount of costly sodium periodate to achieve meaningful yields (Ogawa et al., 1980; Ko et al., 2013). To produce steviol from Ste, enzymatic methods have also been reported (Wan et al., 2012; Ko et al., 2013; Milagre et al., 2009; Nguyen et al., 2016; Chen et al., 2014; Mizukami et al., 1982) (Table 23.2). Steviol yield has been reported to be 20% by using pancreatin with ethanol as a cosolvent at pH 7.0 (Milagre et al., 2009). The yield is 10% with fungal lipase/ethanol at pH 4.0 and 20.8% with *Aspergillus niger* at pH 7.0 for seven days (Milagre et al., 2009). Crude hesperidinase containing β-1,4-rhamnoglucosidase and flavonoid-β-glucosidase from a culture medium of *A. niger* has been used to break the glycoside bonds of Ste into steviol (Mizukami et al., 1982). Among nine screened commercial enzymes produced from *A. niger* (hemicellulose, hesperidinase, and β-glucanase), *Tricoderma longibrachiatum* (β-glucanase), *Aspergillus aculeatus* (Viscozyme L), *Trichoderma reesei* ATCC 26921 (β-glucanase), *Clostridium thermocellusm* (thermostable β-glucanase), or *Penicillium decumbens* (naringinase), and almond β-glucosidase, two enzymes prepared from *A. aculeatus* and *P. decumbens* have been found to be able to hydrolyze the glucosidic linkage of sophoroside at the 13-hydroxyl group or glucose at the 19-carboxyl group, thus producing the following three products from Ste: Ru, steviol monoside, and steviol (Ko et al., 2013) (Table 23.2). Naringinase (EC 3.2.1.40) has an activity of α-rhamnosidase responsible for naringin hydrolysis to produce prunin (4,5,7-trihydroxy flavovone-7-glucoside) and rhamnose. It also contains an activity of β-glucosidase that can hydrolyze prunin into naringenin (4′,5,7-trihydroxyflavanone) and glucose (Ribeiro and Ribeiro, 2008). The naringinase from *P. decumbens* has both activities of α-L-rhamnosidase and β-D-glucosidase for the hydrolysis of naringin to produce naringenin as the final product (Lee et al., 2013). This enzyme exhibits higher hydrolyzing activity against Ste, Ru, steviol mono-glucoside, and steviol mono-glucosyl ester than β-glucobioses. It seldom hydrolyzes RebA containing a β-glucosyl (1–3) unit at the C-13-hydroxyl group of Ste (Ko et al., 2013). The major pathway for steviol synthesis by β-glucosidase from *P. decumbens*is is Ste to Ru to steviol mono-glucoside to steviol. Steviol yield by *P. decumbens* β-glucosidase has been reported to be 64% using 47 mM Ste

TABLE 23.2 Sources of Glycoside Hydrolases Used for Bioconversion of Steviol Glucosides

Sources	Enzyme	pH	*T* (°C)	Substrate	Products	Yields (%)	Reference
Penicillium decumbens	β-Glucosidase	4.0	55	Stevioside	Steviol	64	Ko et al., 2013
Sulfolobus solfataricus	β-Galactosidase (mutant)	4.5	80	Stevioside	Steviol	97.4	Chen et al., 2014
	β-Galactosidase	6.0	75	Stevioside	Steviol	99.2	Nguyen et al., 2016
Aspergillus sp.	β-Galactosidase	4.5	60	Stevioside	Rubusoside	91.4	Wan et al., 2012
Aspergillus aculeatus	β-Glucosidase	5.1	63	Stevioside	Rubusoside	66	Ko et al., 2012
Thermus thermophilus	β-Glucosidase	7.0	70	Stevioside	Rubusoside	92	Nguyen et al., 2014
Streptomycess sp. GXT6	β-Glucosidase	8.5	50	Stevioside	Rubusoside	78.8	Wang et al., 2015
Clavibacter michiganense	β-Glucosidase	4.5	40	Stevioside	Steviolbioside, rubusoside	ND	Nakano et al., 1998
				Rubusoside	Steviolmonoside		
				Rebaudioside A	Rebaudioside B		
				Steviol-19-*O*-glucoside	Steviol		
Flavobacterium johnsonae	β-Glucosidase	7.0	40	Stevioside, rebaudioside	Steviolbioside, rebaudioside B	ND	Okamoto et al., 2000
				Rubusoside	Steviolmonoside, steviol-19-*O*-glucoside		

at 55°C with pH 4.0 (Ko et al., 2013). Recently, β-glycosidase from *Sulfolobus solfataricus* has been used to hydrolyze Ste to steviol with high yield (99.2%) at 75°C for 12 h (Nguyen et al., 2016; Chen et al., 2014) (Table 23.2). This β-glucosidase can hydrolyze β-glycosidic bonds in Ru, Ste, and RebA to produce steviol with different efficiencies (Nguyen et al., 2016). Steviol yields from Ste, Ru, and Reb have been reported to be 98%, 44.2%, and a negligible amount, respectively (Nguyen et al., 2016). Reaction kinetic studies of *S. solfataricus* β-glycosidase (SSbgly) using Ste as substrate have revealed that K_m and k_{cat} values are 17.21 mM and 1.62 s^{-1}, respectively. The k_{cat}/K_m ratio of SSbgly for Ste is 0.094 $s^{-1}mM^{-1}$ (Nguyen et al., 2016). β-Glycosidase from *S. solfataricus*, ahyperthermophilic bacterium grown in volcanic springs with optimal growth at 75–80°C, is one of the most thermostable glycosyl hydrolases (Wu et al., 2013). Currently, among the reported methods, β-glycosidase from *S. solfataricus* has shown the strongest conversion of Ste (>99% of used Ste) to steviol with a yield of over 98%. This simplified purification method for SSbgly by heat treatment can result in activity recovery of >95% (Nguyen et al., 2016). Thus, it is applicable as an efficient and cost-effective steviol production method with the potential for large-scale industrial application.

23.2.2 Production of Rubusoside

Rubusoside (13-*O*-β-glucosyl-19-*O*-β-D-glucosyl-steviol, Ru) is the major component (5.3 mg/g dry leaves (Tanaka et al., 1981) in leaves of *R. suavissimus* S. Lee (Rosaceae), commonly called tiancha in Chinese or Chinese sweet tea, that is cultivated throughout southwestern China. Trace amount of Ru has also been found in *S. rebaudiana* Bertoni (Chaturvedula and Prakash, 2011; Koh et al., 2009). Ru is a good candidate for a natural sweetener due to its sweetness, which is approximately 115 times sweeter than sucrose at a concentration of 0.025% (Koh et al., 2009). On top of its role as a sweetener, Ru also has been used as a solubility enhancer of pharmaceutical compounds to improve their bioavailability (Nguyen et al., 2014, Zhang et al., 2012, 2011a). Because growth of *R. suavissimus* S. Lee has variable annual yields depending on regional climates (Wan et al., 2012), mass purification of Ru is complicated and economically impractical (Ko et al., 2012; Sugimoto et al., 2002). Indeed, Ru costs at least 10 times more than Ste (Ko et al., 2012). Thus, researchers have tried to produce Ru by selectively cleaving the β-1-2-glucosidic linkage of sophorosyl moiety at site C13 of Ste. Wan et al. (Wan et al., 2012) have reported that β-galactosidase from *Aspergillus* sp. can hydrolyze a glucosyl unit of Ste to produce Ru with a yield of 91.4% at 60°C for 72 h, resulting in Ru synthesis of 0.19 g/L/1000 U/h (Table 23.2). Because this enzyme is specific for hydrolyzing Ste, none of its analogs, such as RebA, RebC, and other steviol glycosides in commercial crude *S. rebaudiana* leaf extract, can be hydrolyzed by this enzyme (Wan et al., 2012). Among nine commercial glycosidases from *A. niger*, *T. longibrachiatum*, *A. aculeatus*, *T. reesei*, *Clostridium thermocellum,* and almonds, only β-glucosidase from *A. aculeatus* (Viscozyme L) can hydrolyze the glucosidic linkage at the sophoroside of Ste. In comparison with other β-glycosidases, this β-glucosidase exhibits higher specificities toward Ste and Ru. However, it can rarely hydrolyze the β-1-3-glucosidic linkage at the 19-carboxyl moiety of RebA or steviol mono-glucosyl ester (Ko et al., 2012). By using β-glucosidase from *A. aculeatus*, an Ru yield of 66% has been obtained from a 280 mM Ste at 63°C with pH 5.1 (Ko et al., 2012) (Table 23.2). Nguyen et al. have screened 31 commercial enzymes with mixed activities of pectinase, hemicellulases, α-galactosidases, cellulases, β-galactosidase, and purified recombinant β-galactosidase from *Thermus thermophilus* expressed in *E. coli* for the transformation of Ste to produce Ru (Nguyen et al., 2014; Lim et al., 2016) (Table 23.2). Among them, commercial Sumizyme SPC from *A. niger*, Sumilact L from *A. niger*, Validase AGS from *A. niger*, naringinase from *Penicillium* sp., and recombinant lactase from *T. thermophiles* have been found to be able to convert Ste to Ru with efficiencies of about 59%, 35%, 56%, 51%, and 92%, respectively (Nguyen et al., 2014). By comparing the amount of Ru synthesis per 1000 U/h among reported papers (Wan et al., 2012), lactase from *T. thermophiles* has shown 23.2 and 56.8 times higher yields than the reported yield of Ru (0.19 g/L/1000 U/h) using β-galactosidase from *Aspergillus* sp. (Wan et al., 2012; Nguyen et al., 2014). Lactase from *T. thermophilus* is a thermostable enzyme. Compared to mesophilic enzymes, thermostable enzymes exhibit maximum activity at a temperature range from 70 to 90°C with significant benefits such as increased reaction velocity, decreased contamination of microorganisms, and extended half-lives of enzymes under reaction conditions (Petzelbauer et al., 1999; Maciunska et al., 2000). After heating the solution containing crude β-galactosidase, 78% of mesophilic proteins are removed,

with the recovery of β-galactosidase activity at 89% (Lim et al., 2016), higher than the recovery yield for partial and purified β-glucosidase from *A. aculeatus* (at 44.7% and 1.8%, respectively) (Ko et al., 2012). To reduce costs, reuse the enzyme, continuously process, and reduce autodigestion in large-scale production, alginate beads have been prepared for an immobilized lactase reactor (Nguyen et al., 2014) or double jacket immobilized lactase columns (Lim et al., 2016). The Ru yield using immobilized lactase has been found to be 1.2 times higher than that using a free enzyme (Nguyen et al., 2014). Wang et al. reported that β-glucosidase obtained by *Streptomyces* sp. GXT6 can convert 98.2% of Ste to 78.8% Ru at pH 8.5 and 50°C for 6 h (Wang et al., 2015) (Table 23.2). This enzyme can also hydrolyze sorphorose, laminaribiose, cellobiose, amygdalin, gentiobiose, esculin, and salin. Enzyme kinetic parameters of this β-glucosidase for Ste are as follows: K_m 1.47 mM, $V_{max} = 16.83\ \mu mol/min/mg$, $k_{cat} = 13.18\ s^{-1}$, and $k_{cat}/K_m = 8.97\ s^{-1}\ mM^{-1}$. To produce Ru, β-glucosidase can specifically hydrolyze the glucosyl moiety of sophoroside at position C13 in Ste. Therefore, it is a prospective candidate for the commercial production of Ru.

23.2.3 Production of Steviolbioside

Steviolbioside 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxyl]kaur-16-en-18-oic acid] is a natural sweetener found in *S. rebaudiana* leaves in rare amounts (Ibrahim et al., 2014). Synthetic methods for steviolbioside such as alkaline and enzymatic hydrolysis of Ste have been investigated (Ko et al., 2013; Chen et al., 2016). Steviolbioside is a byproduct in the production of steviol from Ste using purified β-glucosidase obtained by *P. decumbens* naringinase (Ko et al., 2013). Chen et al. have screened the following six commercial or homemade glycosidases and lipase: β-galactosidase from *Kluyveromyces lactis* (Maxilact LG 2000), β-galactosidase from *S. solfataricus*, β-glucosidase from *A. niger* (Novozymes), β-glucosidase from *Penicillium multicolor* (Aromase), *Candida antarctica* (Novozyme 435), and *Rhizomucor miehei* (Lipozyme RM IM) (Chen et al., 2016). Among them, β-galactosidases from *K. lactis* and *A. niger* have shown high specific activity for the glycosyl ester linkage hydrolysis of Ste, producing steviolbioside as the primary product. A steviolbioside yield of 96% has been obtained at 25 mg Ste/mL, 40°C, pH 7.0, and 9000 U/g Ste for 12 h (Chen et al., 2016).

23.2.4 Other Bioconversion Products From Stevioside

β-Glucosidases from *Clavibacter michiganese* and *Flavobacterium johnsoniae* can hydrolyze glucosyl ester linkages at C19 position of RebA, Ste, Ru, or steviol mono-glucosyl ester. They can also hydrolyze the glucosidic bond in the saccharide at C-13 site of RebB and steviolbioside to polymerize steviol glycosides at a lesser degree (Nakano et al., 1998; Okamoto et al., 2000). However, β-glucosidase from *C. michiganese* cannot cleave the glucosyl residue of RebA, Ste, Ru, or steviol mono-glucoside at the C-13 position, resulting in steviol mono-glucoside as the product after its hydrolysis of steviol glucoside (Nakano et al., 1998) (Table 23.2). β-Glucosidase from *F. johnsoniae* can hydrolyze rubusoside to steviol monoside and seviol-19-glucoside, producing steviol as the final product (Okamoto et al., 2000) (Table 23.2).

23.3 APPLICATIONS OF STEVIOL GLUCOSIDES

23.3.1 Natural Solubilizer

Bioavailability is the major challenge facing the design of oral administration for any drug. Several factors affect oral bioavailability, including water solubility, permeability of the drug into cells, the rate of dissolution, presystemic metabolism, and sensitivity to the efflux mechanism (Savjani et al., 2012). Among those factors, poor solubility and permeability are the most frequent causes of low oral bioavailability (Savjani et al., 2012). Poor water solubility was observed in 70% of new pharmaceutically active ingredient candidates in recent years (Kawabata et al., 2011). Drugs, which are poorly soluble in water, are absorbed slowly inside the body; this leads to inadequate bioavailability with both stomach and intestine mucosal toxicity, thus postponing clinical development of the drug (Le Garrec et al., 2004; Savjani et al., 2012). Some steviol glycosides including Ste, Reb A, and Ru have been found to have solubilizing properties (Table 23.3). Zhang et al. reported that 10% Ru can enhance the solubility from 0.8×10^{-2} mg/mL at 0.1% Ru to 8.5 mg/mL at 10% Ru for etoposide (Zhang et al., 2012) and from 0 to 2.32 mg/mL at 10% Ru, respectively (Zhang et al., 2011a,b). Sizes of Ru-etoposide complex and Ru-curcumin complex are 6.3 ± 0.6 nm and 8 nm, respectively. These water-soluble complexes can maintain their anticancer activities (Zhang et al., 2012). They published that paclitaxel solubility in water can be increased from 1.6 to 6.3 mg/mL with 10%–40% Ru at a particle size of 6.6 nm. In addition, over 80% of this complex has remained soluble in the gastric and intestinal fluids (Liu et al., 2015). Moreover, compared to taxol, the complex of paclitaxel-Ru has almost four times greater permeability in the Caco-2 cell monoculture (Liu et al., 2015) (Table 23.3). Nguyen et al. have reported that Ru can enhance the liquiritin solubility in water from 0.98 to 4.7 mg/mL (Nguyen et al., 2014). Furthermore, it can increase the solubility of teniposide (from 0 to 3.42 mg/mL) (Nguyen et al., 2014) and quercetin (from 0 to 7.7 mg/mL) (Nguyen et al., 2015). The Quercetin-Ru complex can also improve the inhibition activity of quercetin against 3CLpro of severe acute respiratory syndrome (SARS) and human intestinal maltase while maintaining its DPPH radical scavenging and mushroom tyrosinase-inhibiting activities (Nguyen et al., 2015). RebA can enhance the water solubility of quercetin from 0 to 1.46 mg/mL (Nguyen et al., 2015) (Table 23.3). Curcuminoids from turmeric powder, *Curcuma longa* L., have scavenging activities against reactive oxygen species (ROS) and free radicals (Ahsan et al., 1999), effective for antidiabetic usage by decreasing blood glucose levels (Nishiyama et al., 2005), and nematicidal activities (Kiuchi et al., 1993). They can also suppress the proliferation of various tumor cells of the head, neck, lung, pancreas, breast, and prostate as well as against leukemia (Sandur et al., 2007). Although curcuminoids are highly safe and well tolerated by patients, even at very high doses ($\leq$12 g per day) without showing toxicity in vivo studies (Shoba et al., 1998; Cheng et al., 2001; Lao et al., 2006), they have not yet been approved as a therapeutic component due to their poor water solubility (Araujo et al., 2010), chemical unstableness in alkaline solutions (Wang et al., 1997; Price and Buescher, 1997), rapid metabolism (Pan et al., 1999), poor membrane permeation (Wahlang et al., 2011), low bioavailability, and insufficiency to reach the blood concentrations required to affect disease markers or clinical end points, even at chronic doses up to 12 g per day (Lao et al., 2006). The Joint FAO/WHO Expert Committee on Food Additives only approved the use of curcuminoids as food additives if they are extracted

TABLE 23.3 Solubilization of Insoluble Compounds Using Steviol Glucosides and Their Properties

Steviol Glucosides	Compound	Solubility (mg/mL)	Size (nm)	Biological Activity of Soluble Complex	Reference
Rubusoside (10%, w/v)	Etoposide	8.46	6.3 ± 0.6	- Reduced the viability of HT-29, MDA-MB-231, and PC3 cancer cells	Zhang et al., 2011a,b
	Liquiritin	4.7	ND	ND	Nguyen et al., 2014
	Teniposide	3.42			
	Curcumin	2.32	8.0	- Reduced the viability of Caco-2, HT-29, MDA-MB-231, and PANC-1 cancer cells	Zhang et al., 2011a,b
	Quercetin	7.7	ND	- Enhanced inhibition activity against human intestinal maltase, 3CL-protease of SARS - Maintained mushroom tyrosinase inhibition activity	Nguyen et al., 2015
Rebaudioside A (10%, w/w)		1.5		ND	
Rubusoside (40%, w/v)	Paclitaxel	6.26	6.6	- Resulted 4.0 times increase in permeability in Caco-2 cells monocultures - Maintained anticancer activity similar with taxol in DMSO	Liu et al., 2015
Stevioside (8%, w/v)	Extracted curcuminoids from turmeric, *Curcuma longa*	11.3	110.8	- Maintained antioxidant activity and inhibition activity against NS2B-NS3pro of dengue virus type 4	Nguyen et al., 2017
Rebaudioside A (8%, w/v)		9.7	95.7		
Stevioside glucosides (8%, w/v)		6.7	32.7		

from natural source materials. From turmeric powder, Nguyen et al. have directly obtained 11.3, 9.7, and 6.7 mg/mL of water-soluble curcuminoids by using Ste, RebA, and stevioside glucosides (SG), respectively (Nguyen et al., 2017). These water-soluble extracts are nano-sized particles with >80% stability at pH 6.0–10 solutions (Nguyen et al., 2017). The prepared water-soluble turmeric extracts with Ste, RebA, and SG showed inhibition activities (IC_{50}) against NS2B-$NS3^{pro}$ of dengue virus type IV with the value of 14.1, 24.0, and 15.3 μg/mL, respectively (Nguyen et al., 2017) (Table 23.3). Wheat bran (WB) is obtained during the milling process as a by-product, and it contains abundant nutrients and bioactive substances such as carbohydrates (60%), protein (12%), fat (0.5%), minerals (2%), phenolic acids, arabinoxylans,

flavonoids, caroteinoids alkylresorcinol, and phytosterols (Onipe et al., 2015; Slavin, 2003; Javed et al., 2012), known to have health-promoting properties by controlling the glycemic index, decreasing the cholesterol level in plasma, and suppressing the growth of human colon cancer cells. Furthermore, they possess potential prevention properties against oxidative, microbial, inflammatory, and carcinogenic activities (Pruckler et al., 2014; Jensen et al., 2006; Liu et al., 2012; Brouns et al., 2012). Lim et al. have extracted water-soluble polyphenol from WB by using Ru, Ste, RebA, and steviol glucosides (Lim et al., 2016). Total phenol contents in a WB extract prepared by Ru, Ste, RebA, and SG have been found to be 1.19, 1.13, 1.23, and 1.13 times higher, respectively, than that of WB extract prepared using water (Lim et al., 2016). DPPH radical scavenging activities (SC_{50}) of water-soluble WB extracts prepared using water, Ru, Ste, RebA, and SG are 8.76 ± 0.3, 4.87 ± 0.3, 5.34 ± 0.22, 7.27 ± 0.1, and 7.82 ± 0.02 mg/mL, respectively. Thus, WB extracts prepared by Ru, Ste, RebA, and SG possess higher antioxidant activities than WB extracts prepared using water (Lim et al., 2016).

23.3.2 Fructose Transporter (GLUT5) Inhibitor

Among various glucose transporters in human cells, GLUT1 is expressed in most tissues (Yoshikawa et al., 2011; Lange and Brandt, 1990). The over-expressed GLUT1 might be relevant to obesity and noninsulin-dependent diabetes (Miele et al., 1997). Unlike GLUT1, GLUT5 is generally expressed in the small intestine and absorbs fructose from the lumen (Ellwood et al., 1993). Overconsumption of fructose is considered to cause deleterious metabolic effects, thus GLUT5 becomes an increasingly important target for human health (Basciano et al., 2005; Rutledge and Adeli, 2007). Unlike glucose, insulin does not regulate fructose in serum (Litherland et al., 2004). An increase in fructose consumption is correlated with production of lipogenesis and triglyceride at an organism level, and it leads to insulin resistance (Basciano et al., 2005, Rutledge and Adeli, 2007). George Thompson et al. have studied the inhibition effect of Ru on the GLUT1 and GLUT5 of humans expressed in insect cell culture and found that Ru can inhibit both GLUT1 and GLUT5 with IC_{50} values of 4.6 and 6.7 mM, respectively, while Ste does not have such inhibition activities (George Thompson et al., 2015). By using the in silico docking method, Rub was found to interact to the active sites of GLUT1 and GLUT5 in distinguishable form due to a key residue of tryptophan in GLUT1 and alanine in GLUT5 (George Thompson et al., 2015). Ru is a natural solubilizer that can combine with other GLUT drugs to enhance their solubility. In addition, it has effects against GLUT1 and GLUT5.

23.3.3 Effect on Renal Function

By interfering with basolateral entry, Ste and steviol can inhibit transepithelial transport of *para*-aminohippurate (PHA) in rabbit renal proximal tubules (Srimaroeng et al., 2005). Ste has no inhibition effect on either PAH (human organic anion transporter 1, hOAT1) or ES (estrone sulfate, hOAT3) absorbance. However, steviol can significantly and amount-dependently inhibit PAH and ES uptake in hOAT1 and hOAT3 (Srimaroeng et al., 2005). IC_{50} of steivol for hOAT1-mediated PAH transport has been reported to be 11.1 μM compared to 62.6 μM in the case of hOAT3-mediated ES absorbance (Srimaroeng et al., 2005). Michaelis-Menten inhibition constants (K_i) for steviol transport mediated by hOAT1 and hOAT3 are reported to be

2.0 ± 0.3 and 5.4 ± 2.0 μM, respectively (Srimaroeng et al., 2005). As low as 1 μM steviol can increase the efflux of [^{3}H]PAH (transstimulated) through both hOAT1 and hOAT3 (Srimaroeng et al., 2005). Therefore, steviol has great promise to minimize the renal removal of anionic drugs and their metabolites (Srimaroeng et al., 2005).

23.4 CONCLUSION

Steviol glucosides have various applications including their use as an alternative sugar for food, as an ingredient for pharmaceuticals, and as solubilizing agents. Traditionally, steviol, rubusoside, steviolmonoside, steviolbioside, and rebaudioside B are in trace amounts in the stevia leaf, limiting their availability. With our method, the soluble complex of steviol glucosides with less-soluble compounds increased its permeability while retaining the biological activity. Therefore, the production of compounds from stevioside by using the immobilized β-glucosidase with high conversion yields has great potential to be used in industrial applications.

Acknowledgments

Authors thank Eugene Sim at Green Bio Sciences and Technology, Seoul National University for improving the English language of the manuscript. This work was partially supported by the Agriculture, Food and Rural Affairs Research Center Support Program (710012031HD220), and by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the High Value-added Food Technology Development Program (116013032HD020) funded by the Ministry of Agriculture, Food and Rural Affairs, Republic of Korea (D. Kim), by the Basic Science Research Program through the National Research Foundation of Korea (NRF) (NRF-2015R1D1A1A01056929; D. Kim, and 2015R1D1A4A01020522; T.T. Hanh Nguyen), and under the framework of International Cooperation Program managed by the NRF (2016K2A9A2A08003613).

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CHAPTER

24

Enzymatic Processing of Juice From Fruits/Vegetables: An Emerging Trend and Cutting Edge Research in Food Biotechnology

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24.1 INTRODUCTION

Fruits and vegetables are an imperative source of dietary nutrients such as vitamins, fibers, carbohydrates, pigments, organic acids, etc. These naturally occurring constituents are assumed to provide an extensive range of physiological benefits through their antioxidant, antiallergic, anticarcinogenic, and anti-inflammatory properties. The availability of fruits and vegetables is seasonal throughout their geographical extent so as to access the nutritional benefits, year-round processing and preservation are a prerequisite. Because the moisture content of fresh fruits and vegetables is more than 70% (weight basis) and they are a highly perishable commodity with a short life span, it is estimated that every year nearly 20%–50% of the fruits/vegetables are lost due to environmental conditions, mishandling during transportation, and a lack of preservation and processing practices (Singh et al., 1994). Thus, intensive care is required in both postharvest storage and during processing techniques. The richness of nutrients in fruits/vegetables encourages the food biotechnologist to convert them into various food products. Juice products are the most typical food items commonly produced from fruits and vegetables that are important not only for human wellness but also from a commercial point of view. During the 1930s, the fruit and vegetable juice industry faced huge difficulties associated with low yields and filtering to obtain an acceptable quality of the final product (Bhardwaj and Pandey, 2011). With advancement in human civilization, different

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https://doi.org/10.1016/B978-0-12-813280-7.00024-4

techniques such as mechanical, thermal, physicochemical, and enzymatic processing have been developed for the extraction of juices. Currently, enzymatic treatments are widely used as an integral constituent of modern juice processing from fruits and vegetables.

Enzymes have been used by humans since time immemorial, either knowingly or unknowingly. They possess diverse biological, physiochemical, and catalytic properties with defined substrate specificity, which enables raw material biotransformation with low/no inhibitor formation, thus manifesting a better quality of the end product (Uenojo and Pastore, 2007). Enzymes are being rigorously and scientifically developed from different sources (microbial, animal, plant) to be used in food preservation and better utilization, even during the off-season. In this milieu, the processing of fruits/vegetables with biocatalysts has improved the production of juices, pulps, purees, jams, jellies, etc. This promotes the increased utilization of processed products with least waste production. Enzymes such as cellulase, amylase, tannase, naringinase, hemicellulose, and pectinases are highly suitable for optimization of the juice treatment process. The use of these enzymes is considered superior to mechanical and thermal comminution processing of several fruit and vegetable masses (Roumbouts and Pilnik, 1978). The main purpose of using cellulase, hemicellulase, pectinases, and proteases, or a cocktail of these enzymes in juice processing is to accelerate juice extraction from raw materials, to increase processing efficiency, and to generate a clear and visually attractive final product. This novel venture is highly encouraging and its performance at the industrial scale needs to be investigated with special emphasis on aspects such as screening for efficient enzyme producers and their inducing agents for enhanced extracellular production. Along with this requirement, there is also a need for concocting a minimum number of steps to be adopted for enzyme extraction so that these enzymes are capable of being utilized in their crude form, which is a key advantage for large-scale application of enzyme-based juice production technologies.

24.2 CONVENTIONAL JUICE PROCESSING

Juice processing involves several sets of operational activities such as peeling, extraction, yield recovery, clarification, and bitterness removal. Basically, this process is required to increase the shelf life, improvise the organoleptic properties, and retain or increase the nutritional property of the juices through such processing. The processing technologies widely practiced in the fruit/vegetable processing industries are thermal processing, mechanical scuffing, and the application of chemicals. Mechanical peeling involves the application of abrasive devices, drums, rollers, knives, milling cutters, etc. Chemical treatments such as the addition of sodium hydroxide solution are mostly used to degrade the pulp layer adhered to the cuticular tissue by acting on the thin waxy layer of the skin (Caceres et al., 2012). This chemical process is also known as lye peeling (Shirmohammadi et al., 2012). Conventionally, the term thermal processing is elaborated by blanching, pasteurization, and heat sterilization techniques that are mostly initiated by heat generation through an electric thermostat or combustion of fuels that finally gets transferred and dissipated by a convection or conduction mode within the entire system. Radio frequency heating, microwave heating, and ohmic and dielectric heating are some of the novel thermal processing methodologies. The basic concepts reside in uniform utilization of heat by a single flow of ohmic electric current; they are practically effective compared to other overheating techniques. The processes mostly reduce

or completely eliminate the microbial load, thereby ensuring the microbiological safety of the product. Besides ensuring the shelf life of the product, researchers also realized the utmost importance of the nutritional content of the product, which forced them to develop novel non-thermal processing technologies that are at the verge of commercialization. High hydrostatic pressure, high-intensity pulsed electric fields, dense phase carbon dioxide (DPCD), ozone, ultrasound, and ultraviolet light are the main weapons of non-thermal processing, which not only overcomes the microbial hurdle but also improves the nutritional quality indices and bioactive content level over conventional heating. Although all non-thermal processing techniques show promising results in improving the retainment of phytochemicals such as vitamins, anthocyanins, and polyphenols, ozone treatment is the last choice because of its high oxidizing property. Despite of all the potential of the processes, there still remains a gap to reach this milestone without compromising the nutritional aspects (Rawson et al., 2011a, 2011b; Pingret et al., 2013).

24.3 NEED OF ALTERNATIVE TECHNOLOGIES FOR FRUIT/VEGETABLE PROCESSING

The retainment of phytochemicals and nutrients in fruit and vegetables broadly depends on the processing of the food. Different types of conventional food processing are reported, such as degradation of appearance, texture, phytochemicals, and nutrients of the food products (Tiwari and Cummins, 2013). The conventional juice extraction process, which involves peeling, slicing, blending, and pressing, is not only energy sapping and time consuming, but also results in a low yield of juice (Aviara et al., 2013). Thermal processes resulted in enzyme inactivation, texture changes, destruction of antioxidants, loss of water-soluble and oxygen-labile nutrients such as vitamin C and B, a reduction in phytochemical content, and a degradation of flavonoids (Çopur and Tamer, 2014). A minimum loss of Vitamin C was observed during treating orange juice with ohmic heat treatment. Heat treatment also decreased the levels of bioactive components such as total anthocyanin, ascorbic acid and carotenoids of mulberry fruit extract, pineapple juice, and cashew apple juice (Vikram et al., 2005). The ultrasound treatment of orange, strawberry, and tomato juices showed the degradation of Vitamin C content; that may be due to free radical formation and the production of oxidative products (Tiwari et al., 2009). A decrease in the phenolic content was observed in sonicated watermelon juice (Rawson et al., 2011a, 2011b). Alighourchi et al. (2008) reported a decrease in the total anthocyanin content. For the peeling of fruits and vegetables, conventional peeling possesses several disadvantages such as high peel loss in mechanical and thermal peeling and the high cost of chemical use in chemical peeling. Bishai et al. (2015) mentioned 15%–40% and 25%–40% peel loss of potatoes in mechanical and steam peeling, respectively. In the conventional evaporation method to concentrate the juice, there are several drawbacks such as a loss of volatile aroma compounds, degradation of nutritional property, color change, and the high energy consumption (Iborra et al., 2016). The conventional filtration process is labor intensive, time consuming, and discontinuously operated. Pap et al. (2010) reported a reduction of anthocyanins and flavonols, to some extent, of black currant juice in the centrifugation and ultrafiltration processes. Ultrafiltration and osmotic distillation of kiwi fruit juice caused a reduction of total antioxidant activity by 4.4% and 11.1%, respectively (Cassano et al., 2006). The debittering of citrus juice using AmberliteIRA-400 (exchange resin) shows a reduction

of antioxidant capacity, the lowest radical scavenging activity, a low inhibitory effect of juice samples on lipoperoxidation, low levels of glutathione oxidation, and DNA damage, which included the oxidation of the purine and pyrimidine bases (Cavia-Saiz et al., 2011). To overcome such drawbacks of conventional processing techniques, researchers developed innovative processing methodologies using enzymes to fill the gap without compromising the organoleptic and nutritional quality of juices. While considering the economics of the process, the cost-effective production of food enzymes using cheap substrates enables the technology to be applied in industries. The process of application of enzymes is not only sustainable but also generates no hazardous toxic by-products, which thereby makes it safe and eco-friendly for society.

24.4 ENZYMATIC PROCESSING

The accelerating demand for novel biocatalysts in the juice industry has uplifted the manufacturing of enzymes from various sources. Enzymes are the biological catalysts with high substrate specificity and a high tolerance to a broad range of pH and temperatures. Enzymatic degradation of the biomaterial depends upon the incubation time, temperature, enzyme concentration, agitation, pH, and type of substrate of the reaction system. Cellulose, hemicellulose, pectin, lignin, and proteins are the main components present within the primary cell wall of fruits and vegetables. During the processing of fruits/vegetables, raw materials are hydrolyzed by different enzymes to their respective monomeric units for the preparation of the corresponding finished products. Cellulase, hemicellulases, and pectinases have been widely used as macerating enzymes in the fruit/vegetable juice industries (Bhat, 2000). Because tropical fruits contain more cellulose and hemicellulose components compared to other fruits, the use of pectinases combined with hemicellulase and/or cellulase is recommended not only for fruit maceration but also to develop cloud stability, texture, and proper concentration of nectars and purees in the final product by reducing viscosity (Kashyap et al., 2001). Enzymatic peeling of fruits/vegetables is also a very promising methodology for avoiding the minimum meat loss (Bishai et al., 2015). Fruit and vegetable juices contain high amounts of concentrated polysaccharides. Due to the presence of these components, juices become highly opaque and viscous after extraction and thus enzymatic treatment becomes necessary in the clarification step to produce haze-free, clear juices. Pectinases have been commonly used in juice processing, mostly during the pressing and straining of juices to reduce opaqueness and viscosity and to remove suspended particles of pectinous substances. Tropical fruits such as mangoes, peaches, guava, bananas, and avocados have mostly been used to produce nectars and purees from fruits. Those products have been diluted or blended with other fruit products to make even more delicious fruit cocktails. Polygalacturonase and propectinase, along with cellulase activities, can partially hydrolyze the propectin of fruits to produce highly viscous purees from fruits (Bhat, 2000). By mixing water, sugar syrup, and citric acid to the juices, the cloud particles settle down and often form gels, resulting in a clear supernatant layer that may reduce the desirability of the products. Therefore, a very important quality factor of juice is that cloud particles should be stable. In the production of fruit juices, the use of exogenous enzymes prior to the pulping stage not only results in cloud stability but also generates higher yields.

24.5 ENZYMES FOR FRUIT/VEGETABLE JUICE PRODUCTION

Enzymes such as pectinases, cellulase, hemicellulases, naringinase, laccase, tannase, amylases, glucose oxidase, etc., have immense application in the food processing industries. They hydrolyze the cell wall of the fruit/vegetable, releasing all nutrients and therefore facilitating improved juice yields, shortening the processing time, and enhancing the sensory characteristics (Mojsov, 2012). Hence, the treatment of a single enzyme or concoctions of preferred enzymes on the substrate is the deciding factor of the final product obtained. Althuri et al. (2017) reported that microbial sources are mostly preferred over plant and animal sources as they do not produce phenolics and other exogenous inhibitors. Hence, the selection of microbial strains that can yield a high quantity of the desired enzyme with minimum by-product formation is a major prerequisite for commercialization. Some of the major enzymes are discussed in the following sub-sections.

24.5.1 Pectinases

Pectinases are one of the few first groups of hydrolytic enzymes used at the commercial level. This group of enzymes includes a range of biocatalysts that bear the potential to cleave high molecular weight pectic polysaccharides into their respective monomeric galacturonic units. By nature, pectic substances are complex biomolecules present as the major components of middle lamellae and within the primary cell wall in complexation with cellulose microfibril and structural protein to form an insoluble complex biomolecule. This complex structural arrangement is responsible for the formation of "cloud particles." This molecular representation is mostly due to the presence of a positively charged protein molecule surrounded by pectin molecules that are negatively charged. The viscosity or cloudiness of the juices gets improved upon the addition of pectinases. Because of their negative charge, pectin molecules repel each other and, in the presence of the enzymes, get cleaved while the protein portion gets exposed. As a result, electrostatic repulsion gets reduced between the particles, which finally forces them to form larger particles and settle down at the bottom (Kashyap et al., 2001). The role of individual pectinases will be elaborated in the following sub-section.

24.5.1.1 Pectinesterases (PE)

Pectinesterases or pectinmethylesterases (PMEs) (E.C. 3.1.1.11) mostly originate from plants, bacteria, and pathogenic fungi belonging to the CAZy class 8 of carbohydrate esterases. PMEs are biologically active in monomeric form and bear a molecular mass within the range 25–45 kDa (Kohli et al., 2015). Although the exact mode of action of PMEs is a matter of controversy, hypothetically there are three modes of action of PMEs that differ on the source of their origin. Single-chain mechanisms and multiple-chain mechanisms are mostly exhibited by PMEs of bacterial and plant origin. Fungal PMEs mostly work by the multiple-chain mechanism. The preference of binding of de-methoxylated pectin justifies the need of free carboxyl residue within the vicinity of the catalytic active site of PMEs. The carboxyl group of an Asp residue present within the catalytic site gets negatively charged and is stabilized by a hydrogen bond of an Arg that cleaves the carbonyl carbon residue of homogalacturonan methyl ester through a nucleophilic attack mechanism. Thereafter, a negatively charged tetrahedral intermediate form gets stabilized by an oxyanion hole generated by the side chain

of Glu. In the second step, an Asp residue performs the role of a proton donor of the active site and the homogalacturonan backbone stays stable within the substrate binding cleft to perform the coming de-methoxylation step (Jolie et al., 2010).

24.5.1.2 Pectin Lyase (PNL)

Pectin Lyase (E.C.4.2.2.10) is also one of several inducible enzymes, mostly produced by the microbial genus of *Aspergillus*, *Penicillium*, and *Fusarium*, although a few reports also mention its presence in plants and animals. PNLs are mainly categorized into PNLA and PNLB. These enzymes show optimum activity in a wide range of pH from 5.5 to 10.5 and temperature from 35 to 65°C. PNLs preferentially catalyze highly esterified pectin in a random fashion through transelimination of glycosidic linkages and produce unsaturated methyl oligogalacturonates as end products. There is no absolute requirement of Ca^+ ions for PNL activity but their catalytic optima gets stimulated by the presence of Ca^+ ions. Research reports reveals that PNLA derived from *Aspergillus niger* produces mono-, di-, tri-, and tetragalacturonates as end products (Yadav et al., 2009).

24.5.1.3 Pectate Lyases

Pectate lyases or polygalacturonate lyases or pectate transeliminases (E.C. 4.2.2.2.) were first discovered and isolated from the culture of *Erwinia carotovora* and species of *Bacillus*. Depending on the distinctive properties and order of discovery, pectate lyases are named Pel A, Pel B, Pel C or Pel 1, Pel 2, Pel 3, etc. According to carbohydrate-active enzyme classification, pectate lyases belong to a bulky class of polysaccharide lyases (PL), where they are further categorized into five families: PL 1, PL 2, PL 3, PL 9, and PL 10. The general mechanism of action of β-elimination is similar in all pectate lyases. They catalyze the cleavage of de-esterified pectin backbone built of the α-1,4-linked polygalactosyluronic acid present in pectic acid by the transelimination reaction, forming unsaturated 4,5-D-galacturonate as the end product (Rodriguez et al., 2002; Patta et al., 2014).

24.5.1.4 Protopectinase

Protopectinase (PPases) or pectinosinase is one of the pectin hydrolases that catalyzes the solubilization of insoluble pectin in the presence of water. The mechanism of action of PPases classifies them into A-type or B-type. The inner site of protopectin containing the polygalacturonic acid is mostly cleaved by A-type PPases whereas the outer site that connects the cell wall components with polgalacturonate are cleaved by B-type PPases. The biological and physicochemical properties of all types of A-type PPases are similar and have a molecular mass of 30 kDa. PPase-S and -L are mostly basic in nature and PPase-F is acidic. PPase-T, -C, and -B have molecular masses of 55, 30, and 45 kDa, respectively. PPase-T has an isoelectric point (pI) of 8.1 and PPase-C and -B have a pI of ~9.0. The hydrolysis of protopectin is catalyzed by all these types of PPases, which in turn helps in reducing the viscosity of the medium (Jayani et al., 2005).

24.5.1.5 Polygalacturonases (PG)

Polygalacturonases (PG), classified into the glycosyl hydrolases family, catalyzes hydrolysis of α-1,4 glycosidic bonds between PGA, generating D-galacturonate as the end product. On the basis of hydrolase activity, they are divided into *endo*-PG or poly (α-1,4 D-galacturonide).

Glycano hydrolase catalyzes the random cleavage of pectic acid whereas *exo*-PG or poly (α-1,4 D-galacturonide) galacturonohydrolase acts in a sequential manner on non-reducing ends of pectic acid, producing mono-galacturonate or di-galacturonate (in some cases) as the end product (Pedrolli et al., 2009). Pectinases can be produced by various kinds of microorganisms such as *Aspergillus* sp., *Bacillus* sp., *Penicillium* sp., *Pseudozyma* sp., *Streptomyces* sp., etc., through submerged fermentation (SmF) and solid-state fermentation (SSF) (Garg et al., 2016). Sittidilokratna et al. (2007) produced polygalacturonase, pectatelyase, and pectinlyase from *Bacillus* sp. strain N10 and *Escherichia chrysanthemi* strain N05. The obtained titers of these enzymes were 135.14, 16.07, and 4.48 unit/mL for polygalacturonase, pectatelyase, and pectinlyase, respectively. Using mosambi peel as a substrate, polygalacturonase was produced through SSF by *Aspergillus awamori nakazawa* MTCC 6652. Partial purification using the activated charcoal of polygalcturonase facilitated a 34.8-fold increase in the activity with 69.8% recovery; it was further employed for apple juice clarification (Dey et al., 2014). On the basis of mode of action, there are three types of pectic enzymes: (i) depolymerizing enzymes (hydrolases, lyases); (ii) de-esterifying enzymes (esterases), and (iii) protopectinase, already discussed earlier. However, on the basis of pH, they are of two types: (i) acidic and (ii) alkaline pectinases. Acidic pectinases are mostly produced from fungal sources such as *A. niger* and are used for the removal of pectic substances from the system by selective hydrolysis of polysaccharides of the middle lamella. The juices commercially produced by industries include (a) juices with clouds (tomato, prune, citrus); (b) sparkling clear juices (grapes, apple), and (c) unicellular products.

Cloudy citrus juices are mostly stabilized by pectic enzymes such as polygalacturonases. Pectin from oranges is less methylated in comparison to apple pectin because of the natural presence of pectin esterase, which removes the methyl groups. Thereby, the controlled application of pectinases is very important for increasing the yield and cloud stability. Banana is widely accepted for the aroma of isoamyl acetate. But banana juice is too viscous and pulpy, so the pectinase application helps in clarifying the juice as well as increasing the yield. While considering sparkling clear, haze-free juices, pectinases are added to improve the straining and pressing of juices. Apple juices are mostly prepared as an unclarified and unfiltered juice containing pulpy particles. So, pectinases are the only enzymes that de-polymerize the esterified pectin and aid in the separation of a flocculating precipitate through sedimentation, which finally produces an amber-colored sparkling clear juice. Unicellular products are mostly produced by organized tissue transformation into intact cell suspension. The resulting product acts as the base material of pulpy juices. Maceraces are the enzymes mostly employed to serve the purpose of transformation, a phenomenon known as maceration. Pectic enzymes in combination with cellulase are able to isolate protoplasts or separate intact cells practically from every plant tissue. Alkaline proteases are mostly of bacterial origin and their application is mostly used for wastewater treatment of the juice processing industries, degumming fiber crops, paper making, and coffee processing. These enzymes do not possess any direct application to the juice processing industries (Kashyap et al., 2001).

24.5.2 Cellulase

Cellulase is a complex of three different enzymes: endoglucanase (E.C. 3.2.1.4), exoglucanase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21). In cellulose hydrolysis, endoglucanase

(EG) and exoglucanase (CBG) work synergistically to transform cellulose into small cellooligosaccharides and then β-glucosidase (BG) hydrolyzes the cellooligosaccharides into simple sugars (glucose) (Sathya and Khan, 2014; Sindhu et al., 2016).

Cellulases are a part of the macerating enzymes complex (cellulase, xylanase, and pectinases) that is highly used for extraction and clarification of fruit/vegetable juices to increase the yield, improve cloud stability, and decrease viscosity of the juices from tropical fruits such as mangoes, peaches, and papayas (de Carvalho et al., 2008). Sreenath et al. (1994) reported that after extraction of pineapple juices, the leftover pulp contains valuable substances. After treatment with cellulase, these compounds can be extracted with a much improved yield. Chen et al. (2011) found that the extraction rate of flavonoids from *Ginkgo biloba* by cellulase can be increased up to 30%. The extraction of syrup from the date palm (*Phoenix dactylifera* L.) usually produces low-quality products due to its unpleasant texture. This problem can be overcomed by adding cellulase, which reduces the turbidity and increases the extraction of soluble solids of interest.

24.5.3 Hemicellulase

Hemicellulase can be classified as xylanase (E.C. 3.2.1.8) and galactanase (E.C. 3.2.1.89), which hydrolyzes the hemicellulose constituents of fruits and vegetables. These enzymes can be procured from GRAS microorganisms (*A. niger, Trichoderma reesei, Cephalosporium sacchari,* and *Agaricus bisporus*). Hemicellulase works best in combination with pectinase and is used to clarify the juices of apples, grapes, and mandarin oranges. Hemicellulase (Xylanases) also helps in biobleaching of palm fruits, resulting in improvements in optical properties such as brightness and color (Martín-Sampedro et al., 2012).

24.5.4 Laccase

Laccase (E.C. 1.10.3.2) is a copper-containing polyphenol oxidase that oxidizes polyphenols, methoxy-substituted phenols, diamines, and other compounds (Thurston, 1994). Laccase belongs to the family of multicopper oxidase that uses O_2 as the terminal electron acceptor for the oxidation of aromatic and non-aromatic compounds of lignin polymer. Based on laccase's features, its four copper ions are placed under three groups: type 1 (T1, blue copper), type 2 (T2, normal copper), and type 3 (T3, binuclear copper), where T2 and T3 together form a trinuclear cluster (Madhavi and Lele, 2009). Giovanelli and Ravasini (1993) and Gökmen et al. (1998) carried out stability tests of ultrafiltrated and laccase-treated samples and found that those treated with laccase have increased susceptibility to browning during storage. On the contrary, Cantarelli (1986) used a mutant laccase from *Polyporus versicolor* to treat black grape juice. He reported that there was removal of 50% of total polyphenols and higher stabilization than the physical-chemical treatment. Artik et al. (2004) studied the effect of laccase application on the clarity stability of sour cherry juice and found that the phenolic content was decreased by around 70%. On the other hand, Ritter et al. (1992) obtained a stable and clear apple juice by applying laccase in conjunction with ultrafiltration. Cantarelli and Giovanelli (1990) reported that by using laccase followed by ultrafiltration and by the addition of additives such as ascorbic acid and sulfites, the color and stability have improved in comparison to conventional treatments.

24.5.5 Glucose Oxidase

Glucose oxidase (E.C. 1.1.3.4), a flavoprotein, belongs to a class of oxidoreductase and catalyzes the oxidation of β-D-glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor with the simultaneous production of hydrogen peroxide. It is a dimeric protein with a molecular weight of 150–180 kDa, and is composed of two identical subunits. Each subunit binds into a domain: one domain binds to the substrate glucose while the other domain binds non-covalently to a cofactor flavin adenine dinucleotide (FAD). Muller (1928) was the first who isolated glucose oxidase from the mycelia of *P. glaucum* and *A. niger*. The most common sources of GOx were *A. niger, P. notatum, P. glaucum, P. purpurogenum, P. amagasakiense, P. variabile,* and *Alternaria alternate* (Caridis et al., 1991). The mechanism can be divided into a reductive and an oxidative step. In the reductive step, GOx catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone, which can be enzymatically hydrolyzed to gluconic acid. Subsequently, the FAD ring of GOx is reduced to $FADH_2$. In the oxidative step, the reduced GOx is reoxidized by oxygen to yield hydrogen peroxide. Glucose oxidase has significant importance in beverage industries as it is highly used for the removal of trace amounts of oxygen and residual glucose from different sources such as fruit/vegetable juices, beer, wine, and soft drinks in order to prolong their shelf life (Reed and Underkoer, 1966). It also imparts color, flavor, and texture to juices.

24.5.6 Amylase

Amylase is an important hydrolase enzyme that has been widely used for many decades and represents one of the most important enzyme groups in the biotechnology field (Naidu and Saranraj, 2013). Depending upon the mechanism of reaction, catalytic activity, and structure, amylolytic enzymes are classified into three families of hydrolases: a) α-amylases (E.C. 3.2.1.2), b) β-amylases (E.C. 3.2.1.3), and glucoamylases (E.C. 3.2.1.1). Further, it can also be classified as *exo-* and *endo*-amylases (Fleuri et al., 2015). Different types of organisms, including animals, plants, and microorganisms, are the source of amylase production. Microorganisms are used for the external production of amylase. For α-amylase production, microorganisms such as *Aspergillus oryzae, A. niger, Bacillus amyloliquefaciens, B. circulans,* and *B. licheniformis* are involved. α-Amylase is used as starch saccharification in different industries and accounts for about 30% of the enzymes in the world market (Fleuri et al., 2015). In the apple juice industry, the main setbacks are turbidity, slow filtration, damage to the filtration membrane, and gelling after juice concentration due to the high starch concentration (15% of this polysaccharide). Carrin et al. (2004) used α-amylases to clarify apple juice (Granny Smith variety) at different degrees of ripeness. As amylases are calcium-containing enzymes, thus treatments with this fortify apple juices with calcium as a healthy and non-fat alternative (Dey et al., 2014). Okoth et al. (2000) used amylase before pasteurization for passion fruit juice production for increased homogeneity and decreased juice turbidity.

24.5.7 Tannase

Tannase, otherwise known as Tannin acyl hydrolase (E.C. 3.1.1.20), was discovered by Tieghem in 1867 (Aguilar et al., 2007). It is an inducible extracellular enzyme produced by

different animals, plants, and microbes such as fungi, bacteria, and yeasts (Hadi et al., 1994; Purohit et al., 2006). Tannase is basically involved in the hydrolysis of hydrolysable tannins by hydrolyzing the ester bonds in tannic acid and facilitating the release of gallic acid and glucose. This hydrolytic action decreases the binding ability of tannin with protein molecules due to the cleavage of the ester bond, thus avoiding the aggregation of macromolecules (Mahapatra et al., 2005). The presence of tannin is responsible for the bitterness, astringency, and color of the juice. In order to remove bitterness causing molecules enzymatic treatment process facilitate over other conventional treatment process without changing unique nutritional property of the juice. Tannase was reported for the debittering of pomegranate juice where 25% tannin degradation occurred and 49% degradation was observed using a concoction of enzymes with no negative impact on biochemical and quality attributes. The conventional industrial practices used to be the addition of gelatin to the fruit juice where the protein has got a strong affinity to bind to the tannin molecules so as to get a precipitation and thus the percentage bitterness is also considerably reduced (Rout and Banerjee, 2006). Sharma et al. (2014) used 2% tannase produced by *A. niger* for the detannification of guava juice (*Psidium guajava*) and reported 59.23% of tannin removal after 60 min (Sharma et al., 2014). Ferreira et al. (2013) applied tannase in orange juice for improvement of the chemopreventive potential. Tannase modified the polyphenolic composition of the orange juice and acted in naringin and hesperidin in the removal of glycosides at 40 °C in a 60 min reaction time. This biotransformation process significantly increases the antioxidant capacity of the juice and thus, acts in a broader and more significant way than other enzymes in the biotransformation of the polyphenols of the orange juice.

24.5.8 Naringinase

Naringinase (E.C. 3.2.1.40) is a hydrolytic enzyme that posses the activity of both α-L-rhamnosidase (E.C. 3.2.1.40) and β-D-glucosidase (E.C. 3.2.1.21). It has numerous important applications in the food and pharmaceutical industries. It is used in the removal of hesperidin haze from orange products, bitterness removal from citrus juice, and enhancing the aroma of grape juices as well as the production of glycopeptide antibiotics, deglycosylation of flavonoids, and gellan depolymerization. Though it can hydrolyze many glycosides, for example, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides, naringin, hesperidin, and rutin, it explicitly hydrolyzes naringin to naringenin. In the first step of hydrolysis, α-L-rhamnosidase splits naringin into rhamnose and prunin (4,5,7-trihydroxy flavanone-7-glucoside) by liberating one molecule of L-rhamnose. In the second step, β-glucosidase converts prunin glucose and naringenin (4,5,7-trihydroxy flavanone) by liberating one molecule of D-glucose (Awad et al., 2016; Srikantha et al., 2016). Naringinase can be produced externally by fungi, yeasts, and bacteria, for example, *A. niger, A. flavus, Staphylococcus xylosus, Williopsis californica*, etc. (Chen et al., 2013). Naringin (4,5,7-trihydroxy flavanone-7-rhamnoglucoside) present in several citrus fruits is responsible for the bitterness of juice. The use of naringinase is very helpful for the hydrolysis of naringin in the tasteless component, naringenin. About 74% removal of naringin was found by using a naringinase concentration of 1.0 g/L in citrus fruit juice with incubation at 40°C for 4 h (Patil and Dhake, 2014). Ni et al. (2014) used pectinase and naringinase to improve pomelo (*Citrus grandis*) juice yield. A concoction of pectinase and naringinase was used for a combined processing treatment of peeling and enzymatic hydrolyzes resulted in a juice yield of 42.3%. Recently, a new technique was applied for the debittering of grape

fruit juice using controlled immobilized naringinase on electrospun cellulose acetate nanofibers, where positively charged naringinase and negatively charged alginate were alternately coated on negatively charged electrospun cellulose acetate nanofibers. This process successfully converted 22.72% naringin to prulin and removed 60.71% limonin (Huang et al., 2017).

24.5.9 Limonate Dehydrogenase

Limonate dehydrogenase is a hydrolysable enzyme used in different citrus juice processing industries for the removal of the bitterness-causing agent. Limonin is the bitterness-causing agent present in citrus juice and causes delayed bitterness. It is a highly oxygenated triterpene derivative in the group of limonoids. In citrus juice, low pH conditions facilitate the conversion of limonoate A-ring lactone to limonin and thus causes delayed bitterness. Not only is this phenomenon a great hindrance to the citrus juice processing industry, but it also affects customer acceptability. This problem can be overcome by either preventing the formation of limonin precursors prior to harvest or by the biotechnological removal of limonin (Verma et al., 2010). Limonoate dehydrogenases are involved in the dehydrogenation of the hydroxyl group at C-17 of limonoids and create non-bitter 17-dehydrolimonoids. It hits all limonoids, excluding deoxylimonin and deoxylimonate, if their D-ring is open (Munish et al., 2002; Hasegawa, 1999). *Pseudomonas putida* is characteristically able to produce intercell limonoate dehydrogenase, which was used for limonin removal from citrus juice. Limonoate dehydrogenase from *P. putida* with the presence of NAD as cofactor show a nine-fold reduction in limonin content (Verma et al., 2010)

24.6 FUTURE TRENDS AND PERSPECTIVES

Enzymes are widely used in processing aids to improve the final product quality and increase the yield while reducing the overall cost. The integration of this biological catalyst is a well-established method, but there is still scope for further improvement in enzyme production. Researchers are putting in consistent effort to make this application of enzymes more effective and diversified (Fernandes, 2010). Multidisciplinary studies related to enzymes should be performed to design a new process or improve the available process, develop novel enzyme preparations with functionally synergistic effects, and develop new methods for easier and cheaper production of these enzymes to fulfill the demands of the fruit and vegetable processing industries. Solid-state fermentation is one of the promising techniques, bestowing advantages for the production of industrially important commercial food enzymes. Furthermore, thorough research is required for the optimization of the process, which could be carried out for pilot plant studies based on the laboratory development followed by commercialization.

24.7 CONCLUSION

As far as the processing of food products is concerned, the use of some conventional processes can alter the quality of the food products by depleting their natural constituents. Thus, there is a requirement for advanced processing mechanisms that can serve products

without compromising their natural quality. In this scenario, the use of enzymes in the juice industry has increased significantly because of the specificity of enzyme action, the retainment and enhancement of nutritional property, high yield, and easy handling as well as their cost-effective and environmentally friendly nature. Thus, enzyme-mediated juice processes are gaining more impetus in serving enhanced nutraceutical properties, sensory attributes, and increased shelf life of fruit juice. A degree of care and ingenuity is often required to adapt these sensitive biological catalysts to industrial processes, and the amalgamation of basic biochemical knowledge and modern biotechnology is opening up new areas of application, especially for enzymes of microbial origin. Rigorous and dedicated research efforts are constantly being made to make the application of enzymes in juice processing effective and diversified. Still, advanced and precise research is required for the selection and mixing of enzymes with juice to ensure proper kinetic activities for optimum functionality and productivity.

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CHAPTER

25

Non-*Saccharomyces* Yeasts: An Enzymatic Unexplored World to be Exploited

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25.1 INTRODUCTION

Since the French microbiologist Louis Pasteur discovered the transformation of grapes into wine, vinification involving the participation of yeast has been analyzed in detail. About 130 years later, however, there are still some areas that should be studied in more detail (Pretorius, 2000). One aspect to be improved is that of many non-*Saccharomyces* yeasts not usually associated with the juices. These yeasts, present in all fermentations, have high metabolic activity and are very active. In fact, their metabolites can have a big impact on the final quality of the alcoholic beverage. Initially, these yeasts were considered a source of problems related to the microbial production of wine. Today, winemakers use indigenous yeasts as a basic part of the authenticity of their wines and they will allow the yeast to incorporate various desirable characteristics. Every time we find more evidence to support this, we note that increasing the number of scientific contributions can improve the knowledge of the role in fermentation of non-*Saccharomyces* yeasts, also called low fermentative capacity yeasts (Padilla et al., 2016).

Non-*Saccharomyces* yeasts have been isolated from the fruit cuticles, but also on the cellar equipment (Martini, 1993). Although they are present in an environment very different than the juice or beverage, the types of microorganisms found in the fruit will modify the ecology of the subsequent fermentation, particularly in the early steps. Microbes are considered to colonize around the stomata of the fruit, which secretes small amounts of exudate (Johnson, 2013). *Kloeckera* and *Hanseniaspora* (its sexual anamorph) are the most widespread apiculate yeasts, frequently accounting for more than half the yeast cells on ripe fruits (Pretorius et al., 1999). Other genera founded on berries are *Zygosaccharomyces*, *Pichia, Candida*, *Wickerhamomyces, Metschnikowia,* and *Torulaspora* (Kurtzman, 2012). Numerous other yeasts

https://doi.org/10.1016/B978-0-12-813280-7.00025-6

are also present, some of which have an impact on the sensory characteristics of beverages, such as *Kluyveromyces*, *Sporidiobolus*, and *Hansenula*. These yeasts are the most frequently found in the juice before inoculation with *Saccharomyces cerevisiae*. Among healthy berries, the *Saccharomyces* species is difficult to be isolated (Boynton and Duncan, 2014). Several authors have described a sequential process of supremacy by the various low fermentative capacity yeasts during the fermentation, followed by *S. cerevisiae*, which finishes the process. Non-*Saccharomyces* yeasts can be detected during vinification and their supremacy during the first stages can modify the final wine characteristics (Jolly et al., 2003) (Table 25.1).

For many years, the importance of low fermentative capacity yeasts in alcoholic beverages was limited and eradicated by using selected *S. cerevisiae* strains: non-*Saccharomyces* were considered to be spoilage yeasts (Andorrà et al., 2010). Nevertheless, in the last 30 years, there has been an increasing interest in the beneficial participation of non-*Saccharomyces* yeasts in vinification (Gil et al., 1996). Some of the compounds produced by these yeasts are beneficial and contribute to the quality of the alcoholic beverages when they are used in cofermentations with *S. cerevisiae* (Mateo et al., 1991). When pure low fermentative capacity yeasts are used in cultures with *S. cerevisiae*, their undesirable properties are not expressed or can be changed by the metabolism of the *S. cerevisiae* yeasts (Ciani and Comitini, 2011). Numerous strains identified as some non-*Saccharomyces* species have been broadly arrayed regarding the progress of some metabolites changing the aroma of the beverage. Assorted studies on the growth and

TABLE 25.1 Main Non-*Saccharomyces* Yeasts Isolated From Fruits and Juices

Aureobasidium pullulans	*H. uvarum*
Brettanomyces sp.	*Issatchenkia terricola*
B. anomalus	*Kluyveromyces thermotolerans*
Candida guilliermondii	*Metschnikowia pulcherrima/C. pulcherrima*
C. molischiana	*Pichia angusta*
C. stellata	*P. anomala*
C. utilis	*P. capsulata*
C. zemplinina	*P. guilliermondii*
Debaryomyces castellii	*P. kluyvery*
D. hansenii	*P. membranifaciens*
D. polymorphus	*Saccharomycodes ludwigii*
D. pseudopolymorphus	*Schizosaccharomyces pombe*
D. vanriji	*Sporidiobolus pararoseus*
Hanseniaspora sp.	*Torulaspora delbrueckii*
H. guilliermondii	*Trichosporon asahii*
H. osmophila	*Wickerhamomyces anomalus*
H. vineae	*Zygosaccharomyces bailii*

metabolites produced between *Saccharomyces* and non-*Saccharomyces* yeasts in assorted cultures have revealed their effect on quality. Aromatic profile and flavor depend on the strains and the inoculation approaches (Sadoudi et al., 2012). Furthermore, an abundant quantity of research is informative about enzyme activities in vinification (Maturano et al., 2012).

25.2 PRODUCTION OF EXOCELLULAR ENZYMES BY NON-*SACCHAROMYCES* YEASTS

Recent research in the alcoholic beverage industry follows different objectives in the biochemical, microbiological, and agronomic phases. The applied microbiology has three different hallmarks: the sensory, industrial, and fermentative assets of microbial strains. The enzymatic characteristics of the diverse yeasts implied in the alcoholic fermentation process have been assayed for a long time (Belda et al., 2016). A great number of non-*Saccharomyces* yeasts secrete a range of enzymes that may be of technological use for the biotechnological production (Van Rensburg and Pretorius, 2000). Yeasts that produce the more important number of exocellular enzymes are included in the genera, *Hanseniaspora*, *Pichia* (*Wickerhamomyces*), *Candida*, and *Metschnikowia*. The enzymes that these yeasts secrete include lyases, glycosidases, lipases, pectinases, esterases, and proteases (Mateo et al., 2011).

25.2.1 β-Glucosidases

Glycosidically, precursors of volatiles are varied and complex, mainly concerning the aglycone part. The sugar fragments consist of β-glucosides and some diglycosides: α-arabinosyl- β-glucosides, α-arabinosyl-β-glucosides, α-rhamnonosyl-β-glucosides, β-glucopyranosyl-β-glucosides, β-apiosyl-β-glucosides, and β-xilosyl-β-glucosides. The aglycon portion is often composed of terpenols, but other flavor precursors such as phenolic acids, C-13 norisoprenoids, cyclic or linear alcohols, and possibly volatile phenols can be found (Fig. 25.1). The yeast producing these enzymes has been reviewed by Padilla et al. (2016).

The most abundant glycosides in fruit juice are apiosylglycosides (up to 50%), followed by rutinosides (5%–15%), and lastly glucosides (3%–10%). All glycosidic precursors do not exist in all vegetal species and the original source provokes great differences regarding their amounts. The glycoside flavor potentiality remains rather constant during fermentation and also in drinks. These conclusions opened a new area of rigorous investigation on the chemistry of glycoconjugated flavor components in order to industrially use this significant flavor source (Mateo and Jiménez, 2000; Maicas and Mateo, 2005). Glycosides of terpenes can be hydrolyzed by an enzymatic method to improve beverage flavor, releasing free aromatic complexes from the original glycosides. Cleavage of glycosidic precursors is produced by several enzymes that act consecutively according to two steps: initially, α-arabinosidase, β-apiosidase, or α-rhamnosidase make the hydrolysis of the terminal sugar moiety (rhamnose, apiose, or arabinose) and the corresponding β-glucosides are liberated in the medium; then, the action of a β-glucosidase origins the deliverance of the monoterpenol molecule. Nevertheless, disaccharide glycosides can also be hydrolyzed following a one-step enzymatic procedure. Using grapes as a source, some enzymes catalyzing these reactions have been isolated (Maicas and Mateo, 2005). Enzymatic hydrolysis of glycoside extracts from

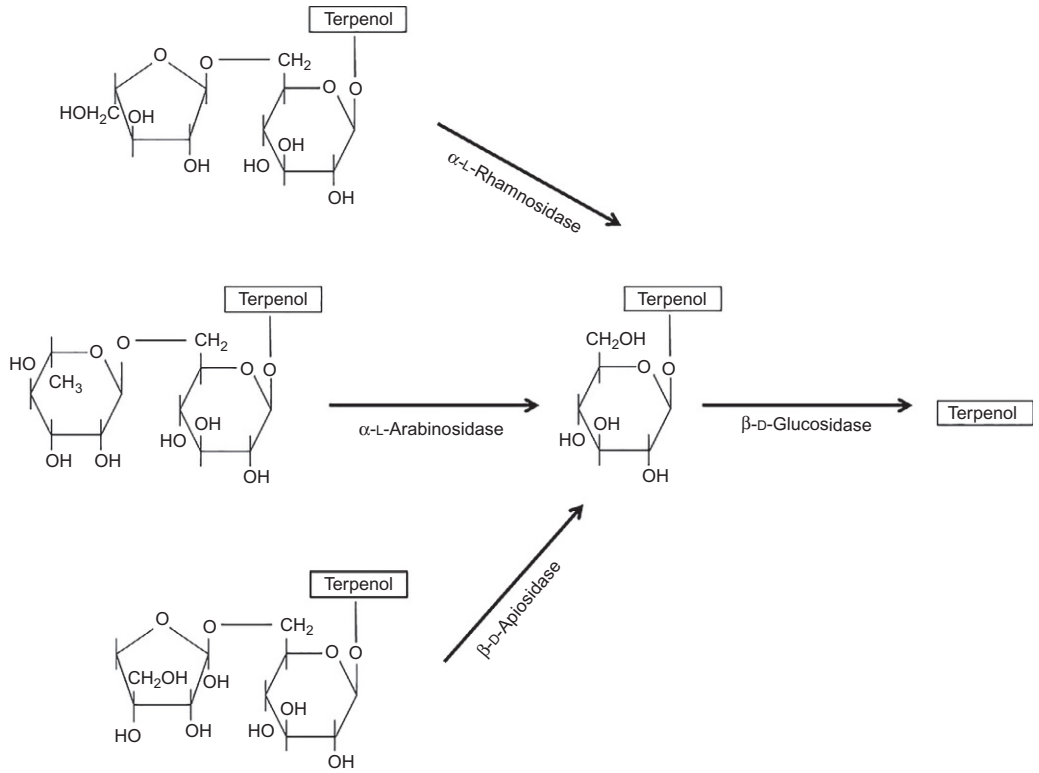

FIG. 25.1 Mechanism of enzymatic hydrolysis of terpenyl glycosides.

Chardonnay, Riesling, Muscat, Semillon, Sirah, and Sauvignon grapes has activated the liberation not only of terpenes, but also C-13 norisoprenoids: 3-hydroxy-β-damascenona and 3-oxo-β-ionol (Gunata et al., 1990a).

Only a few results are obtained regarding the glycosidic activity of yeast isolates and the applied properties of the proteins. Low β-apiosidase. α-arabinosidase, or α-rhamnosidase activities were described in *S. cerevisiae* but different assays have been developed to clone these corresponding genes purified from diverse microbes in the yeast *S. cerevisiae* (Bisson and Karpel, 2010). However, data on β-glucosidase activity on *Saccharomyces* are inconsistent. The first outcomes showed that *S. cerevisiae* had little activity (Gunata et al., 1990b) but Delcroix et al. (1994) found three enological strains presenting high β-glucosidase activity. On the other hand, Darriet et al. (1988) have shown that enzymes situated in the periplasmic space of *S. cerevisiae* were able to hydrolyze terpene glucosides of Muscat grapes; they found also that the activity of this β-glucosidase was glucose-independent. Mateo and Di Stefano (1997) detected β-glucosidase activity in various *Saccharomyces* strains, assaying its hydrolytic activity on *p*-nitrophenyl-β-glucoside (*p*NPG) and glucosides of Muscat juice. This enzymatic activity is induced by the presence of bound β-glucose as the only carbon source in the medium and is a characteristic of the yeast strain. This β-glucosidase

is linked with the yeast cell wall, and is quite glucose-independent but inhibited by ethanol. These effects could open new pathways regarding other glycosidases in *S. cerevisiae*; α-arabinosidase, α-rhamnosidase, or β-apiosidase activities could be induced in yeast by altering the composition of the medium, including inductive mixtures as well as in filamentous fungi (Dupin et al., 1992). Interest in the health benefits of red wine has increased in the last several years due to the occurrence of resveratrol, which can be found mainly as a glucoside form. Yeast-endowed β-glucosidase activity improves free-resveratrol concentration in wine without altering its composition or sensorial properties. After screening 308 non-*Saccharomyces* yeast strains for β-glucosidase, Gaensly et al. (2015) found 14 yeasts that increased the resveratrol concentration up to 102% without any noteworthy difference; nine of these yeast strains also created high ethanol content. During the alcoholic fermentation of the *Vitis labrusca* grape must, four autochthonous *Hanseniaspora uvarum* β-glucosidase-producer strains displayed adequate oenological characteristics and hydrolyzed resveratrol-glucosides.

The enzyme β-glucosidase seems not to be encoded by the *S. cerevisiae* genome (Ubeda-Iranzo et al., 1998). In contrast, non-*Saccharomyces* yeasts identified as species from the genera *Pichia, Hansenula*, *Hanseniaspora, Debaryomyces,* and *Candida* possess various degrees of β-glucosidase activity and can play a role in releasing volatile substances from nonvolatile precursors (Spagna et al., 2002). Fermentation of the Chardonnay grape must with mixed cultures of *S. cerevisiae* and *Debaryomyces. pseudopolymorphus* occasioned an increased concentration of the terpenols nerol, citronellol, and geraniol in wine (Cordero Otero et al., 2003). Similarly, cofermentation of Muscat grape juice with *S. cerevisiae* and *Debaryomyces vanriji* produced wines with an increased concentration of various terpenols (Garcia et al., 2002). On the other hand, wines only fermented with *S. cerevisiae* allow beverages with high quantities of terpene compounds compared to mixed cultures of Sauvignon Blanc grape juice produced with *Torulaspora delbrueckii*/*S. cerevisiae* and *Candida zemplinina*/*S. cerevisiae* (Sadoudi et al., 2012). The β-glucosidases from low fermentative capacity yeasts, such as *Candida wickerhamii, Pichia anomala,* and *Candida molischiana*, were found to be more tolerant to hostile conditions (high ethanol or sugar levels, low temperatures, low pH values,) and tended to be more specific for glycosides than those from other yeast species (Jutaporn et al., 2009). Efforts have previously been made to improve the aroma of alcoholic beverages using non-*Saccharomyces* yeasts and their glycosidases (Gonzalez et al., 2013). Screening autochtonous yeasts showing glycosidase activities and their use in the fermentative process may permit industries to produce beverages with higher varietal, agreeable, and typical aroma profiles. Consequently, it is useful to discover the potential of autochtonous yeast biodiversity from a single enological ecosystem for specific and rich β-glucosidases. The availability of petri dishes with media containing *p*-nitrophenyl-β-glucopyranoside (*p*NPG), cellulose-congo red, or 4-methylumbelliferyl-β-glucuronide (4-MUG) can contribute to screening β-glucosidase-producing strains (Wang et al., 2013). Grossmann et al. (1987) reported a yeast of the *Hansenula* species isolated from unfinished wines that has an inducible β-glucosidase activity, but this protein was inhibited by sugars. Other yeast isolates such as *C. wickerhamii* and *C. molischiana* also possess activities against different β-glucosides and they were low-influenced by the aglycon nature (Gunata et al., 1990c). β-Glucosidase from *C. molischiana* was immobilized to Duolite A-568 resin, displaying comparable physicochemical properties to those of the free enzyme. The immobilized enzyme

was found to be very stable under wine conditions and could be used for several repeated hydrolyses of the bound aroma (Gueguen et al., 1997). Through screening almost 400 strains identified as belonging to 20 yeast species, it was determined that the isolates of the species *Debaryomyces polymorphus*, *Debaryomyces castelli*, *Kloeckera apiculata*, *Debaryomyces hansenii*, and *Hansenula anomala* showed β-glucosidase activity (Rosi et al., 1994). A single strain of *D. hansenii* showed the highest extracellular enzymatic activity as well as some wall-bound and intracellular activity and, during exponential growth, its synthesis was improved by O_2 presence and repressed by high sugar concentration. The best condition for this enzyme was pH 4.0–5.0 and 40°C. Hydroxyapatite was used to immobilize this enzyme using a one-step procedure. The immobilized enzyme showed a lower activity than the purified free enzyme, but was much more stable than the enzyme in a cell-free supernatant (Riccio et al., 1999). Their studies have revealed the ability of several wine yeasts to hydrolyze norisoprenoids, terpenoids, and benzenoids glycosides; among these yeasts, *H. uvarum* was able to hydrolyze both glycoconjugated forms of pyranic and furanic oxides of linalool (Fernandez-González et al., 2003). Different authors have also proposed the central role of low fermentative capacity species of yeasts in liberating the glycosidic fraction of fruit flavor compounds (Mendes et al., 2001). Different *Pichia* and *Wickerhamomyces* isolates, identified as *Pichia fermentans*, *Wickerhamomyces anomalus*, and *Pichia membranifaciens*, were isolated from grapes and wineries in the Requena-Utiel region of Spainand characterized by molecular and physiological procedures (sequencing and PCR-RFLP). They proved to be the most important species to be used as an enzyme source because they shows tolerance to levels higher than 10% of ethanol and glucose, making them of pronounced interest in vinification use (Madrigal et al., 2013). The sensorial features of Muscat-based wines are connected to the amounts of terpenols, so an increase of such a concentration, obtained by the action of glycosidic enzymes produced by *Hanseniaspora*, is expected. *H. vineae* and *H. uvarum* isolates have been proposed to be worthy yeast candidates to be used in industrial vinification procedures to improve wine characteristics (Table 25.2) (Lopez et al., 2014). Optimal conditions to induce β-glucosidase activity have also been determined (López et al., 2015).

The use of autochtonous yeast isolates with β-glucosidase activity has also been examined in three Chinese regions. The effects of diverse conditions on the synthesis of β-glucosidase showed that one single *Trichosporon asahii* strain produced a higher quantity of β-glucosidase enzymes than the other yeasts when acidic media were used. Nevertheless, *S. cerevisiae* and *H. uvarum* isolates displayed higher β-glucosidase synthesis when a high content of glucose conditions was used. Additionally, when assays on the β-glucosidases stability and activity were performed under the influence of hostile factors, results showed that the enzyme from the *T. asahii* strain had a stronger low-pH-value resistance than β-glucosidases obtained from other yeasts (Wang et al., 2016). A quantitative colorimetric assay was performed by Hu et al. (2016) to screen yeasts from various regions of Korea to detect β-glucosidase production. Three isolates, identified as *Rhodotorula mucilaginosa*, *P. membranifaciens*, and *H. uvarum*, were selected. The β-glucosidic enzyme from the *H. uvarum* strain showed the highest activity under fermentative conditions among the selected yeasts. The extract from *H. uvarum* containing glycosidase activities displayed catalytic specificity for some terpenes and C13-norisoprenoids aromatic glycosides. It was able to enhance sweet, fresh, nutty, and berry aroma properties in beverages.

TABLE 25.2 Terpenes in Muscat Wine Inoculated With Selected *Hanseniaspora* Isolates (μg/L)

		***Hanseniaspora* Inoculated**		
	Control[a]	***H. uvarum***	***H. vineae* I**	***H. vineae* II**
Oxide A[b]	29.7 (1.2)[c]	30.4 (2.1)	33.7 (3.2)	26.9 (3.4)
Linalool	20.0 (0.9)	40.4*(3.9)	47.4*(3.4)	38.2*(5.3)
Ho-trienol	24.0 (3.2)	51.3*(5.3)	35.1*(4.2)	24.9*(0.6)
2-Phenylethanol	1890.2 (43.4)	3057.5*(39.8)	2747.8*(26.8)	2568.5*(45.6)
Terpineol	53.3 (3.4)	67.2*(4.7)	65.1*(1.2)	54.5 (3.9)
Nerol	24.6 (2.8)	25.8 (1.1)	23.4 (3.1)	26.3 (1.2)
Geraniol	59.8 (5.0)	61.3 (3.7)	56.9 (1.7)	62.8 (1.7)
Diol 1[d]	43.2 (4.7)	87.9*(2.1)	80.2*(2.1)	81.2*(3.2)
4-Vinylphenol	63.2 (1.2)	89.7*(2.4)	75.7*(5.8)	62.1 (0.9)
Endiol[e]	nd	58.8*(2.1)	52.0*(3.4)	34.1*(4.2)
Diol 2[f]	12.0 (0.6)	13.4 (0.9)	7.8 (2.6)	10.1 (0.9)
2-Phenylethyl acetate	28.0 (4.1)	56.2*(7.2)	23.3 (1.2)	25.8 (4.7)
2-Methoxy-4-vinylphenol	89.0 (6.1)	103.0*(5.3)	105.4*(6.5)	94.1 (2.9)

nd: not detected.
[a] *Wine obtained by spontaneous fermentation.*
[b] *Cis-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol.*
[c] *Standard deviation (n = 3). ANOVA one factor, significant difference is indicated as * ($p < 0.05$).*
[d] *2,6-Dimethyl-3,7-octadien-2,6-diol.*
[e] *2,6-Dimethyl-7-octene-2,6-diol.*
[f] *2,6-Dimethyl-2,7-octadien-1,6-diol.*

25.2.2 Xylanases

Xylanolytic enzymes of microbial origin have received great attention due to their probable industrial applications for sustainable fuel-ethanol fabrication from xylan. They constitute the major component of hemicelluloses found in the cell walls of monocots and hard woods and represent one of the richer biomass resources. Two main reactions proceed during cleavage of the xylan backbone: 1,4-β-xylosidases are exoglycosidases that cut terminal xylose residues from the nonreducing extreme of short-chain xylooligosaccharides whereas endo-1,4-β-xylanases (1,4-β-xylan xylanohydrolase) hydrolyze internal β-(1 → 4)-xylosidic links in the apolar xylan backbone to produce xylooligosaccharides, water-soluble compounds (Polizeli et al., 2005). Additional enzymes such as α-glucuronidase, α-arabinofuranosidase, and acetyl xylan esterase act to eliminate side-chain substituents. 1,4-β-Xylosidase plays an important role in xylan degradation, considering that xylanases are not capable of completely hydrolyzing xylans. A high variety of microorganisms produce 1,4-β-xylosidase, but only a reduced number of yeast species and a restricted number of isolates can produce it (Linden and Hahn-Hagerdal, 1989).

In a selection and characterization program, >100 isolates of non-*Saccharomyces* yeasts have been considered and four of them were selected because of their capacity to produce both high β-xylosidase and β-glucosidase enzymes (López et al., 2015). They were to identified as *W. anomalus*, *H. uvarum*, *H. vineae*, and *P. membranifaciens*. The induction procedure was enhanced to be carried on a YNB-medium, inoculated with 10^6 cfu/mL, added with 5% xylan (*w*/*v*) and statically incubated at 28°C for at least 48 h. All the isolates had optimum activity at pH 5.0–6.0 for both enzymes. Each yeast exhibited a characteristic profile of enhancement, inhibition, or insignificant effect for both β-glucosidase and β-xylosidase. Aroma molecules released from glycosidic precursors treated with pure cultures of each of the previously mentioned isolates were also calculated, displaying a terpene concentration increment when beverages were inoculated with the selected non-*Saccharomyces* isolates. Romero et al. (2012) described an 1,4-β-xylosidase tolerant to ethanol from a *P. membranifaciens* isolate grown at 28°C on xylan. The xylosidase was obtained by protein purification on a Sephadex G-100 and DEAE-cellulose columns. The relative molecular mass of the protein was calculated to be 50 kDa by protein electrophoresis. The activity had a V_{max} of 7.5 μmol/min mg protein and a K_m of 0.50 mmol/L, using *p*-nitrophenyl-β-xylopyranoside as the substrate. The enzymatic characteristics (thermal and pH stability, ethanol tolerance, and scarce inhibition by glucose) make this molecule an interesting microorganism to be employed in the production of xylose by an enzymatic pathway and improvement of hemicellulose degradation for bioproduction of ethanol.

25.2.3 Proteases

Proteases were classified on the basis of three-dimensional structure, amino acid composition of the active center, and the catalytic mechanism into four classes: aspartic endopeptidases, serine endopeptidases, metalloendopeptidases, and cysteine endopeptidases, following the NC-IUBMB commission. Each class of protease has a precise ability to cut a specific peptide bond and shows a characteristic group of active amino acid molecules organized in a characteristic configuration to determine the catalytic site (Tyndall et al., 2005). The aspartic proteases produced by low fermentative capacity yeasts have a tertiary structure with two approximately symmetric loci with each lobe displaying an aspartic acid molecule to form the catalytic site. The activity of the aspartic proteases is dependent on pH conditions, in opposition to other types of proteases (Borelli et al., 2008). Aspartic endopeptidases (E3.4.23.x) are extensively distributed in the biosphere. A number of these enzymes are composed of 320–350 amino acids with molecular weights from 30.000 to 55.000 Da and isoelectric points (pI) ranging from 3.0 to 4.5 because of the high quantity of acidic amino acid residues (about 13%) in the enzyme. About the substrate specificity, they show a predilection toward peptide compounds and proteins with large hydrophobic side chains on either side of the scissile bond (Rawlings and Bateman, 2009). However, there are eight subfamilies within the aspartic proteases with the sequence Asp-Thr(Ser)-Gly at their active site, according to the MEROPS and Protein Data Bank (PDB). The groups differ according to the amino acids in the catalytic site, the quantity of disulfide links in the structure, the position of the aspartic acid residues in the peptide chains, the optimal pH of the enzymes varying from acidic to neutral, and substrate specificity (Rawlings et al., 2011). The capacity of yeasts to produce protease enzymes was described by different authors. The importance of these enzymes is based on their potential to degrade haze

proteins in juices and to produce nitrogen sources for microorganisms (Lagace and Bisson, 1990). Haze production is one of the most important problems for alcoholic beverage production. This alteration occurs in juices with reduced content in polyphenols content due to the coagulation of protein molecules in alcoholic beverages from adverse storage conditions. They can either precipitate to form a sediment or flocculate, producing haze in recipients (Pocock and Waters, 2006). The presence of haze reduces the economical value of the beverage because consumers may perceive it as a microbial alteration (Waters et al., 2005). In the industry, the haze is removed by bentonite but, under certain conditions, it may have some unfavorable effects on the quality because some flavor, color, and aroma compounds may disappear together with proteins (Waters et al., 2005). Because of these effects, other methods to remove haze-causing proteins have been proposed, including the application of proteolytic enzymes (Rosi and Costamagna, 1987). Dizy and Bisson (2000) have shown that some strains of *Hanseniaspora* synthesized proteases in juice and changed the protein content of the beverage.

Exocellular proteolytic activity of non-*Saccharomyces* yeasts may increase the nitrogen concentration in juices for the microorganisms grown during alcoholic fermentation (Cramer et al., 2002). A lack in nitrogen content may conduce to stop fermentations. On the other hand, yeasts can produce volatile fatty acids, higher alcohols, and esters, compounds contributing to the fermentation aroma of beverages, as primary metabolites from amino acids and sugar metabolism (Swiegers et al., 2005). Proteolytic activity of some *Hanseniaspora guilliermondii* strains has been studied as a biotechnological solution to reduce turbidity due to proteins in alcoholic beverages (Strauss et al., 2001). Our findings shows that different *Hanseniaspora* isolates can synthesize proteolytic enzymes (Mateo et al., 2015) but the protease activity in *Wickerhamomyces, Pichia* and isolates was too low (Madrigal et al., 2013), according to results found by other researchers (Strauss et al., 2001). They proposed that *Hanseniaspora* yeasts could be a useful microorganism to synthesize this protein, but different results have been published. Many of these studies have conducted with *H. uvarum* (*K. apiculata*) isolates and, on the basis of the results obtained, exocellular protease of this microorganism has low activity. On the other hand, the assays made by these authors were performed with *H. uvarum* strains in acidic pH buffers and we have shown that the protease from *Hanseniaspora* yeasts is pH dependent, showing maximum values at pH 6.0. We have identified 26 *Hanseniaspora* isolates obtained from grapes by molecular techniques and they were assayed for exocellular protease synthesis. Isolates belonging to different *Hanseniaspora* species showed the highest activity. Protease was ethanol, fructose, and glucose content-independent, but divalent cations affect its activity; these data support that they were aspartic proteases. The β-mercaptoethanol effect suggests the importance of disulfide links to maintain the native structure of the enzyme. So, these enzymes are suitable to be used in industrial processes at pH 7.0 in the bread, meat, and cheese industries (Mateo et al., 2015).

25.2.4 Pectinases

Pectinases are enzymes that depolymerize pectins, substances present in the cell walls of vegetables (Ismail, 1996). They degrade pectic substances, which are mainly composed of galacturonic acid subunits partially esterified with methyl groups joined by α(1–4)-glycosidic bonds. They have different applications in the textile and food industries (Henriksson et al., 1999), such as coffee and tea fermentation, extraction of essential oils, and clarification of fruit

juices (Jayani et al., 2005). These enzymes are used in fermentative processes to increase juice extraction by degrading interfering polysaccharides, increasing the release of aroma and color compounds along all fermentative processes. At the same time, they can help to improve filterability, liquefaction, and clarification, releasing more organoleptic compounds contained in the fruit skins, thereby making a positive improvement to the beverage characteristics (Van Rensburg and Pretorius, 2000). Most commercial pectinase preparations used in the food industry are obtained from molds, in particular the *Aspergillus* species (*A. niger*, *A. wentii*, and *A. oryzae*) and *Rhizopus* (Acuna-Arguelles et al., 1995), Microorganisms categorized as "generally regarded as safe" (GRAS) produce high quantities of these enzymes. Microbial pectinases are divided into depolymerizing and saponifying proteins. Depolymerizing enzymes are polymethylgalacturonases, pectin lyases, polygalacturonases, and pectate lyases. Saponifying enzymes are mainly pectinesterases (Whitaker, 1990). However, these molds secrete other enzymes that may produce adverse reactions, such as the liberation of undesiderable volatile phenols in the production of fruit juices or the production of arabinofuranosidase, which can cause turbidity. Pectic enzymes from molds usually contain a mixture of pectinolytic enzymes, such as xylanases, cellulases, and hemicellulases, that are useful for the maceration and extraction of juice. However, for the stabilization of the cloud in orange juice, only one type of pectinolytic enzyme is required (Alimardani-Theuil et al., 2011). As an alternative to these mold enzymes, some autochtonous yeasts producing exocellular pectinolytic proteins could be used. This option could avoid the use of expensive foreign pectinases and improve the quality of alcoholic beverages because these yeasts can increase their sensory characteristics (Fleet, 2008). Many *S. cerevisiae* strains usually used in alcoholic beverage industry have no capacity to hydrolyze pectic substrates. However, certain strains of *S. cerevisiae* have been found to degrade polygalacturonic acid, which is important for the fermentation of plant-derived substrates (Fernández-González et al., 2005). When the enzyme extracted from *Saccharomyces bayanus* is added to grape must, the effects on turbidity are the same as when a commercial enzyme complex is added (Gainvors et al., 1994). Interest in the application of non-*Saccharomyces* wine yeasts has increased in recent years, but little attention has been dedicated to the ability of these yeasts to secrete pectic enzymes (Charoenchai et al., 1997; Strauss et al., 2001). Pectinases have been isolated from various yeast species of the genus *Kluyveromyces*, *Cryptococcus*, *Rhodotorula*, *Aureobasidium pullulans*, and *Candida* (Merín et al., 2011), especially polygalacturonases (Birgisson et al., 2003). So, yeasts provide a different source for the industrial production of commercial pectinases. They were proved to be advantageous over molds regarding the production of pectinases because of their unicellular nature, simple growth, and nonrequirement of an inducer in the growth medium. In addition, genetics may increase enzyme production, thus suggesting that the commercial enzymatic complexes by yeasts can be promising (Jia and Wheals, 2000). Regarding the production of pectinase, yeasts usually do not secret pectin methyl esterase (PME) and, therefore, their pectinases can be used to clarify fruit juice and alcoholic beverages without producing methanol (Schwan and Wheals, 2004). Pectinases produced by yeasts are not a mixture, as earlier reported in microorganisms (Pedrolli et al., 2008), and they have shorter fermentation cycles. Therefore, they are considered GRAS organisms and are more preferable as production strains. Nowadays, low temperature active enzymes are attractive for use in industry, as colder conditions hamper spoilage. Further milder conditions avoid changes in nutritional and organoleptic properties (Nakagawa et al., 2004). Low temperature

active pectinases are helpful in fruit juice clarification while thermostable enzymes are useful in juice extraction (Belda et al., 2016). After screening >100 non-*Saccharomyces* isolates in our laboratory, only very low pectinase activity has been detected (Madrigal et al., 2013; Lilao et al., 2015). Nevertheless, new screenings have allowed us to obtain some isolates, belonging to GRAS species *Candida ulmi* and *C. melibiosica*, with remarkable pectinase activity.

25.2.5 Carbon-Sulfur Lyases

Although a high diversity of volatile compounds is involved in alcoholic beverage aroma, only some of them are responsible for the varietal aroma and some thiols (sulfur-containing compounds with functional groups such as esters, alcohols and ketones) were identified whose olfactory characteristics represent many of these specific aromas: 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-mercapto-4-methylpentan-2-ol (4MMP). Although these thiols are found in low concentrations in wine, their perception thresholds are quite low and therefore play a key role in some prefermentative aromas (Tominaga et al., 1998). The thiols are produced by yeast from precursors during fermentation but they are not detected in juice (Dubourdieu et al., 2006). Different factors affect the concentration of precursors in juices and, therefore, the volatile thiol concentrations in the final product: agronomical practices and prefermentation operations such as skin contact and fermentation conditions (Masneuf-Pomarède et al., 2006). However, yeast strain is the main factor in volatile thiol release during alcoholic fermentation (Swiegers et al., 2009). Thus 3MH and 4MMP are produced from cys-3MH and cys-4MMP and by cleavage with β-lyase enzymes. 3MH is the precursor of 3MHA by yeast acetylation (Swiegers et al., 2007).

The synthesis of thiols from cysteine precursors was very low, suggesting that the concentration of these precursors represents only a small percentage of total varietal thiol production (Subileau et al., 2008). Subsequently, glutathionylated forms of both 3MH and 4MMP (Roland et al., 2010a) have also been found in juice. Glutathionylated 4MMP was converted to 4MMP by yeast at the same efficiency as the cysteinylated precursor and contribute 20% of the total 4MMP (Roland et al., 2010b). Glutathionylated 3MH can also be converted to thiols by yeast (Roland et al., 2010a), but the efficiency is lower than the cysteinylated forms. This transformation by yeast implies synthesis of the cysteinylated form as an intermediate (Grant-Preece et al., 2010). The conversion ratio of glutathione to cysteine precursors in beverages for 3MH can rise 100-fold (Roland et al., 2010a), so that the relative contribution of the two different forms of precursors to 3MH in wine is not clear. Schneider et al. (2006) suggested that there may be different metabolic synthesis reactions because labeled mesityl oxide or E-2-hexenal added to grape juice became incorporated into 4MMP or 3MH, respectively. Adding either glutathione or E-2-hexenal to grape juice increased varietal thiols in the alcoholic beverage (Roland et al., 2010c). Strains in the *Saccharomyces* genus were assayed for their ability to produce volatile thiols from their precursors (Howell et al., 2004). Several commercial *S. cerevisiae* strains have been evaluated for their ability to synthetize varietal thiols. While differences for the 4MMP release have been found, no significant differences have been found for 3MH production (Howell et al., 2004). Nevertheless, some authors have proposed the beneficial influence of non-*Saccharomyces* yeasts and their theoretical applications in the alcoholic beverage industry, mainly in mixed-culture fermentation with *S. cerevisiae* (Moreira et al., 2008). Anfang et al. (2009) showed the impact of two non-*Saccharomyces* species, *C. zemplinina* and *Pichia*

kluyveri, on the release of 3MH and its acetate. It seems that cofermentations using *P. kluyveri* with *S. cerevisiae* may increase 3MHA concentrations in some wines. On the other hand, the role of 15 non-*Saccharomyces* strains from seven species was evaluated on 3MH and 4MMP release in Sauvignon Blanc must and a model medium after partial fermentation. The importance of non-*Saccharomyces* strains on 4MMP release was low compared with *S. cerevisiae* isolates. In contrast, most of the non-*Saccharomyces* strains assayed were able to produce similar or higher quantities of 3SH from its natural or synthetic precursors, except *Issatchenkia orientalis*, *C. zemplinina*, and *H. uvarum*. Yeasts belonging to the species *T. delbrueckii*, *Metschnikowia pulcherrima*, and *Kluyveromyces thermotolerans* had a higher capacity to release 3SH from its precursor form. Nevertheless, additional fermentation studies are required to obtain a clear result of species-associated behavior or strain effects, including different non-*Saccharomyces* isolates.

25.2.6 Lipases and Esterases

Lipases and esterases are enzymes widely present in microorganisms and are included in the general class of carboxylic ester hydrolases (EC 3.1.1). Lipolytic enzymes are central industrial catalysts due to critical features, such as broad substrate specificity, stability in organic solvents, and no requirement for cofactors (Jaeger et al., 1999). Industrial interest in lipases is increasing with applications in laundry detergents and in the dairy industry while low attention has been paid to esterases from yeasts (Basaran and Hang, 2000). Actually, the presence of esterase was mainly associated with basidiomycetes while the occurrence of lipase was linked to ascomycetes (Buzzini and Martini, 2002). Some authors suggested that all lipases and esterases contain an active serine amino acid (Jaeger et al., 1994). This fact forms a turn in the protein structure, called a nucleophilic elbow, and is usually located between a β-strand and an α-helix. Nevertheless, more recent research has revealed that other motifs do exist (Jaeger et al., 1999) that contain a catalytic triad composed of aspartic acid, histidine, serine, or glutamate (Dodson and Wlodawer, 1998). Esterases hydrolyze monomeric soluble substrates in aqueous solutions, and conform to Michaelis-Menten kinetics while showing high specificity toward substrates with chain lengths of 2–10 carbon atoms. Lipases display "interfacial Michaelis-Menten" kinetics, hydrolyze emulsified substrates, and show specificity toward substrates with chain lengths of 10 or more carbon atoms (Holland et al., 2005). Ratledge and Tan (1990) reviewed the synthesis of extracellular lipases by yeasts. *Candida stellata*, *C. pulcherrima*, *C. krusei*, and *T. delbrueckii/C. colliculosa* have the ability to synthetize exocellular lipolytic activities. Lipids originating from the fruits or autolytic reactions of yeasts could be degraded by these proteins, releasing free fatty acids into the juice that may affect beverage quality. Moreover, the production of medium-chain fatty acids (C8 to C10 acids) could inhibit the development of *S. cerevisiae* and malolactic bacteria. However, the ability to hydrolyze tributyrin, the substrate used for the *in vitro* detection of esterases, does not necessarily imply that these yeast enzymes would degrade fruit lipids; further research is needed to determine this possibility (Charoenchai et al., 1997). Our work showed that *Pichia* and *Wickerhamomyces* isolates isolated from grapes have low lipase and esterase activities and are not suitable for relevant enzymatic production. Lipase activity was a more widespread property but only one-half of the isolates of *P. fermentans* and *P. membranifaciens* showed this activity. Esterase activity seemed to be a characteristic of *W. anomalus*. Esterase and lipase activities were very low in all isolates assayed; slight differences in lipase activity were found, but *W. anomalus* gave the highest values (Madrigal et al., 2013). The lipases obtained from yeasts isolated from different

origins have been studied by Marquina et al. (1992) and Rodríguez-Gómez et al. (2010). They reported Portuguese and Andalusian *Candida boidinii* strains exhibiting this activity. *Candida adriatica* is considered useful as it can hydrolyze the bitter tasting secoiridoid compound of the oil (Zullo et al., 2013). Moderate to high lipase activity has been detected in 50% of isolates, most of them identified as *Candida boidinii*, *C. adriatica*, *C. molendinolei*, *C. norvegica*, or *C. oleophila* and *Saccharomyces paradoxus* (Lilao et al., 2015). Esterase activity was not detected in all yeast species assayed. Other authors reported similar results (Rodríguez-Gómez et al., 2010).

25.3 FUTURE PERSPECTIVES

The commercial use of non-*Saccharomyces* cultures in the alcoholic beverage industry is still low, taking into account that the main reason for reevaluating non-*Saccharomyces* yeasts was to obtain products showing the autochthonous features of a geographical area. So, the sensory and oenological characterization of autochthonous *Saccharomyces* and non-*Saccharomyces* isolates as well as the ecological studies will provide different microorganisms to be used in mixed starter cultures for the production of typical beverages (Teixeira et al., 2015). Different researchers have shown the influence of non-*Saccharomyces* yeasts and, based on these studies, the alcoholic beverage industry is considering the use of controlled mixed fermentations. Indeed, industrial mixed starters with selected non-*Saccharomyces* strains and *S. cerevisiae* can improve varietal and fermentative aroma, but also they have an interest in color stabilization (Loira et al., 2015), control of the spoilage microbiota (Oro et al., 2014), reductions of the alcoholic degree of fermented beverages (Morales et al., 2015), or release of mannoproteins (Domizio et al., 2014). Remarkably, a new red wine-making technology has been developed as an alternative to the traditional malolactic fermentation, based on the combined use of two non-*Saccharomyces* yeast strains (Benito et al., 2015). Non-*Saccharomyces* yeasts represent a real field to be explored in the attempt to increase quality in different biotechnological processes. These "spoilage" microorganisms could open a new gate to find new approaches to understand the whole process of fermentation and the biosynthesis of secondary metabolites to increase the sensitive characteristics of foods. This is a new example to remark the importance of understanding the ecological biodiversity of fermentation and the correct way to carry on the process instead to simplify all biotechnological processes or try to use GMO as the only way to optimize biological reactions occurring in obtaining of fermentative products.

Acknowledgments

This work was funded by Universitat de València (grant INV-AE-336499) and Generalitat Valenciana, Conselleria d'Educació, Investigació, Cultura i Esports (grant AICO/2016/079).

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CHAPTER

26

Fructosyltransferases and Invertases: Useful Enzymes in the Food and Feed Industries

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26.1 INTRODUCTION

Enzymes are well-established biotechnological products that have been successfully used in different industries, becoming a growing multimillion dollar market (Anbu et al., 2017; Chauhan et al., 2017; Gaur et al., 2017; Honda, 2017; Mano et al., 2017; Ushasree et al., 2017). >30% of this market is attributed to enzymes and their products used in the food industry involving functional foods and beverages, beer, dairy products, dietary supplements, sweeteners, and syrups (Bali et al., 2015; Patel et al., 2017). It is expected to reach a value of $2.3 billion by 2020 (Demain and Sánchez, 2017; Patel et al., 2017). On the other hand, the industrial use of enzymes guarantees low energy consumption and a reduced reaction time with low influence on the environment. Fructosyltransferases-FTase [EC 2.4.1.9] and/or β-fructofuranosidases-Ffases [EC 3.2.1.26] obtained from microorganisms and plants are responsible for the production of a number of compounds with a great impact in the food industry. So-called fructans such as inulins, fructoligosaccharides (FOS), and levans (Ackerman et al., 2017; González-Garcinuño et al., 2017; Singh et al., 2017) are also considered functional foods. The FOS is produced in nature from sucrose by tranfructosylation activity and stands

https://doi.org/10.1016/B978-0-12-813280-7.00026-8

out as the fructan of higher demand in the functional sugars market. This is largely due to its dual utility as a prebiotic and low-calorie sweetener as well as other well-documented benefits to human and animal health (Lima et al., 2017). These compounds are natural carbohydrates formed by 1–3 residues of fructose linked to a molecule of sucrose and have been given "Generally Recognized as Safe" (GRAS) status by the US Food and Drug Administration (USFDA). The FOS maintains the main chemical and organoleptic properties of sucrose, but unlike it, the FOS is not cariogenic and is not digested by the enzymes present in the small intestine of humans or other monogastric animals, hence their hypocaloric nature. When they reach the large intestine, they are selectively used as a carbon source by *Bifidobacteria* and *Lactobacilli*, the main beneficial bacteria residing there, hence their prebiotic capacity. The production of high oligosaccharides yielding a specific chain length from simple raw materials such as sucrose is a technical challenge. Therefore, the industrial production of short-chain FOS (scFOS) is attracting the attention of researchers due to the pharmaceutical importance of these compounds.

Levan is an extracellular homopolysaccharide, a biologically active fructan polymer consumed as a functional food in Japan and Korea. It is a naturally occurring homopolymer of fructose, which can be found in plants and many microbial strains (Silbir et al., 2014); it is also produced by transfructosylation activity. This fructose polymer has many outstanding properties for food and other industries (Öner et al., 2016). On the other hand, sucrose inversion can be achieved by acid hydrolysis or by using invertase (EC 3.2.1.26) or exoinulinase (EC 3.2.1.80). Sucrose hydrolysis yields an equimolar mixture of fructose and glucose, commercially known as invert sugar. The invert sugar syrup is sweeter than sucrose and easier to incorporate in food and pharmaceutical preparations because it does not show the crystallization problems of its precursor in highly concentrated solutions. The enzymatic process produces food-grade syrups without brown color and undesirable by-products such as hydroxymethylfurfural obtained through nonenzymatic methods. In view of the great demand for FOS, levan, and invert syrups as food ingredients in the food industry, the opportunity exists for the screening and identification of novel strains capable of producing new enzymes with transfructosylation activity and for developing improved and less-expensive production methods. In this review, we discuss fructosyltransferases and invertases as well as the beneficial effects of FOS, levan, and invert sugar and how they can play a key role in the food market. Bear in mind, though, that more effective and less costly production methods can be a main advantage in the food industry.

26.2 FRUCTOSYLTRANSFERASES

Nowadays, the food industry has developed different alternatives for sweeteners, and among them, we have levan and inulin, including FOS. These two types of fructose-rich molecules, the so-called fructans, are produced by the action of different types of frutosyltransferases (FTFs). Fructans are fructose-rich molecules with linear or branched chains. At the points of branching, the anomeric carbons of two fructofuranoses are connected to the oxygens 1 and 6 of the same fructose residue. The number and size of the branches is variable and depends mainly on the origin of each fructan (Banguela and Hernández, 2006). Three criteria are essential for fructan classification: the predominant type of binding, the

degree of polymerization (GP) of the molecule, and its origin. According to these, the most familiar terms in the literature to name these molecules are kesto-n-osas, inulin, levan, fleín, and graminan. Kesto-n-osas are fructose oligomers that are formed by one or more fructose residues attached to a sucrose. Kestotriose (GP = 3), kestotetraose (GP = 4), and kestopentaose (GP = 5) are the most abundant oligofructans or fructo-oligosaccharides in nature with a vegetable or microbial origin (Yun, 1996). Among the kestotrioses, we find 1-kestotriose or 1-kestose, 6-kestotriosa or 6-kestose, and 6G-kestotriose or neokestose. The most common kestotetraoses in nature are 1-kestose, 1-kestotetraose or nistosa and 1,6-kestotetraose or bifurcose (Fig. 26.1A). Inulins ($G_{1\text{-}2}F_{1\text{-}2}F_n$) have the fructosyl residues mostly bound by β(2→1) bonds constituting linear chains that rarely exceed 70 fructosyl units. These fructans are found in plant species belonging to Astereales and Liliales (Hendry and Wallace, 1993). There are inulins produced by fungi; however, some bacteria are also found to produce them with a size ranging from 6 to 9×10^7 Da (Olivares-Illana et al., 2002). Inulins of short chains of up to five residues of fructose are called fructo-oligosaccharides. Among them, as mentioned above, 1-kestose and nystose are the most popular although other thrisaccharides such as 6-kestose and neokestoses are also found in natural polyfructans. Levans ($G_{1\text{-}2}F_{6\text{-}2}F_n$), on the other hand, have fructose residues bound by β (2→6) bonds, as shown in Fig. 26.1B(A and B), and their origin is mainly bacterial and constitutes polymers of high mass.

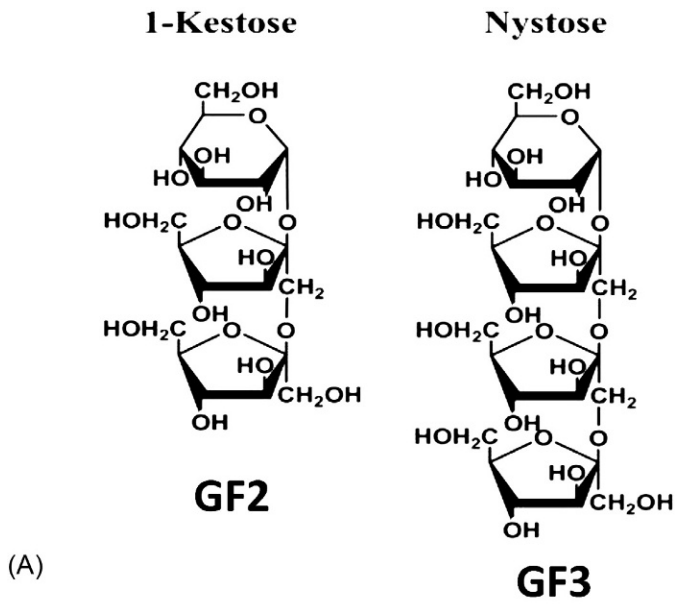
Fructans, due to their properties, are currently used in the production of different high-demand products such as ice cream, soft drinks, juices, yogurt, etc. It is estimated that in the American diet, 2.6 g of inulin or 2.5 g of oligofructosaccharides are consumed per day on average (Moshfegh et al., 1999) while in Europe the daily consumption is 4–12 g. According to these facts, there is a great interest in the search for enzymes and processes that allow obtaining these compounds on an industrial scale. In nature, the synthesis of any type of fructan begins with a transfructosylation reaction carried out by a fructosyltransferase, where sucrose plays the double role of donor and first acceptor of the fructose residue. The presence of sucrose as the precursor molecule has been verified in most of the fructans (microbial or vegetable) whose structure has been determined. However, the shape and extent of the fructan chain may vary depending on the fructosyltransferase origin involved in this process, as will be further explained.

26.3 NOMENCLATURE AND CLASSIFICATION OF FRUCTOSYLTRANSFERASES

The nomenclature given in the literature to fructosyltransferases (FTFs) is based on both the substrate specificity, referred to as the donor and acceptor molecules of the fructose residue, and the catalytic action of the enzyme itself.

26.3.1 Sucrose 1-Fructosyltransferase (1-SST, EC 2.4.1.99)

It cleaves the glycosidic bond of sucrose and transfers the fructose residue to the C-1 position of the fructosyl end of another sucrose molecule, forming the 1-kestose. This enzyme can transfer one or two other fructose residues to the synthesized 1-kestose and thereby form the



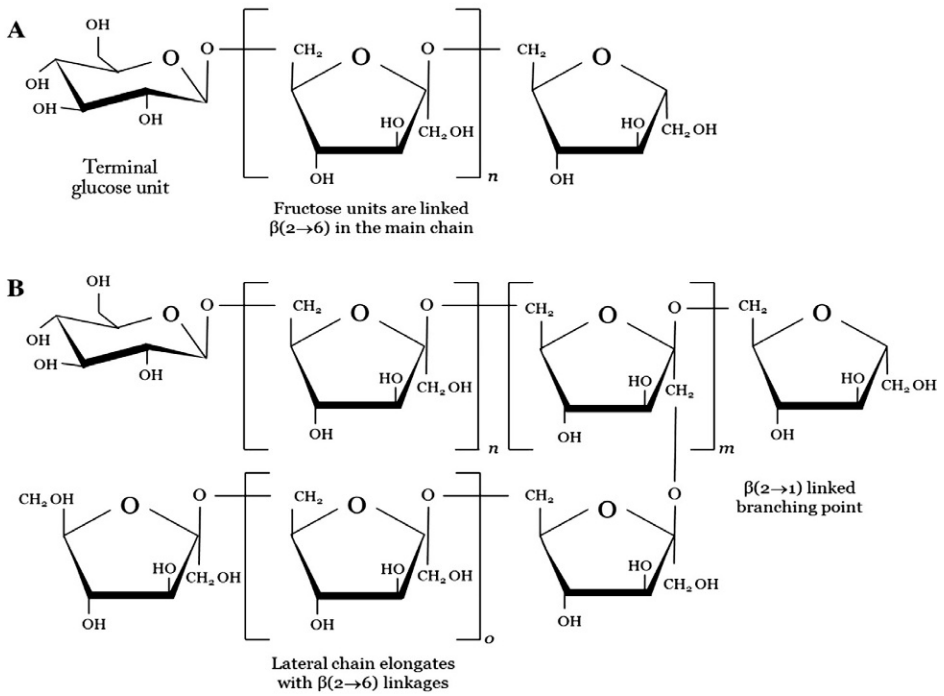


FIG. 26.1 (A) FOS structure with fructose units joined by β- 2-1 linkages. GF2 represents 1-kestose and GF3 1-nystose. (B) Levan structure with fructose units joined by β- 2-6 (A), and β- 2-1 (B) linkages.

nystose or fructosylnysteine, respectively. 1-SST is the first enzyme of the fructan synthesis pathway in plants (families *Asteraceae* and *Liliaceae*) (Vijn and Smeekens, 1999), and is the only fructosyltransferase present in fungi (Rehm et al., 1998).

26.3.2 Fructan 1-Fructosyltransferase (1-FFT, EC 2.4.1.100)

It transfers the terminal fructose residue from a donor fructan (substrate), generally 1-kestose, to the C-1 position of the fructosyl end of another acceptor fructan, allowing chain elongation (Vijn and Smeekens, 1999).

26.3.3 Fructan 6-Fructosyltransferase (6-SFT)

It forms 6-kestose from sucrose and polymerizes it to levan. It cleaves the glycosidic bond of the sucrose and transfers the fructose residue to the C-6 position of the internal fructosyl residue of the 1-kestose to form the bifurcose. The 6-SFT is present only in some species of Gramineae, where it also polymerizes, by means of β (2→6) bonds and in combination with 1-FTF, the chains of the fructans named flein and graminan (Vijn and Smeekens, 1999).

26.3.4 Fructan 6-Glucose-Fructosyltransferase (6G-FFT)

It cleaves the link between the two fructose residues present in 1-kestose and transfers terminal fructose to the C-6 position of the glucosyl residue of a sucrose to form the neokestose. It has been identified only in species of the family *Liliaceae*, such as for example, onion, garlic, etc.

26.3.5 Levansucrase (Sucrose 6-Fructosyltransferase, EC 2.4.1.10)

It cleaves the glycosidic bond of sucrose and transfers the fructosyl residue at the beginning to another sucrose to form 1-kestose and/or 6-kestose. In the case of transfructans, the transfructosylation reactions are polymerized by means of bonds (β (2→6)) to high molecular mass molecules.

26.3.6 Inulinsucrase (Sucrose 1-Fructosyltransferase)

It cleaves the glycosidic bond of sucrose and transfers the fructosyl residue first to another sucrose and then to the synthesized 1-kestose, which continues to polymerize into successive transfructosylation reactions to form high molecular mass inulins. This enzyme has been identified in some bacteria such as *Streptococcus mutans*, *Leuconostoc citreum*, and *Lactobacillus reuteri* (Olivares-Illana et al., 2002; Van Hijum et al., 2002), and is named fructosyltransferases. All these enzymes, considering their ability to recognize and hydrolyze β-fructosyl bonds, belong to the group of β-fructofuranosidases that also include invertases (EC 3.2.1.26), inulinases (EC 3.2.1.7), and levanases (EC 3.2.1.65). Contrary to the proposal of Naumov and Doroshenko, to gather all β-fructosidases in a single superfamily of glycosylhydrolases (Naumov and Doroshenko, 1998), the fructosyltransferases of bacterial origin, in their majority, are found in a new family (Family 68) of glycosylhydrolases (http://www.cazy.org/Glycoside-Hydrolases.html). On the other hand, the FTFs of fungi

and plants as well as the rest of the β-fructofuranosidases compose the family 32 of the glycosylhydrolases. They gather in this family because all present primary structures of high similarity, including the consensus sequence H-x (2)-[PTV]-x (4)-[LIVMA]-[NSCAYG]-[DE]-P-[NDSC][GA]3 where x may be any amino acid. Some authors argue that the great similarity between invertases and vegetable fructosyltransferases at the biochemical and molecular levels constitutes strong evidence of the possible evolution of plant fructosyltranferasas from invertase in events mediated by relatively few mutational changes (Vijn and Smeekens, 1999). In summary, bacterial fructosyltransferases, levansacarase, and inulosucarase, belong to the 68 family of glycoside hydrolases (GH), and together with members of the GH32 family (fructosyltransferases, invertase, sucrose-6P hydrolases, and eukaryotic fructanases), comprise the clone GHJ of β-fructofuranosidase, as defined in the CAZy database (http://www.cazy.org/). Interestingly, all act following a ping-pong mechanism, which involves the formation and subsequent hydrolysis of a covalent fructosyl-intermediate enzyme always with an overall retention of the anomeric configuration of the fructosyl residue.

26.3.7 Fructosyltransferases Produced in Plants and Fungi

The synthesis of fructans in plants occurs in the vacuole (Darwen and John, 1989) and is catalyzed by the combined action of at least two fructosyltransferases with different substrate specificities. Some members of the *Asteraceae* family (*Helianthus tuberosus*, *Chichorium intybus*, *Cynara scolymus*) synthesize linear chain inulin with a polymerization degree (PD) ranging from 10 to 70 units, depending on the plant species using a first enzyme, sucrose: sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) whose function is to irreversibly convert sucrose into 1-kestose and other oligofructanes (GP≤5). These, in turn, serve as substrates for β (2→1) fructan: β (2→1) fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100), which extends the inulin chain in a reversible reaction where the intermediary 1-kestose acts as the main donor and acceptor of the fructose residue. Both 1-SST and 1-FFT of *H. tuberosus* were purified from tubers in dormancy and characterized (Lüscher et al., 2000). 1-SST and 1-FFT were also purified from *C. intybus* root and their physical and catalytic properties were similar to those of the enzymes of *H. tuberosus* (Van den Ende and Van Laere, 1996). Onion (*Allium cepa*) and tulip (*Tulipa gesneriana*), members of the Liliacea family, produce neoinulins. In the synthesis of this special type of fructans participate 1-SST, 1-FFT and a new enzyme, fructan: fructan 6-glucose fructosyltransferase (6G-FFT). After 1-SST synthesizes 1-kestose from sucrose, 6G-FFT transfers the terminal fructose residue of 1-kestose to C-6 of the glucosyl residue of a sucrose, which acts as an acceptor molecule to form neokestose ($F_{2\text{-}6}G_{1\text{-}2}F$), which is then polymerized by β (2→1) bonds by 1-FFT producing neoinulins of 6–8 units. Genes encoding onion 1-SST and 6G-FFT were cloned and expressed in other plants. Experiments with recombinant enzymes have shown that 6G-FFT does not hydrolyze sucrose, and only in combination with 1-SST is able to produce neokestose (Vijn et al., 1998). Barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and other members of the family Poaceae (Gramineae) accumulate fructans of the flein and graminan type and, to a smaller amount, inulin. Inulin synthesis occurs according to the 1-SST/1-FFT model described for the Asteraceae family. A new enzyme, sucrose: fructan 6-fructosyltransferase (6-SFT), was purified from barley and as later identified as being responsible for the formation of flea and β (2→6) chains of the graminin molecule.

The isolated 6-SFT presented two isoforms (pI 4.9 and 5.1), each composed of two subunits of 49 and 23 kDa encoded by a single gene. In the presence of sucrose, both isoforms functioned as a hydrolase and only formed small amounts of 6-kestose. In the presence of sucrose and 1-kestose, this latter substrate assumed the role of acceptor of the fructose residue, forming the bifurcated tetrasaccharide (Sprenger et al., 1995). Contrary to plants, fructan production in fungi is catalyzed only by sucrose: sucrose 1-fructosyltransferase (1-SST), which causes the synthesis and accumulation of 1-kestose, nystose, and fructosylnystole in proportions that vary depending on the origin of the enzyme (Hendry and Wallace, 1993). *Aspergillus foetidus* 1-SST was characterized and was further found that the *1-sst* structural gene represents a single copy in the *A. foetidus* genome and encodes a 537 aa precursor protein (59.1 kDa) with an N terminal signal peptide of 19 aa residues that is cut during the secretion process. The 1-SST deduced from this study shows regions conserved with different β-fructofuranosidases, but within them it shares a higher homology with levanases despite not showing hydrolytic activity on levan (Rehm et al., 1998).

26.3.8 Fructosyltransferases Produced in Bacteria

In bacteria, fructan production is carried out by a single multifunctional enzyme able to convert sucrose in both inulin or FOS and levan. If the synthesized product is inulin, then the responsible enzyme is an inulosucrase, but if products are high molecular weight fructans such as levan, then the responsible enzyme is called levansucrase. However, some levansucrases form both β-(2,1) fructosyl-fructose linkages to produce inulin-type FOS or β-(2,6) linked chains to produce levan.

All levansucrases catalyze the transfer of fructosyl residues from sucrose to a variety of acceptor molecules such as water (release glucose and fructose), sucrose (synthesis of 1-kestose or 6-kestose), the synthesized β-(2,6)-linked oligofructans (elongation of levan), and the released monosaccharides (synthesis of sucrose) and fructose (synthesis of inulobiose or levanbiose). Levansucrases are widely spread in gram-positive and gram-negative bacteria, as described below.

26.4 LEVANSUCRASES IN GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

Levansucrasases have been reported in gram-positive bacteria showing, in all cases, a precursor protein produced with a signal peptide cleaved during secretion (Waldherr et al., 2008). In gram-negative, only *Gluconacetobacter diazotrophicus* levansucrase has a 30-amino acid N-terminal signal peptide for secretion cleaved off during transport to the periplasm (Hernández et al., 1999); the rest of the analyzed levansucrases sequences lack this type of signal. In some cases, the presence of intermediates of the enzyme in cytoplasm and periplasm has been observed. Öner and coworkers (Öner et al., 2016) summarized gram-positive and gram-negative bacteria that produce levan extracellularly from sucrose-based substrates. Like other microbial extracellular polymeric substances (EPS), its production is significantly affected by fermentation conditions such as temperature, pH, oxygen concentration, bioreactor

configuration, and culture medium. Generally, the polymer production is associated with microorganism growth.

26.4.1 Strategies for Recombinant Expression of Levansucrase and Other Fructosyltransferase Genes in Yeasts

Although *E. coli* is a biotechnologically suitable host for the recombinant production of levansucrase (Öner et al., 2016) and other fructosyltransferase genes, *Saccharomyces cerevisiae* and *Pichia pastoris* both have the GRAS status. Basic and productive research can be carried out using these two yeasts, as they both have the ability to efficiently secrete recombinant proteins to the culture medium and therefore are biotechnologically more suitable for the industrial production of these enzymes. However, there are few reports in the literature on the use of yeasts for the production mainly of microbial levan. *Bacillus subtilis* levansucrase was used as a model to study the protein secretion in the yeast *Saccharomyces cerevisiae* (Scotti et al., 1996). The first successful study on levan-producing strains of the yeast *S. cerevisiae* was reported by Franken and coworkers (Franken et al., 2013). Besides, *P. pastoris* was also used to express *G. diazotrophicus* levansucrase coding gene lsdA by using inducible and constitutive promoters, although at different levels (Trujillo et al., 2002, 2001). In both cases, the enzyme was secreted, although when the inducible promoter was used, part of the recombinant protein remained in the yeast periplasmic space. Interestingly, mature native *G. diazotrophicus* levansucrase has an N-terminal pyroglutamic acid and a disulfide bridge as unique modifications (Betancourt et al., 1999). However; expression of the lsdA gene in *Pichia pastoris* yielded an active but glycosylated enzyme that behaved similarly to nonglycosylated LsdA when substrate specificity, fructo-oligosaccharide (FOS) production, sucrose hydrolysis, or levan formation reactions were carried out under different experimental conditions (Trujillo et al., 2004, 2001). Also, *L. mesenteroides* levansucrase was successfully secreted in this yeast (Kang et al., 2011). Recently, the yeast *Kluyveromyces lactis* has also been used for recombinant production of *Aspergillus terreus* fructosyltransferase (Spohner and Czermak, 2016).

26.4.2 Recombinant FOS and Levan Production

Several bacterial strains produce levan naturally. In the above section, we also described the recombinant production of levansucrases. However, the recombinant production of this fructose-rich polymer directly has been mainly produced using transgenic plants. The strategy of converting naturally occurring nonfructan crops into levan producer plants was aimed at two main purposes: to discover the catalyzing specificities of distinctive fructan biosynthesis pathways and the establishment of production systems. These experiments were initially carried out by introducing the *B. subtilis* gene encoding levansucrase in tobacco and potato plants (Ebskamp et al., 1994). From here, other crops have been used to carry out these strategies (Banguela and Hernández, 2006). The highest transgenic levan yield reported so far for a soil-grown plant was obtained when *G. diazotrophicus* levansucrase was produced in tobacco and the resulting levan accumulated at levels between 30% and 70% (*w*/w) dry weight in mature leaves of different transgenic clones (Banguela et al., 2011). In general, plants expressing bacterial fructosyltransferase genes exhibit aberrant phenotypes. However,

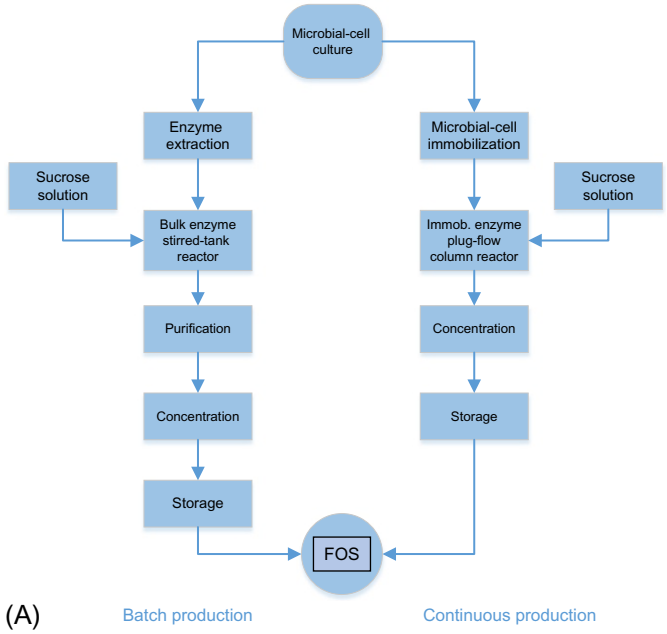
reports have been found in which the accumulation of short-chain linear levan (DP3 to N40) in amounts of 20–75 mg g^{-1} (fresh weight), did not cause visible alterations of the plant phenotype (Matsuhira et al., 2014). For a cost-effective production of commercial FOS, the *H. tuberosus 1-SST* gene was expressed in a sugar beet where GF2, GF3, and GF4 reached up to 73.8, 33.7, and 5.7 mmol g^{-1} (fresh weight), respectively (Sévenier et al., 1998). Other studies using plant fructosyltrnasferases for recombinant FOS production have been reported (Banguela and Hernández, 2006).

26.4.3 Current FOS and Levan Production Systems

The global prebiotics market is expected to grow ~12.7% in the next 8 years, so manufacturers are developing new alternatives to obtain sustainable and efficient processes for application on a large scale (Mano et al., 2017). The FOS demand increases at a rate ~15% per year (Panesar and Bali, 2016) and constitutes ~10% of the natural sweeteners in the market. Therefore, it is strongly desirable to find out cost-effective industrial ways to produce them (Mussatto et al., 2015, 2013). FOS production has been carried out mainly by chemical or enzymatic hydrolysis of inulins (Rastall, 2010), or by enzymatic methods using fructosyltransferases (Yoshikawa et al., 2007). The first industrial method stablished in Europe used chicory roots, where fructan accumulation can reach ~20% of its fresh weight. After root harvesting, fructans are extracted by hot water and then by subsequent purification steps and spray-drying procedures, a white powder is obtained that will then be submitted to partial hydrolysis to produce an FOS reach syrup. A disadvantage of this agronomic procedure is that after root harvesting, inulin can be degraded by fructan exohydrolases. This type of FOS is marketed by the Belgian firm Orafti Ltd. under the name Raftilose, and by the Dutch firm Imperial-Suiker Unie under the name Frutafit. In these commercial products, the main components are kestose (GF_2), nistose (GF_3), fructosylnistose (GF_4), bifurcose (GF_3), inulobiose (F_2), inulotriose (F_3), and inulotetraose (F_4).

The second method takes advantage of the advances in industrial enzymology applied to the large-scale production of FOS by enzymatic means (Fig. 26.2A and B).

Commercial production of FOS was first developed using enzymatic fructosyl transfer on sucrose (Hidaka et al., 1986), and since then, β-fructofuranosidase has been isolated especially from the fungi *Aspergillus niger* (Nguyen et al., 2005), *A. japonicus* (Mussatto et al., 2009), *A. oryzae* (Chang et al., 1995), *Aureobasidium pullulans* (Yoshikawa et al., 2007, 2006), and *Fusarium oxysporum* (Nishizawa et al., 1980). In this sense, the Japanese company Meiji Seika Kaisha Ltd. produces FOS from sucrose 60%–70% (*w*/*v*) as the substrate, using a bioreactor based on *Aspergillus niger* cells entrapped in calcium alginate gels. With this continuous process that produces 4000 tons of FOS per year, FOS content reaches 55%–60% (w/v), ranging from DP 3 to DP 4–5. The final product after chromatographic purification is an FOS-rich syrup that is further sold as syrup or as a white powder obtained by spray drying. In order to reduce production costs, not only cells but also enzyme immobilization techniques have been used. Fungal β-fructofuranosidase has been covalently immobilized by using different inorganic supports (Ganaie et al., 2014). Due to some disadvantages of current immobilization methods related to poor enzyme activity because of diffusion rates, the use of nanoparticles offers some solutions, mainly due to relative low mass transfer resistance (Kim et al., 2006). Solid-state fermentation (SSF) is also attractive for FOS production because of its low



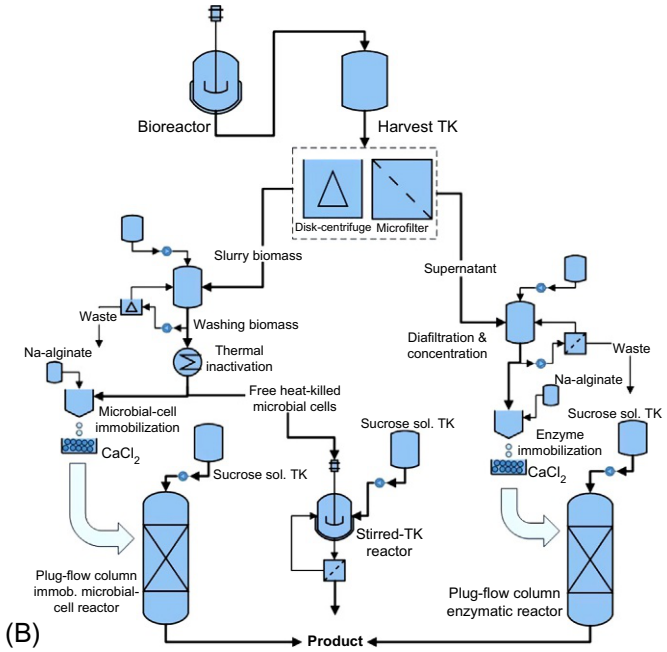


FIG. 26.2 (A) Diagram of batch and continuous bioprocess for FOS production. (B) Industrial bioprocesses for FOS production, using batch or continuous bioprocess with immobilized enzyme or cells.

production costs and high productivity at an industrial level (Mussatto and Teixeira, 2010). Plant fructosyltransferases have also been used instead of fungal enzymes for industrial FOS production. Recently, a patent was released for 1-kestose production using a recombinant plant enzyme produced in *P. pastoris* (Pérez Cruz et al., 2014). This method allows conversions >55% in FOS, where 1-kestose is represented by >90%. Other industrial alternative for FOS include solid-state fermentation utilizing various agroindustrial by-products (Flores-Maltos et al., 2016). Unlike FOS production, there is no commercially attractive technology for levan production. The use of levansucrase to transform sucrose into levan is an expensive and industrially unprofitable process because of the viscosity of the solution where the reaction is carried out due to the synthesized polymer. However, the production of this polymer has been reported from different substrates (Abou-taleb et al., 2015) and different international companies such as Natural Polymers Inc., Real Biotech Co., Ltd., and Advance Co., Ltd., are in charge of its industrial production (Öner et al., 2016). Recently, a new source of inulin and FOS useful for industrial, technological, and nutritional applications can be obtained from the industrial waste of *Stevia rebaudiana* bioprocesses (Lopes et al., 2017; Sanches Lopes et al., 2016). Otherwise, fusion proteins could be a promising strategy for industrial FOS production. Enzymatic production of levan type-fructooligosaccharides (L-FOS) with a DP from 2 to 10, through simultaneous synthesis and hydrolysis reactions, has been investigated. This strategy was accomplished by a new enzyme called $LevB_1SacB$, resulting from the fusion of SacB, a levansucrase from *Bacillus subtilis*, and $LevB_1$, an endolevanase from *B. licheniformis* (Porras-Domínguez et al., 2017). This could be the beginning of new production strategies based mainly on enzyme engineering.

26.4.4 FOS and Levan in the Food Industry

In the food industry, levan is recognized for its bifidogenic effect. It can be used as a human or animal prebiotic, which can significantly modulate the colonic microbiota by stimulating the growth of lactic acid bacteria such as *Bifido bacteria* and at the same time protecting the colon from carcinogens (Srikanth et al., 2015). Levans can also be used as industrial gums (Abou-taleb et al., 2015). Also, levan hydrolysis produces syrups rich in fructose, a sweetener of high demand in the international sweeteners market (Divya and Sugumaran, 2015), fat substitutes (Santos et al., 2014), and fillers (Abou-Taleb et al., 2014). A recent patent application (Xiao et al., 2014) describes how to make yogurt containing levan to be consumed as a functional food. In addition, levan has been used in food nanotechnology as a coating for micronutrient delivery and at the same time acting as a functional prebiotic to be consumed by intestinal microbes (Bondarenko et al., 2015). The most commonly used prebiotics to develop foodstuffs, including baby foods, yogurts, breads, and creamy milk, are inulin, FOS, and galacto-oligosaccharides (GOS) (Rößle et al., 2011). Also, FOS has been used as a substitute for phosphate and dextrose in whole muscle cooked hams (Resconi et al., 2016). The European Commission's Scientific Committee on Food determined that the addition of FOS to infant formulas did not present major concerns (Arslanoglu et al., 2007). Some randomized trials have been conducted to prove the effect of FOS in infant food (Bettler and Euler, 2006). Fecal bifidobacteria counts increased significantly in an FOS-supplemented group when compared with nonsupplements and the counts of bifidobacteria reached the range of a breastfed reference group. A multicenter trial regarding FOS-supplemented infant formula safety was

carried out in the United States, demonstrating that infant growth was maintained without any adverse effects (Scholtens et al., 2006). Inulin, FOS, and honey combined with *Lactobacillus acidophilus* and *Bifidobacterium lactis* were assayed in five petit-suisse cheese formulations and were tested in vitro with human fecal slurry. The fastest fermentation and high lactic acid production as well as the promotion of increased growth rates of bifidobacteria and *lactobacilli* were achieved. Other experiments using FOS in cheese have been reported (Rodrigues et al., 2012). The composition and rheology were evaluated for cow milk samples fermented with and without inulin at different temperatures ranging from 4°C to 6°C, showing an increment in the total solid content of the milk fermented with inulin (Debon et al., 2012). In a clinical study, partially hydrolyzed guar gum and FOS were incorporated in biscuits and further assessed in human volunteers, showing that the consumption of biscuits with added FOS and PHGG gave a significant increase in fecal bifidobacterial numbers (Mantzouridou et al., 2013). Other applications of inulin-containing wastes are related to producing biofuels and biobased chemicals (Hughes et al., 2017).

26.5 INVERTASES

Invertases are another group of enzymes that are useful in the food industry (EC 3.2.1.26), responsible for sucrose inversion yielding D-glucose and D-fructose (Lincoln and More, 2017), as shown in Fig. 26.3.

26.5.1 Nomenclature and Classification of Invertases

Sucrose (α-D-glucopyranosyl β-D-fructofuranoside) is one of the most abundant products in nature. Not only is it the main compound derived from photosynthesis and the predominant molecule of carbon translocation in most plants, but it also plays a central role in the plant's biological functions and responses to environmental stress (Vargas and Salerno, 2010). In plants, glucose and fructose are involved in signaling pathways in which sucrose concentration functions as a key sensor of the nutritional status of plants. Therefore, invertase plays a key role in the control of cell differentiation and development. Although animals, including man, show a marked preference for diets containing sucrose, their genomes do not code for invertase. Instead, they use a different and unrelated enzyme to hydrolyze sucrose, sucrose-glucosidase (EC 3.2.1.48). The genomes of human intestinal microorganisms such as *Bacteriodes thetaiotamicron* (Xu, 2003) and *Bifidobacterium longum* (Schell et al., 2002) have

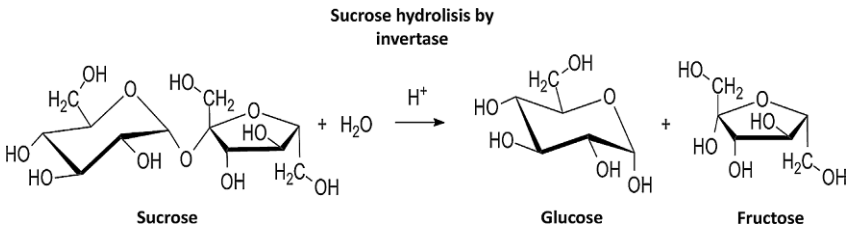


FIG. 26.3 Sucrose hydrolysis by invertase activity, yielding xmolar amounts of glucose and fructose molecules.

invertase genes, demonstrating that these organisms benefit from the consumption of sucrose by humans. The use of sucrose as a source of carbon and energy depends on the rupture of the α-1-β-2-glucosidic bond by the action of invertases that irreversibly hydrolyze the disaccharide in glucose and fructose. There are two types of enzymes that hydrolyze sucrose: the typical invertases named β-fructosidases (β-D-fructofuranosido fructohydrolase, EC 3.2.1.26) and the α-1,4-glucosidases glucosidases (α-D-glucoside glucohydrolase, EC 3.2.1.20) and oligo-α-1,6-glucosidases (EC 3.2.1.48) with a wide range of substrate specificity. Invertase can be classified into two classes according to its activity, initially differentiated by the optimum pH in vitro: (i) acidic invertase (Ac-InVS, EC 3.2.1.26, β-fructofuranosidases) with an optimum pH of between 4.5 and 5, and (ii) alkaline/neutral invertase (A/N-InVS) with an optimum pH in the range of 6.5–8.0 (Tymowska-Lalanne and Kreis, 1998). Invertases are defined as enzymes that hydrolyze sucrose in glucose and fructose, as shown above. Some invertases are reported to be highly specific to sucrose, such as the alkaline invertebrate carrot (Lee and Sturm, 1996), but the strict specificity for a substrate is an exception among these enzymes. In contrast, most β-fructosidases have a relatively broad specificity of substrates and can hydrolyze not only sucrose but also β-fructosid bonds in one or more of the following saccharides: sucrose 6-phosphate, raffinose, inulin, and levan (Lee and Sturm, 1996). Depending on their preferred substrates, β-fructosidases are often named in the literature as sucrose, invertase, fructanase, inulinase, or levanase. Invertases are found in the GH32 family of glycosyl hydrolases according to sequence-based classification (afmb.cnrs-mrs.fr/CAZY). This family, which includes >370 members of plant, fungal and bacterial origin, contains not only invertase but also other fructofuranosidases such as inulinase (EC 3.2.1.7), levanase (EC 3.2.1.65), and *exo*-inulinase (EC 3.2.1.80), and transfructosidases such as sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) and fructan: fructan 1-fructosyltransferase (EC 2.4.1.100) (Alberto et al., 2004). Glycosyl hydrolases or glucosidases are a broad group of enzymes that displays a wide variety of protein folding and substrate specificities. They share a common feature, two critical sites of acid residues, which constitute the catalytic mechanism responsible for the breakdown of glycosidic bonds. In yeast invertase, these two invariant residues have been identified as an aspartate located near the N-terminal that acts as a nucleophile and a glutamate that acts as the general acid/base (Reddy and Maley, 1996). With no known exception to date, the molecular mechanism appears conserved among members of the same sequence-based family. The analysis of the sequence coupled to structural comparisons has revealed significant similarities between representatives of different families, accompanied by a conservation of the catalytic machinery and the stereochemical reaction result, reflecting an old divergence of a common ancestor to acquire new substrate specificities.

26.5.2 *Thermotoga maritima* Invertase

Among >50 different species of known prokaryotic hyperthermophiles (with optimal growth temperature of at least 80°C), only a few have been reported to belong to the bacteria domain (Counts et al., 2017). It is believed that these high temperature-resistant organisms retain ancestral characteristics in their biomolecules and metabolic pathways. *T. marítima* is by far the best source for thermostable invertase for industrial invert sugar syrup production (Martínez et al., 2014). *T. maritima* is a bacterium that does not form spores, in the form of bacillus, that are strictly anaerobic and heterotrophic. It was originally isolated

from geothermal marine sediments and its optimal growth temperature is about 80°C. Phylogenetically, *Thermotoga maritima* seems to be one of the oldest lineages in *Eubacteria*. The *T. maritima* genome is a single circular chromosome of approximately 1.8 megabases in size, which codes for an estimated 1877 proteins. Because its genome has the highest percentage (24%) of genes similar to the archaea genes, *T. maritima* has become ideal for studying the organization of domain and the identification of new protein structures in *Eubacteria* and *Archea*, so that this microorganism has become a study model of hyperthermophilic bacteria. *Thermotoga maritima* invertase (BfrA) hydrolyzes sucrose, raffinose, inulin, and fructose polymers with a β-(1–2) terminal linkage to a glucose molecule, releasing fructose in each case. All substrates of BfrA have in common a fructosyl moiety bonded by β-(2–1) or β-(2–6) bond to the remaining parts of the saccharides. BfrA shows similar catalytic efficiency for the hydrolysis of sucrose and inulin with kcat/K_M values around $4.1\times10^4\,M^{-1}s^{-1}$ and $3.1\times10^4\,M^{-1}s^{-1}$, respectively (at 75°C, pH 5.5) (Muñoz-Gutiérrez et al., 2009). BfrA has an optimum temperature of 90–95°C (in 10 min trials) and was extremely insensitive to thermoinactivation. For 5 h at temperatures up to 80°C and pH 7, the enzyme retained up to 85% of its initial activity. Thus, BfrA is the most thermostable β-fructosidase described to date (Liebl et al., 1998). A *T. maritima* invertase molecule is composed of two individual modules, a catalytic five-sheet β-propellant module (residues 1–295) bound by 10 residues to a β-sandwich C-terminal module (Menéndez et al., 2013).

26.5.3 Recombinant Expression of *Thermotoga marítima* Invertase

T. maritima β-fructosidase (BfrA) is the most thermoactive and thermostable sucrose hydrolyzing enzyme so far identified. The use of molecular techniques has allowed us to efficiently manipulate microbe and plant genes to obtain them in large quantities and to satisfy the high demand for them. Different invertases from different plant species have been expressed in *E. coli* and *Pichia pastoris* for their molecular and functional characterization (Menéndez et al., 2013).

A thermostable biocatalyst is based on immobilized *P. pastoris* cells that express the gene encoding for *T. maritima* invertase (Martínez et al., 2017). The advantage of expressing a gene from a thermophilic organism is that it allowed these authors to kill *P. pastoris* cells by heat over 70°C while maintaining the activity of the periplasmic invertase. The immobilized biocatalyst completely hydrolyses sugar cane into highly concentrated solutions operating at high temperatures in batch reactors and fixed beds. The kinetic models that govern and predict the reaction times of this biocatalyst, operating at low and high sucrose concentrations, were also studied as well as the conditions for the industrial production of inverted syrup (Martínez et al., 2017) that guarantee stable production of invert sugar syrup in a cost-effective manner.

26.5.4 Invert Syrups in Food Industries

The most significant application of invertase is to produce sugar syrups. The production of high fructose syrup, high fructose corn syrup, and high glucose syrup extensively involves invertases (Nadeem et al., 2015). The production of glucose or fructose syrups by acid treatment has several drawbacks in the finished product, such as texture and taste alteration and

formation of the cancerigen 5-hydroxymethyl furfural (Hull, 2010). Therefore, enzymatic hydrolysis is preferred due to the benefits of greater specificity, clear products, higher yields, and a greater degree of purity. Because fructose is sweeter than sucrose, commercial invertas have a high value in confectionery industries for invert sugar production. In addition, the traces of fructoligosaccharides that are generated as a result of the transfructosylation reaction add beneficial medicinal properties for diabetics and increase the absorbance of iron in children (Lincoln and More, 2017). This sugar has the same properties of honey, so that as it does not crystallize, it is ideal for sweetening ice cream because it remains malleable and creamy with a very soft texture. Therefore, it is also used in chocolate confectionery, namely, *fondant*, jellies, caramels, and toffees (Lincoln and More, 2017). It is also used in pastries and bakery items as it helps fermentation and retains moisture so that makes the breads or cakes remain tender for longer. It is widely used in the food industry, especially in the manufacture of ice cream through its anticrystallizing power.

Invert sugar is also used in the ice cream shop because of its antifreezing power, that is, it avoids recrystallization, such as glucose or dextrose, providing the ice cream with a soft, smooth, and creamy texture. In addition, it is also useful in manufacturing drug formulae or drugs such as cough syrups, digestive aid tablets, nutraceutics, baby foods, and formulations of animal feed such as food for livestock and honeybees (Lincoln and More, 2017).

26.6 CONCLUDING REMARKS

In this chapter, the most recent aspects related to fructosyltransferases and invertase based on the practical applications of the authors have been discussed. Because consumers are increasingly interested in their health, they hope that the foods they eat are not only palatable but also safe and healthy. That highlights the importance of further research and development into new functional food-producing enzymes as well as following the search for new species of microorganisms or plants that produce more efficient enzymes.

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CHAPTER

27

Nutritional and Nutraceutical Improvement by Enzymatic Modification of Food Proteins

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27.1 INTRODUCTION

For centuries, enzymes produced by microorganisms (bacteria, yeasts, and fungi) have been used in food manufacturing. The history of food enzymes dates back to early 1880 when humankind utilized microbial proteases and amylases for the production of soy-derived foods. Rennet, a natural enzyme mixture from the stomach of calves, has been used in cheese making for centuries. Rennet is a source of protease that coagulates milk and separates it into cheese (solid) and whey (liquid). For centuries, enzymes produced by yeast have been used to make wine by fermentation of grape juice while papain from raw papaya was used for meat tenderization and in brewing to prevent a chill-haze formation by digesting proteins.

The advent of large-scale application of enzymes in the food industry has drastically set the trend for usage of the enzymes in designing and processing of various foods. Presently, the most established products in the biotechnology industry are enzymes. The global food enzymes market is expected to grow at a compound annual growth rate (CAGR) of more than 7% by 2020, with dedicated usage in the food and feed industries, which includes dairy, beverages, brewing, dietary supplements, etc. (Berka and Cherry, 2006; Ogawa and Shimizu, 2002). Other than the food industry, enzymes are also used in industries such as leather, pulp, detergent, textiles, and personal care.

Food enzymes help in the fortification of several foods to enhance their nutritional value. These enzymes support digestion and the metabolism by assisting in the breakdown of complex proteins, carbohydrates, and fats present in foods into simpler molecules. Proteins form

https://doi.org/10.1016/B978-0-12-813280-7.00027-X

the fundamental and integral part of food components, both nutritionally and functionally. Enzymes are being used to modify proteins through hydrolysis. Enzymatic hydrolysis is a valuable and flexible tool to improve the nutritional and functional properties of proteins.

27.2 USE OF ENZYMES IN FOOD APPLICATIONS

27.2.1 Enzymatic Hydrolysis

Protein hydrolysis is carried out by chemical and enzymatic methods. Most of the enzymes used for protein hydrolysis are from animal sources (such as pancreatin and pepsin), plant sources (such as papain from papaya, ficin from fig, and bromelain from pineapple), and microbial sources (such as Alcalase).

Proteolytic enzymes hydrolyze proteins at the optimum temperature and pH and usually target specific peptide cleavage bonds, resulting in digestion consisting of amino acids and peptides of varying size. Enzymes from animal sources are more specific to their site of action compared to plant enzymes, which are more broadly specific in their action. For example, the enzyme pepsin will cleave at the phenylalanine or leucine bond. Papain has a broad specificity, cleaving bonds at phenylalanine, arginine, and lysine. Pancreatin cleaves at tryptophan, arginine, tyrosine, leucine, phenylalanine, and lysine bonds. Proteins incubated with microbes lead to hydrolysis by fermentation, during which proteolytic enzymes are secreted. Proteases from microbial sources offer a wide variety of enzyme activities. Proteases from bacterial, algal, fungal, and yeast sources are produced on a large scale and usually only require simple purification steps, which can be used for an industrial application such as the production of peptone.

27.2.2 Enzymatic Modification

Enzymatic modification improves the functional property of the protein. In addition to providing nutrition, proteins present in foods also provide specific functional properties that facilitate processing and serve as the basis of product performance. The functional properties of food proteins indicate the physicochemical nature, which governs the behavior of the proteins in foods. The most important functional properties of food proteins, in general, are solubility, emulsification, and foaming (Kinsella, 1982).

Conditions for enzymatic hydrolysis are usually mild but recently the use of high pressure for hydrolysis has also gained interest. The functional properties of the protein depend on hydration and gelation as well as the interfacial and aggregation properties. Enzymatic hydrolysis of proteins enhances the functionality due to the ability to expose the protein structure and reduce the average molecular weight as well as increase the ionic strength, molecular charges, and protein-to-protein interactions (Jeewanthi et al., 2015). The optimum degree of hydrolysis (DH) of proteins is required to obtain improved functionality. The main problem associated with proteolytic hydrolysis of proteins is the production of a bitter taste, coagulation, and the high cost of enzymes. It is important to consider the type of enzyme, hydrolysis conditions, and control of DH. Enzymatic modification of heat-processed soy flour exhibited improved functional properties (Radha et al., 2008).

In a study, a limited proteolysis of gluten has shown to improve the foaming capacity but also shows a decrease in the stability of the foam. The same phenomenon has been observed in the hydrolysates of purified fractions of gliadins (Chobert et al., 1996). Enzyme-modified proteins have advantages in terms of better solubility and digestibility while acting as flavor enhancers and substitutes for the protein. The protein hydrolysates with improved protein digestibility form an important ingredient in geriatric and pediatric foods as well as sports/energy drinks.

27.2.3 Enzymatic Extraction

Various types of enzymes are used for the extraction of bioactive/proteins for food applications. Various food matrices held tightly together are released slowly by the action of enzymes. The enzyme-assisted extraction of volatiles from cumin (*Cuminum cyminum* L.) seeds demonstrated that enzymes facilitate the extraction of cumin oil with the increase in oil yield, with little change in either the flavor profile or the physicochemical properties of the oil (Sowbhagya et al., 2011).

The authors reported that the enzyme-assisted protein extraction method has shown a better extractability and a higher recovery with the minimum use of solvent compared to the nonenzymatic methods described in the literature (Vergara-Barberán et al., 2015). Rice bran was enzymatically modified, which resulted in the inactivation of lipase. In addition to improving the retention of the vitamin B-complex and the antioxidants, enzymatic modification also enhanced the dispersibility and digestibility of rice bran (Vallabha et al., 2015).

27.3 GENERATION OF NUTRACEUTICAL PROTEIN HYDROLYSATES/BIOACTIVE PEPTIDES

Protein-rich resources generate protein hydrolysate/bioactive peptides, which can be used in functional food formulations with the potential for health benefits. Bioactive peptides (sequence of aminoacids) with a positive health impact can be generated by enzymatic (endogenous/microbial) or chemical hydrolysis, which is otherwise encrypted within the sequence of the parent protein. Bioactive peptides are, generally, food-derived peptides that have physiological hormone-like effects in humans. A lot of work on the bioactive peptides from food sources such as milk proteins, oilseed proteins, and marine products has been carried out. Hypertension is one of the independent risk factors for cardiovascular diseases. The enzyme Angiotensin I-converting enzyme (ACE) plays a crucial role in this by promoting the formation of the vasoconstrictor Angiotensin II and inactivating the vasodilator bradykinin. Some of the food-derived peptides act as ACE inhibitory peptides and significantly reduce blood pressure (Erdmann et al., 2008). These peptides could be used to replace the synthetic drugs used for the treatment of hypertension. The structure-function relationship of these inhibitory peptides has been studied extensively. These inhibitory peptides, apart from inhibiting ACE, may also act by releasing vasodilatory substances such as nitric oxide or by binding to opioid receptors (Somers et al., 2000). The peptides released from amaranth seed proteins after enzymatic digestion show inhibitory activity against

dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5). An enzyme known to deactivate incretins, hormones involved in insulin secretion, has been reported by Velarde-Salcedo et al. (2013).

The peanut protein hydrolysate has shown ACE inhibitory and antioxidant activity and the degree of hydrolysis influences the functional properties of proteins (Jamdar et al., 2010). The effect of lowering blood pressure was observed using the Australian canola protein hydrolyzed by alcalase and pepsin in spontaneously hypertensive rats. These hydrolysates may serve as useful ingredients to formulate antihypertensive functional foods and nutraceuticals (Alashi et al., 2014). ACE inhibitory peptides are generated using many microbial species. Sour milk fermented by *Lactobacillus helvetica* contained ACE inhibitory tripeptides (Ile-Pro-Pro and Val-Pro-Pro) that, on ingestion, lowered the blood pressure in mildly hypertensive subjects (Tuomilehto et al. 2004). Fermentation of soy protein with *Lactobacillus casei* spp. pseudoplantarumuse produced ACE inhibitory peptides, where the peptide sequence identified showed an important role for glutamine (Q) and threonine (T) residues in ACE inhibition (Vallabha and Tiku, 2014).

Several studies have demonstrated the ability of proteins to inhibit lipid oxidation in foods. Food sources such as milk (casein and whey) and soy on hydrolysis have shown to exert antioxidant activity. The literature shows a reverse relationship between antioxidant intake and diseases. The antioxidant activity of bioactive peptides can be attributed to their radical scavenging, inhibition of lipid peroxidation, and the metal ion chelation properties of peptides. The peptide structure and its amino acid sequence may play a role in contributing to antioxidative activity. Peptides containing high amounts of histidine and hydrophobic amino acids seem to possess antioxidant potency. These peptides may minimize free radical-induced damage to the cells and also inhibit lipid oxidation in foods. The incorporation of soy, whey, casein, and egg yolk hydrolysates in foods such as tuna, pork, and beef has shown inhibition of lipid oxidation (Diaz and Decker, 2005; Pena-Ramos and Xiong, 2003; Sakanaka et al., 2005; Sakanaka and Tachibana, 2006).

The ex vivo digestion of palm kernel hydrolysate exhibited a potent ACE-inhibitory activity (IC50 50 mg/mL) as well as anticancer activity against human colon epithelial cancer HT-29 cells and hepatocarcinoma HepG2 cells. Two resistant peptides, RADVFNPR and KLPLVERIP, were identified in the hydrolysate (Tapal et al., 2016). Hypercholesterolemia is another risk factor for cardiovascular diseases. Soy protein hydrolysates reduce total cholesterol levels much more effectively than soy protein. Two peptides from the soybean β-conglycinin, on their interactions with the catalytic site of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR), demonstrated that they behave as competitive inhibitors of HMGCoAR activity with a statin-like mechanism (Lammi et al., 2015). Peptides in soybean protein hydrolysate (SPH) are reported to have a hypocholesterolemic effect by dietary upregulation of LDL-R transcription, consequent to an enhanced catabolism or a reduced synthesis of intracellular cholesterol, thereby effectively stimulating LDL-R transcription in the human liver cell line and reducing the blood cholesterol level (Cho et al., 2007).

Milk-derived whey proteins/hydrolysates/peptides exert hypocholesterolemic effects in different animal models. The incorporation of whey protein in the diet was observed to significantly reduce the total cholesterol levels in rats fed with cholesterol-free and cholesterol-enriched diets; β-lactoglobulin tryptic hydrolysate also exhibited a similar effect (Hsieh et al., 2015; Nagaoka et al., 2001). The study on defatted corn protein hydrolysate after in vitro incubation with gastric proteases demonstrated a bile acid binding capacity, suggesting

that corn hydrolysate using flavourzyme may be used as a potential cholesterol-reducing agent. (Kongo-Dia-Moukala et al., 2011). Post-oral administration of various dosages of high and low arginine fractions from rice bran protein hydrolysate remarkably regulated hypertension in DOCA salt-induced hypertensive rats (Vallabha et al., 2013).

Many dietary proteins have shown an effect on lowering both blood pressure cholesterol, based on their amino acid profiles, especially the arginine: lysine (Arg:Lys) ratio in hypertension, atherosclerosi, and hypercholesterolemia conditions (Tovar et al., 2002). The effect of the Arg:Lys ratio has been investigated in the protein form and free amino acid form on hypertension in hypercholesterolemic Wistar rats. The Arg:Lys ratio in both the protein form (MPI) and the free amino acid form strongly affects the metabolic pathways of hypertension with a moderate effect on hypercholesterolemia (Vallabha et al., 2016). Proteins containing low ratios of methionine-glycine and lysine-arginine show an effect on lowering blood cholesterol. The intake of variable ratios of Arg:Lys or Gly:Met offered a beneficial influence on the lipid profile and plasma levels of selected cardiovascular disease markers such as ADMA, SDMA, and homocysteine in hypercholesterolemic rats (Venkatesh et al., 2017).

27.3.1 Challenges in Application of Peptides and Hydrolysates

In order to obtain low-cost and good-tasting foods with an extended shelf life, the food industry makes use of nonhealthy additives. Increased consumer awareness of food, diet, and health is leading to increased demand for natural ingredients such as bioactive peptides in food products for consumption of healthy foods. To commercialize the use of health-beneficial bioactive peptides as food ingredients, one needs to overcome the challenges. The main drawbacks associated with enzyme-assisted hydrolysis of proteins involve the high cost of enzymes, the generation of bitter peptides, and the hygroscopic property of hydrolysate. A reduction in the bitterness of hydrolysate can be achieved by the control of DH, the selective separation of bitter peptides, treatment with *exo*-peptidases, methods to mask or adsorb the bitter taste, and modification of taste signaling.

27.4 ENZYMES AS A PREDICTIVE TOOL TO ASSESS THE POTENTIAL ALLERGENICITY OF PROTEINS

Proteins are an important part of the human diet, and they comprise both animal- and plant-derived sources. From a nutritional point of view, protein is required to be digested by proteolytic enzymes of the gastrointestinal tract into the constituent amino acids. The allergenicity of food proteins is attributed to the resistance of gastrointestinal digestion initiating an immune reaction. About 1%–2% of adults and 6%–8% of children are affected by food allergies and approximately 90% of all food allergies are associated with proteins (Ladics, 2008; Metcalfe et al., 1996; Sampson, 1997). A rigorous safety assessment process for an allergenic potential of the expressed protein exists for genetically modified (GM) crops (Ladics, 2008). Food proteins that induce allergic sensitization, react with IgE antibodies, and induce allergic reactions are known as food allergens (Schnell and Herman, 2009).

To assess the stability of protein to the human gastric environment as well as its nutritional value, the pepsin digestibility method was developed (Marquez and Lajolo, 1981;

Nielson, 1988; Thomas et al., 2004; Zikakis et al., 1977). Later, protein digestibility using pepsin was used for the evaluation of the potential allergenicity of novel dietary protein (could be derived from GM foods) (Metcalfe et al., 1996). The present assessment strategy focuses on the "weight of evidence" approach by considering factors such as the source of protein, the protein's amino acid similarity with that of known allergens, pepsin digestibility, and clinical testing as outlined by the Ad Hoc International Task Force on Foods Derived from Biotechnology (Codex Alimentarius Commission, 2003; Thomas et al., 2004). From the safety point of view, highly digestible proteins have reduced the potential for allergenicity but still, proteolytic-cleaved peptides of allergens may bind to IgE. Proteolytic enzymes were used for the identification of protein segments that may bind to IgE and the first known report on the application of the in vitro pepsin digestibility assay was used to evaluate food protein allergens by Astwood et al. (1996) (Thomas et al., 2004).

Genetically modified proteins (by recombinant DNA technology) are evaluated for potential allergenicity by the "weight of evidence" approach recommended by the Codex Alimentarius Commission (2003) that includes digestibility by pepsin (Thomas et al., 2004). For this purpose, a consistent and reproducible common protocol for evaluating the in vitro digestibility of proteins was developed by testing at different laboratories using the same proteins (consisting of ovalbumin, bovine serum albumin, concanavalin A, ribulose diphosphate carboxylase, α-lactoglobulin, ovomucoid, horseradish peroxidase, phosphinothricin acetyltransferase, Ara h 2 (a peanut conglutin-like protein), and soybean trypsin inhibitor) (Thomas et al., 2004). Digestion was performed at pH 1.2/2.0 with a ratio of 3:1 pepsin to protein, w:w (10 U of pepsin activity/μg of protein) (Thomas et al., 2004). A sampling of hydrolysate was done at different time intervals and assessed from stained SDS-PAGE gels. From the experiment, it was observed that results obtained across laboratories were more consistent at pH 1.2 (91% agreement) than at pH 2 (77%) (Thomas et al., 2004). This established the method for the in vitro protein digestibility evaluation, which gave better reproducibility and consistency in results using the same proteins at different laboratories (Thomas et al., 2004). The allergenic potential of new food proteins is determined by the results of in vitro digestion experiments, based on the relationship between gastrointestinal digestion and food allergies (Schnell and Herman, 2009).

Enzymatic digestion of food protein is essential from a nutritional as well as a safety point of view. Through the processes of digestion, food proteins are cleaved in the GI tract. Extensive cleavage of these dietary proteins makes them lose antigenicity; therefore, resistance to digestion is considered an indicator of potential allergenicity (Schnell and Herman, 2009). The exact site of food protein reactivity for antigen recognition is still not known. However, it is believed that most food allergens, after moving through the stomach in an intact form with antigenicity potential, are absorbed in the intestine (Schnell and Herman, 2009). Most of the studies on the allergenic potential of new food proteins are done using purified protein by in vitro simulated gastric digestion, but the potential effect of the prepared food matrix as a whole component must be assessed (Schnell and Herman, 2009).

Ingestion of a novel protein in a food ingredient may increase the exposure of consumers to the proteins, which could be allergenic. Because of increasing awareness, many new foods and food formulations are being subjected to allergenicity test (Goodman et al., 2007). As a part of the safety evaluation of novel protein components for allergenic food, protein digestibility is evaluated by the pepsinolysis method to assess the allergenic risk for the consumer

(Goodman et al., 2007; Ladics, 2008). New additional approaches such as animal models or an ex vivo system mimicking an in vivo process may be utilized. However, with the advancement of science and technology, until now new approaches have not been assessed for evaluation and validation for predicting protein allergenicity (Ladics, 2008).

27.5 FOOD PROTEIN DIGESTIBILITY BY EX VIVO AND IN VITRO STUDIES

Ex vivo (derived from the Latin word "out of the living") means that which takes place in an external environment (outside an organism) under natural conditions. Ex vivo digestion (EVD) of proteins refers to the digestion of proteins outside the gastrointestinal system using human gastrointestinal enzymes. Human gastrointestinal juice contains a complex mixture of isoforms of proteases, amylases, and lipases in combination with bile salts, bilirubin, inhibitors, and other minor components that affect protein degradation (Almaas et al., 2011; Dunn, 2002; Furlund et al., 2013; Scheele et al., 1981). In order to simulate the human enzymatic digestion, gastrointestinal juices aspirated from human volunteers are being used in the EVD digestion method. To understand the mechanisms involved in food protein digestion, use of the EVD model would mimic the human digestion pattern. The most challenging physiological parameters in the digestion of food proteins are the variation in GI enzymes, acid, ionic concentration, bile salt, and substrate availability, and the digestion time in the gastric and intestine (Furlund et al., 2013). The advantage of using human juices is that they are more likely to mimic an in vivo digestion. Therefore, the method is referred to as the EVD model.

Gastrointestinal (GI) digestion of a dietary protein is influenced by the physicochemical characteristics of the protein. A complex combination of mechanical, physicochemical, and physiological processes is involved in the GI digestion of proteins in humans. Digestion of dietary proteins in the gastrointestinal system involves stomach and intestinal proteolytic enzymes. The protein digestion begins in the stomach, where pepsin breaks down the protein into smaller peptides. Pepsin exhibits maximum activity at acidic pH 2.0 and is inactivated at pH 6.5 in the duodenum. The pH in the duodenum is gradually increased to 5–7.5, due to the secretion of bicarbonate and pancreatic juices. The increased pH inactivates the gastric enzymes and gives the optimal activity of the duodenal enzymes (trypsin/chymotrypsin). When the semidigested peptide mix reaches the duodenum, the intestinal enzymes continue to break down the peptides. The final stage of digestion of proteins occurs on the surface of intestinal enterocytes by brush border enzymes, where peptides are hydrolyzed to amino acids as well as di- and tripeptides. The nutrients are then absorbed by the enterocytes of the jejunum and ileum and can be further degraded by intracellular proteases before entering the blood stream.

The type of food matrix, the form of food particles, the digestion steps (starting from mouth to the large intestine), the enzyme specificity, and its optimum conditions must be considered during the development of in vitro or ex vivo methods (Devle et al., 2014; Hur et al., 2011; Minekus et al. 2014). An in vitro digestion method can be used as a screening tool for different foods, as simulating in vivo digestion may not be complete (Hur et al., 2011). With simplified methodology and minimized experimental variables, a standardized in vitro method for digesting food proteins is required to attain a reproducible experiment. The COST

Action INFOGEST protocol has standardized an international method of in vitro digestion simulating human gastrointestinal conditions (Minekus et al., 2014). Furthermore, different parameters such as time, temperature, and the use of enzymes are described. The method has been validated in three interlaboratory studies where some parameters concerning protein digestion have been clarified (Egger et al., 2016). These studies also showed that the consensus method has led to an increased consistency and better comparability of in vitro digestion (IVD) studies. The IVD of proteins under simulated gastrointestinal conditions is performed using commercial enzymes such as pepsin and pancreatin to determine the probable of bioactive peptides. In vitro studies for protein hydrolysis have been carried out using the commercial enzymes, mainly from porcine or bovine origin. The commercial enzymes purified from different animal sources are likely to vary in functionality, specificity, and stability (Furlund et al., 2013).

Due to the growing interest in revealing how food proteins are digested in the human digestive tract, EVD using human gastrointestinal juices and IVD using commercially available enzymes have been the subject of study. Proteins digested with nonhuman and human enzymes seem to generate different peptides with regard to both sequence and length (Eriksen et al., 2010; Furlund et al., 2013). Many researchers have opined that, due to the complex protein digestion process, EVD results differ from IVD. The commercial enzymes appear to digest proteins (lactoferrin, caprine whey proteins) more efficiently compared with human digestive juices when used at similar enzyme activities. This could lead to conflicting results when comparing human in vivo protein digestion with digestion using purified enzymes of nonhuman species (Eriksen et al., 2010; Furlund et al., 2013). Comparative reports of peptide profiles identified in digests/hydrolysates of salmon proteins and caprine whey proteins obtained with the use of human gastrointestinal juices/commercially available enzymes suggested that the hydrolysis pattern differed in both enzymes due to different sources (Almaas et al., 2011; Darewicz et al., 2014; Borawska et al., 2016). The peptides generated in salmon protein varied in the amino acid sequences produced by EVD and IVD, resulting from the specificity of the two enzymes (Borawska et al., 2016). Due to the complexity in human gastrointestinal enzymes, the cleavage pattern in the protein chains differs compared to purified nonhuman enzymes (Almaas et al., 2011).

During the food protein digestion process, protein fragments (bioactive peptides) may be generated in the GI tract that impart biological activity and impact the physiological activity of the body. The degradation of protein in the GI tract should be taken into consideration when health effects are proposed (Furlund et al., 2013). The EVD profile is closer to in vivo than in vitro experiments. Studies on the generation of biologically active peptides by EVD are relatively few. Caprine whey protein hydrolysate exhibited better antibacterial activity using human gastrointestinal enzymes compared to nonhuman gastrointestinal enzymes (Almaas et al., 2006, 2008, 2011). In order to screen the health potential of carp muscle tissue proteins, salmon protein, and oil palm kernel protein, various biological activities in EVD have been performed (Borawska et al., 2015, 2016; Tapal et al., 2016). Relatively few studies on food protein digestion using human gastrointestinal enzymes have been reported. The research on digestion studies suggests that human digestive enzymes should be preferred over pure commercial enzymes from other species when mimicking human digestion (Almaas et al., 2011; Borawska et al., 2014; Borawska et al., 2016; Eriksen et al., 2010).

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CHAPTER

28

Plant-Derived Enzymes: A Treasure for Food Biotechnology

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28.1 BASIC FUNDAMENTALS OF FOOD PROCESSING

Food processing is a transformation practice in the beverage and food industry to make the raw foodstuff of animal and plant origin suitable for consumption. On the basis of their difference from one another, the processed food materials can be distributed into highly processed foods, minimally processed foods, and processed foods (Ohlsson and Bengtsson, 2004; Monteiro et al., 2011). The processing methods are performed through a wide range of biological and chemical agents such as enzymes. The utilization of biological agents in food processing dates back to 6000 BCE and includes bread making, beer brewing, cheese making, and wine making. The first known purposeful microbial oxidation dates from 2000 BCE with the engendering of vinegar (Schäfer et al., 2002; Vasic-Racki et al., 2006). Even though enzyme usage was a part of the routine, nobody was aware of these reactions or the chemistry behind them. The most popular processed enzymes, invertase and pectinases, were used in foods from the 1930s; the usage of invertase initiated the immobilized enzymes in the 1960s. Since then, the large-scale application of enzymes in the food industry, the trends for designing and implementing them in processes, and the production of goods based on them have increased rapidly. Currently, these products are mainly recognized in the biotechnology industry. The revenue from this industry increased from $1.3 billion in 2002 and will likely reach $7 billion in 2013 (Bon and Ferrara, 2007; Leisola et al., 2002). A component of this market is devoted to usage in the food industry, and it includes brewing, dietary supplements, dairy, beverages etc. (Berka and Cherry, 2006; Ogawa and Shimizu, 2002). This is followed by the leather, textile, pulp, detergent, personal care, paper, and feed industries (Christopher and Kumbalwar, 2015).

https://doi.org/10.1016/B978-0-12-813280-7.00028-1

28.2 TYPES OF PLANT-DERIVED ENZYMES

Plant-derived enzymes include amylase, invertase, papain, bromelain, ficin, lipoxygenase, etc. These enzymes have played an important part in food production, including syrups, bakery products, alcoholic beverages, dairy products, etc. Besides the use of the plant as a factory for enzyme production, it can also serve as a raw material (enhancer) for the enhancement of microbial enzyme activity employed in the food industry (Fig. 28.1).

28.2.1 Proteases

Comprising about 60% of the total enzyme market, peptidases or proteases establish the major group in the bioindustry, particularly in the food, pharmaceutical, and detergent industries, with a long range of their uses. Protease proteolysis is the catabolism of protein by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain making the protein (Gupta and Khare, 2007; Kalpana et al., 2008). Because the appropriate producers of protease enzymes for commercial exploitation are nontoxic and nonpathogenic, they are considered safe and ecofriendly (Gupta et al., 2002).

It is well known that proteases are universal, being constituted in a wide diversity of sources such as animals, plants, and microorganisms, and that they are physiologically essential for living organisms (Rao et al., 1998). Plant-based protease production and its use are dependent on the availability of agricultural land and certain climatic conditions. Some of the well-known plant-based origin proteases are papain, bromelain, ficin, keratinases, etc. (Abidi and Limam, 2008; Shankar et al., 2010).

The use of proteases in the food industry goes back to ancient times. They have been regularly used for several purposes, such as the preparation of soya hydrolysate, meat tenderization, cheese making, and baking (Cheong et al., 1993). They have occupied the maximum pertinent place among industrial enzymes. Plant proteases have been used in food science, medicines, and detergent manufacturing for several years, but due to their higher production costs, their production is diminishing as compared to those of microbial origin. For industries, new proteases with new and more attractive physicochemical properties are still

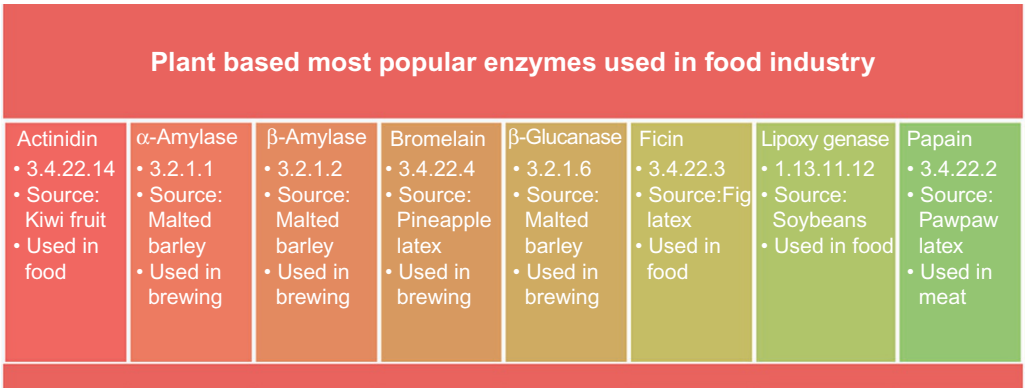

FIG. 28.1 Plant derived enzymes in food biotechnology.

TABLE 28.1 Uses of Plant-Derived Proteases

Types of Proteases	Plant Sources	Uses
Actidin	Kiwi fruits	Food, dairy products, and meat tenderization
Bromelain	Pineapple latex	Brewing
Papain	Papaya latex	Meat tenderization
Ficin	Fig latex	Food

developing. No doubt, genetic engineering and DNA technology will play a significant role in their production (Feijoo-Siota and Villa, 2011) (Table 28.1).

28.2.1.1 Actidin

EC number:	3.4.22.14
Source:	Kiwi fruit, pineapple, mango, banana, and papaya
Used in:	Food, dairy products, and meat tenderization
Molecular weight:	32 kDa
Substrate:	Dairy substrate
Product:	Fresh cheese

Actidin, a novel sulfhydryl protease (Cysteine protease), was originally isolated from the gooseberry (kiwi fruit). It is used commercially to tenderize meat by enhancing the deprivation of the myofibrillar proteins into peptides chemical processes in the meat industry (Varughese et al., 1992). Even at more concentrations, actidin prevents the surface mushiness and shows mild tenderizing activity. Due to the lower inactivation temperature (60°C) of this enzyme, it is easier to regulate the tenderization process without overcooking (Eshamah et al., 2014; Tarté, 2009). In postmortem aging, it activates the m-calpain during the process (Ha et al., 2012). Actidin has various applications in the food industry because it is beneficial over other plant proteases such as papain and ficin (Fig. 28.2).

28.2.1.2 Bromelain

EC number:	3.4.22.4
Source:	Pineapple latex
Used in:	Brewing
Molecular weight:	33,000 D
Substrate:	Myofibrillar proteins and collagen
Product:	Overtenderization of meat

Bromelain is a type of protease enzyme obtained from the aqueous extract of unripe fruits and stems of pineapples (*A. comosus* from Bromeliaceae family). Bromelain is a mixture of

FIG. 28.2 Actidin (PDB database).

phosphatases, peroxidases, glycoproteins, glucosidases, cellulases, and different proteases as well as carbohydrates. The plant stem contains major proteases termed the ananase or "stem bromelain" (EC 3.4.22.32) while the pineapple fruit juice protease is termed the "fruit bromelain" (EC 3.4.22.33).

Two additional CPs were identified only in the stem through comosain and active site-directed affinity chromatography: ananain (EC 3.4.22.31) (Napper et al., 1994; Rowan et al., 1988, 1990). Crude commercial bromelain from the pineapple stem has been purified by the sequential use of ammonium sulfate fractionation, gel filtration, and ion exchange chromatography. A total of 0.87 g of purified bromelain is obtained from 10 g of the starting material (Devakate et al., 2009; Murachi et al., 1964; Vanhoof and Cooreman, 1997; Wharton, 1974). The optimal pH for stem bromelain activity is 6.0–8.5 for most of its substrates, and its optimal temperature range is 50–60°C. Fruit bromelain has far greater proteolytic activity compared to stem bromelain as well as a wider specificity for peptide bonds (Feijoo-Siota and Villa, 2011; Polaina and MacCabe, 2007).

Bromelain is the main enzyme for tenderization (causes overtenderization by breaking collagen and myofibrillar proteins) of meat in industries with an organized environment, and it is beneficial for the assurance of the microbiological purity and quality. In the processing of adult beef, bromelain revealed the greatest results at 10 mg/100 g meat, for 24 h at 4°C, followed by increasing the temperature at the rate of 1°C/min until it reached 70°C. Bromelain is commercially available in a powdered form. It is predicted that 95% of the enzymes in the United States are obtained from plants such as papain and bromelain, whereas microbially derived tenderizers are not utilized broadly (Ionescu et al., 2008) (Fig. 28.3).

28.2.1.3 Papain

EC number:	3.4.22.2
Source:	Pawpaw latex
Used in:	Meat
Molecular weight:	23,000 Da
Substrate:	Cuts the protein chains in the fibrils and also in the connective tissue
Product:	Tenderizing the meat

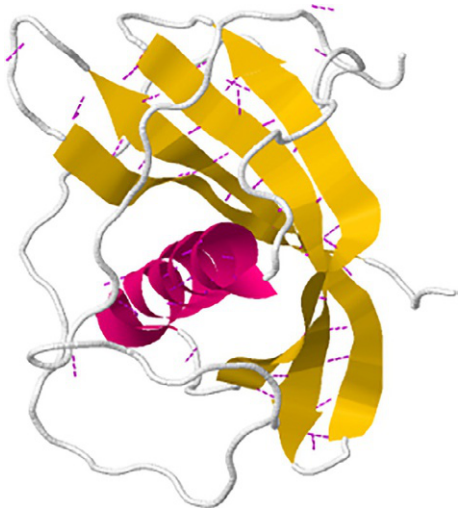

FIG. 28.3 Bromelain (PDB database).

Papain (EC 3.4.22.2), another cysteine protease enzyme, contains a trivial latex element (5–8%). It was the first to be isolated and crystallized in 1879 (Drenth et al., 1968; Kamphuis et al., 1984). Papain exhibits endopeptidases, aminopeptidases, dipeptidyl peptidases, esterase, amidase, transamidase, thiolesterase, and transesterase activities moreover its protease activity (Barbas and Wong, 1987; Johnston, 1956). The optimum pH (5.0–7.0) of papain differs with the substrate's nature, being 7.0 when casein is utilized as the substrate. In comparison, it is tremendously temperature stable compared to other proteases. The effective activity of this enzyme is established over the temperature variety of 10–90°C (Feijoo-Siota and Villa, 2011; Whitehurst and Van Oort, 2010) (Fig. 28.4).

28.2.1.4 Ficin

EC number:	3.4.22.3
Source:	Fig latex
Used in:	Food
Molecular weight:	25KD
Substrate:	Cleaves protein at tyrosine and phenylalanine residue
Product:	Produce hydrolysates and controlled tenderization

Ficin, or ficain (EC 3.4.22.3), is extracted from *F. glabrata*'s dried latex (Sgarbieri et al., 1964). It also exists in another species of ficus such as *Ficus elastica* and *Ficus carica*. Crude ficin has significant commercial importance but few studies have been carried out on the ficin isolated from the latex of another species of *Ficus*. A green fig of 10–15 g comprises only about 100–150 mg ficin (Uhling, 1998). Naturally, in latex isolated from a *Ficus* tree, ficin occurs in multiple forms (Jones and Glazer, 1970) that are distinguishable by ion-exchange chromatography (Kramer and Whitaker, 1969; Liener and Friedenson, 1970; Malthouse and Brocklehurst, 1976; Williams and Whitaker 1969). The optimal pH range for ficin is from 5.0–8.0 and the

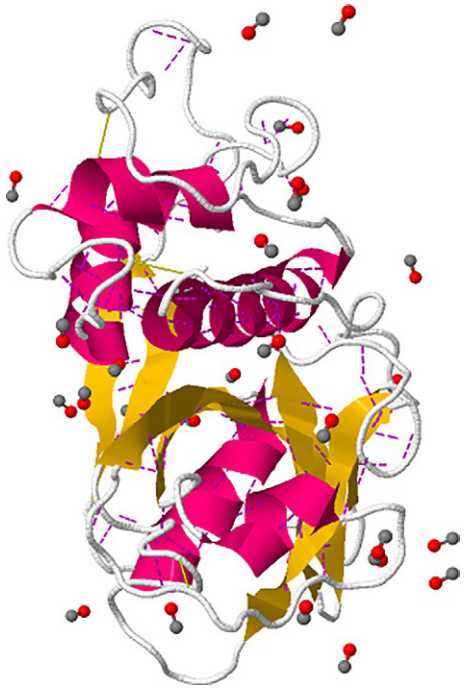

FIG. 28.4 Papain (PDB database).

optimum temperature is 45–55°C (Polaina and MacCabe, 2007). Until now, only three fragments of ficin have been studied (one fragment around the catalytic Cys, another fragment around the catalytic His, and an N-terminal fragment). Due to amino acid sequences, Cys was found to resemble the corresponding sequence in papain for the neighboring residues of the active site (Devaraj et al., 2008; Feijoo-Siota and Villa, 2011) (Fig. 28.5).

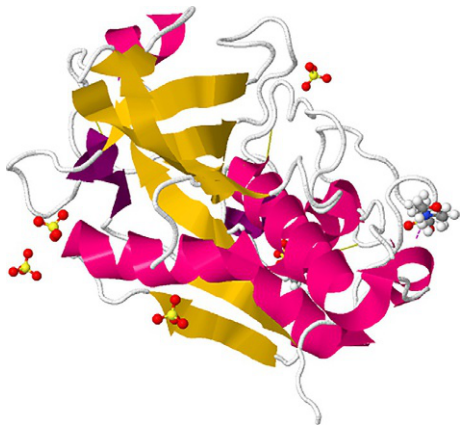

FIG. 28.5 Ficin (PDB database).

28.2.2 α-amylase

EC number:	3.2.1.1
Source:	Malted barley
Used in:	Brewing
Molecular weight:	51.0–54.0 kDa
Substrate:	Starch and glycogen
Product:	Glucose and maltose

α-Amylases are extracellular, industrially important starch conversion enzymes that catalyze the starch and hydrolyze the alpha 1,4-glycosidic linkages to release glucose. The optimum pH of α-amylases varies from 2 to 12 and they are thermostable. These enzymes have diversified applications in the food, starch liquefaction, sugar, paper, and pharmaceutical industries. Amylolytic enzymes are widely applicable in the food industry, such as in the manufacture of high fructose corn syrups, glucose syrups, and maltose syrups. These enzymes are also used in the reduction of turbidity to produce clarified fruit juice; the reduction of the viscosity of sugar syrups for longer shelf life, saccharification, and solubilization of starch; and to delay the staling of baked products (Christopher and Kumbalwar, 2015).

In the process of beer fermentation, alcohols are made by the conversion of sugars. Sugars are traditionally manufactured by a technique called mashing in which various grains are allowed to react with enzymes from germinated barley (malt) to produce starch (Saxe, 2010; Takamoto et al., 2004). Then again, fermentable sugars are produced from starch with a combination of industrially produced enzymes (amylase, protease, etc.), meaning the malting process can be avoided, saving energy and agricultural land (Fig. 28.6).

28.2.3 β-amylase

EC number:	3.2.1.2
Source:	Malted barley
Used in:	Brewing
Molecular weight:	223.8 kDa
Substrate:	Starch, glycogen
Product:	Beta-maltose

β-amylase was also known as *4-alpha-D-glucan malto hydrolase* with its systematic name. In polysaccharides, it hydrolyzed the alpha-D-glucosidic linkages and from the nonreducing ends of the chains, removed the maltose parts (Balls et al., 1948; French, 1960; Manners, 1962). β-amylase acts on glycogen, starch, oligosaccharides, and related polysaccharides and by inversion produces beta-maltose. Throughout the maturing period of fruit, this enzyme breaks down starch into maltose, and gives a sweet flavor to mature fruit. In plant storage and vegetative tissues such as seeds, nodes and tubers, and leaves, amylases are extensively

FIG. 28.6 α-Amylase (PDB database).

distributed (Dunn, 1974). Prior to seed germination, this enzyme exists in an inactive form. β-amylase shows extensive amylolytic activity in the stems and leaves of the plant (Dreier et al., 1995). The β-amylase enzyme is mainly found in cereals such as wheat and barley and in sweet potatoes (Okon and Uwaifo, 1984) (Fig. 28.7).

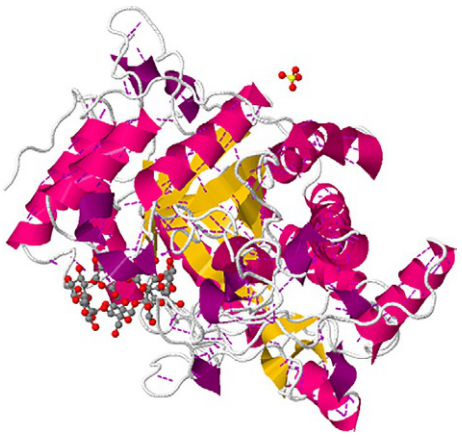

FIG. 28.7 β-Amylase (PDB database).

28.2.4 Lipases

EC number:	3.1.1.3
Source:	Lupin, avocado, pine nut, walnut, lentils, coconut, chickpea, oats, mungbean, castor bean, and eggplant
Used in:	Food
Molecular weight:	40–50 kDa
Substrate:	Glycerol backbone of a lipid substrate
Product:	Monoglycerides and two fatty acids

Lipases are the enzymes that alter the lipid properties by changing the position of fatty acid chains and exchanging one or more fatty acid chains with new chains in the glyceride. This precise property modifies lipids to a higher fat value from a relatively inexpensive and less-desirable lipid (Sharma et al., 2001). Lipases can cause esterification and interesterification while catalyzing the hydrolysis of fats and oils. This esterification and interesterification process are used to attain value-added products by the lipolytic alteration of oils and fats. This fatty acid and positional lipase specifically show higher industrial potential than the bulk production of fatty acids through hydrolysis. Lipase is a flexible enzyme having a potential role in the food, pharmaceutical, leather, detergent, cosmetic, textile, and paper industries (Houde et al., 2004). An immense number of fat-clearing enzymatic lipases are produced at an industrial scale. The commercial lipases produced are used for the processing of other foods and flavor development in dairy products such as fruit, milk products, meat, baked foods, vegetables, and beer. Industrial applications of phospholipases have been used in the treatment of egg yolk for the production of mayonnaise and emulsifiers, for the oil-degumming stage in the fining of vegetable oils, and in lecithin alteration. This enzyme is also used in the processing of sauces such as bernaise, hallandise, and cafe de Paris. Lipases are effectively applied as a catalyst for ester synthesis. These esters are used as flavoring mediators in the food industry and produced from short-chain fatty acids. For the synthesis of ester, the widely used lipases are immobilized on silica and microemulsion-based organelles (Ghosh et al., 1996; Sharma et al., 2001) (Fig. 28.8).

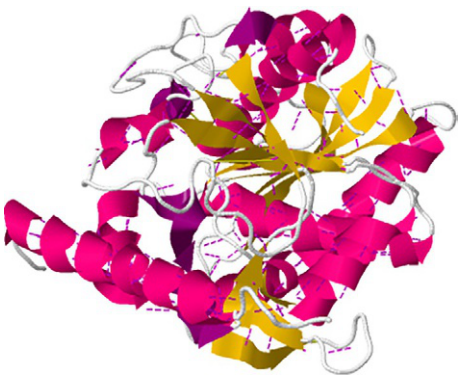

FIG. 28.8 Lipases (PDB database).

28.2.5 Lipoxygenase

EC number:	1.13.11.12
Source:	Soybeans
Used in:	Food
Molecular weight:	94,038 Da
Substrate:	Polyunsaturated fatty acids
Product:	Fatty acid hydroperoxides

Lipoxygenases are enzymes classified in the class oxidoreductases (linoleate means oxygen, LOXs) present in plants, fungi, and animals. They are the iron cofactor with the huge collection of monomers containing dioxygenases that catalyze the breakdown of PUFA (Gardner, 1991). Lipoxygenases were characterized and crystallized in 1947 by Theorell et al. Lipoxygenases produced by plants have a noteworthy importance in the food industry. Lipoxygenases generate approximately 9832 aromas and flavor in several plant products (Theorel et al., 1947). Lipoxygenases also act as bleaching agents and have an important role in the baking industry through raising the mixing tolerance and increasing the texture of the dough (Nicolas and Potus, 1994) (Fig. 28.9).

28.2.6 Invertase

EC Number:	3.2.1.26
Source:	Phloem of higher plants
Used in:	Food
Molecular weight:	205 kDa
Substrate:	Sucrose
Product:	Glucose and fructose

FIG. 28.9 Lipoxygenase (PDB database).

Invertase occurs both inside and outside the cell. It is produced by the submerged controlled aerobic fermentation of a nontoxigenic, nonpathogenic strain of *Saccharomyces cerevisiae*, extracted after washing and autolysis (Uma et al., 2010). Other names include glucosucrase, Saccharase, beta-h-fructosidase, beta-fructosidase, sucrase, invertin, fructosylinvertase, maxinvert L 1000, acid invertase, and alkaline invertase. The systematic name is beta-fructofuranosidase. The enzyme has a broad range of industrial applications such as the production of confectionaries with liquid contents, as in the case of some chewing gums. It also facilitates the formation of ethanol from cane molasses. However, the use of invertase is very limited because another enzyme, glucose isomerase, can help in the conversion of glucose to fructose at a cheaper cost (Uma et al., 2010). For taste and health reasons, the food industry requires highly purified invertase for use. It is also used in the preparation of digestive aid tablets, chocolates, infant food formulas, the assimilation of fortified wines etc. (Christopher and Kumbalwar, 2015) (Fig. 28.10).

28.2.7 Pectinase

EC No.:	3.1.1.11
Source:	Commonly all fruits
Used in:	Clarification of fruit juices and wines
Molecular weight:	37KDa
Substrate:	Pectin in fruits
Product:	Fruit juice from fruits

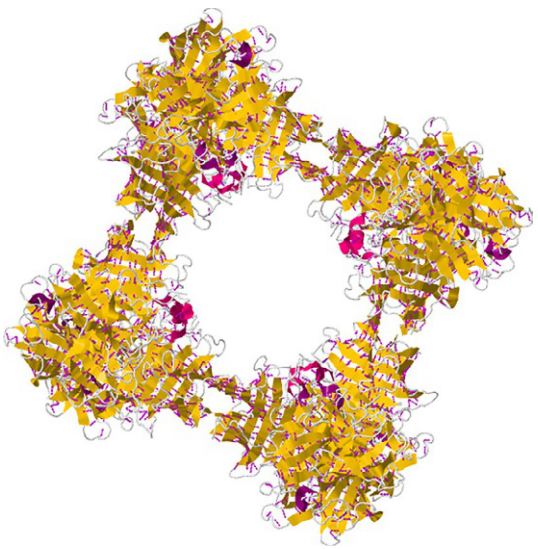

FIG. 28.10 Invertase (PDB database).

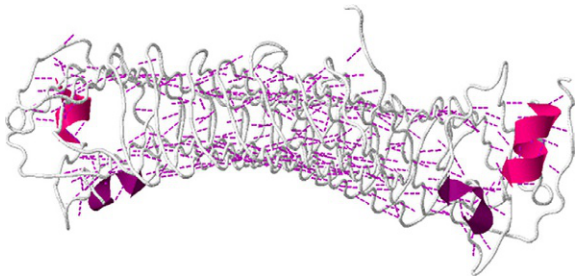

FIG. 28.11 Pectinase (PDB database).

The pectic enzyme includes pectolyase, pectozyme, and polygalacturonase. It is used in processes involving the degradation of plant materials. Pectin is a complex polysaccharide found in fruits. It is responsible for haze and precipitate formation in juice and has a negative impact on the yield when fruits are converted to juice (Whitaker, 1984). Pectin-degrading enzymes are widely used for the extraction and clarification of fruit juices and wines (Tressler and Joslyn, 1971). The significance of pectin in fruit juice technology and in wine making has been brilliantly brought out by Hickinbotham and Williams (1940).

Processing with enzymes makes the juice clear by breaking down the pectin and allowing the suspended particles to settle down. It also removes unwanted changes in bouquet, color, and stability. These enzymes are also supportive in other processes, for instance in the manufacture of fruit purees, the deskinning of orange segments, and wine clarification. The production costs of clarified juice would have a higher production yield and be greatly competitive in comparison with other established processes. For the tropical fruit juice industries, it represents a real substitutional method to spread production and enhance market share (Tapre and Jain, 2014) (Fig. 28.11).

28.3 PRODUCTION OF FOOD ENZYMES FROM PLANTS

The α-1,4-glycosidic bonds of starch containing amylose and amylopectin units is hydrolyzed by the enzyme α-amylases. It is extensively used in various industries such as paper, pulp, sugar, textile, food, brewing, starch liquefaction, and alcohol. A gene-encoding thermostable α-amylase was isolated from *Bacillus licheniformis*. It was expressed in transgenic tobacco plants. A chimera having the α-amylase gene of *Bacillus licheniformis* was transformed into tobacco protoplasts, showing much higher expression levels that are promising for commercial applications (Pen et al., 1992).

Due to a low concentration in the seeds, essential amino acids such as lysine and threonine are particularly important in cereal crops (Fornazier et al., 2003). Aspartate serves as the common precursor to these amino acids, along with methionine and isoleucine (Azevedo and Lea, 2001). In plants, the aspartate metabolic pathway at key enzyme steps is strongly regulated (Azevedo et al., 1997). Among them, aspartate kinase, homoserine dehydrogenase, dihydrodipicolinate synthase, and threonine synthase have been identified in a number of plant species (Azevedo et al., 1992; Teixeira et al., 1998; Vauterin et al., 1999).

TABLE 28.2 Applications and Benefits of Papain

Industries	Application/Benefit
Medicinal	Healing of burn wounds
	Dressing for wound debridement
	Hypertension, medication for kidney stones, urinary tract disorders, analgesic, abdominal pain during menstruation, dysentery, fever, and diarrhea
	Enhances the production of hydrogen and the degradation of glucose, proteins, and lipids
Food	Meat tenderizer, beer chill proofing,
	Cheese production, extraction of flavor and color compounds from plants
Textile	Detergents (laundry, dishwashing) and bloodstain remover

Papaya, which is a latex-containing plant, used this as its strategy to defend against pathogens. The latex of papaya is a milky-like thixotropic fluid (exhibiting a stable form at rest but becoming less viscous when stressed) having about 15% of dry matter where 40% of the dry matter is mainly constituted by enzymes, mainly cysteine endopeptidase. Altogether they account for more than 80% of the whole enzyme fraction. The endopeptidase enzyme in papaya in latex is stored in its inactive form. It rapidly changes into active mature enzymes after the liberatation of latex from the plants (Azarkan et al., 2003). According to Ambri and Mamboya (2012), the greener the fruit, the additionally active the papain. The salt precipitation method is the conventional way previously applied in papain purification (Table 28.2).

28.4 ENZYMES IN FOOD PROCESSING

The tenderness and softness of meat have been recognized as the most significant factors affecting the perception of taste and consumer satisfaction (Miller et al., 2001). Tenderness is a complex trait. Usually, the two chief structural features of muscle that influence tenderness are connective tissue contribution and integrity of the myofibrils. Only five enzymes are considered as having a "Generally Recognized as Safe" status by US agencies, namely papain, ficin, bromelain, *Aspergillus oryzae* protease, and *Bacillus subtilis* protease. Actinidin and zingibain are good enzymes for tenderization (Feijoo-Siota and Villa, 2011; Naveena et al., 2004; Sullivan and Calkins, 2010).

Studies revealed that papain showed the greatest ability to improve tenderness in meat. Bromelain degraded collagen more than the contractile proteins and increased tenderness. Ficin degrades both myofibrillar and collagen proteins (Sullivan and Calkins, 2010). Papain and bromelain are also used in the manufacture of different sauces (Díaz et al., 1996) and dry cured ham (Feijoo-Siota and Villa, 2011; Scannell et al., 2004).

In the food processing industry, the majority of enzymes used are for the break down and modification of biomaterials. Novel, economic, and green technologies are the prime areas of demand in the fat and oil modification industries (Gupta et al., 2003).

28.5 ENZYMES IN BREWING

The extensive application of enzymes to brew with high amounts of inexpensive raw materials such as barley focuses on future aspects of enzymes in the brewing industry. In barley, starch has to be broken down into fermentable sugars before the yeast can make alcohol. Thus, conventional brewing contains an extra step, namely malting, in which enzymes needed for the degradation of starch into fermentable sugars are produced, compared with wine making. The majority of enzymes are produced during the germination, for example, α-amylases and proteases, while some enzymes are already present in the barley, for example, β-amylases. In the final malt, entire enzymes essential for the conversion of "grains" into a fermentable liquid are present. However, the malt enzymes do have some boundaries. They can only work at certain pH values, temperatures, etc., and the performance might be too low to do a proper job in a proper time. In comparison, commercial exogenous enzymes can be designed to have more enzymatic power to work at preferred temperatures and pH values. Supplementation of exogenous enzymes can make brewing faster, more consistent, and easier at various steps during the process. Barley malt is the traditional source of enzymes used for the conversion of cereals into beer. There will be several undesirable consequences if too little enzyme activity is present in the mash, such as the extract yield will be too low, wort separation will take too long, fermentation process will be too slow, too little alcohol will be produced, beer filtration rate will be reduced, and the flavor and stability of the beer will be inferior.

Thus, in order to prevent these problems, exogenous enzymes are employed to supplement the malt's own enzymes. Furthermore, to shorten the beer maturation time and to produce beer from cheaper raw materials, industrial enzymes are used to ensure better adjunct liquefaction and to produce low-carbohydrate beer ("light beer").

The method to chill-proof beer by the application of proteolytic enzymes was patented by Leo Wallerstein in 1911 (De Clerck, 1969). In the brewing industry, crude bromelain or papain (Jin and Toda, 1988; Kennedy and Pike, 1981) and ficin (Priest and Stewart, 2006) are also employed in order to obtain good colloidal properties at low temperatures, thus eliminating cloud formation (Jones, 2005). At present, because additive-free beers still prevail in some European countries, papain is not used widely (Feijoo-Siota and Villa, 2011).

28.6 ENZYMES IN BAKING

In the production of baked goods, enzymes can be added individually or in complex mixtures at a very low level that may act in a synergistic way. Baking comprises the use of enzymes from three different sources (Di Cagno et al., 2003):

1. The endogenous enzymes in flour.
2. Enzymes associated with the metabolic activity of the dominant microorganisms.
3. Exogenous enzymes added in the dough.

In the baking industry, there is a rising focus on lipolytic enzymes. Because the enzymes degrade polar wheat lipids to produce emulsifying lipids, recent findings suggest that phospholipases can be used to supplement or substitute traditional emulsifiers (Collar et al., 2000; Kirk et al., 2002). Lipase was primarily used to enhance the flavor content of bakery products

by liberating short-chain fatty acids through esterification. Along with flavor enhancement, it also prolonged the shelf life of most of the bakery products. Texture and softness could be improved by lipase catalyzation (Loboret and Perraud, 1999). All hydrolytic enzymes, including lipase, were found to be effective in increasing the specific volume of breads and reducing the initial firmness (Keskin et al., 2004). By hydrolysis of butterfat with suitable lipase, increased butter flavor for baked goods was generated (Uhling, 1998).

In the baking industries, proteases are used to hydrolyze gluten so the baking mass may be easily prepared. These enzymes may find a natural niche for industrial application because of their rapid rate of reaction, optimal pH, and temperature as well as the lack of pentosanase or amylase side activities (Polaina and MacCabe, 2007). At present, new products with higher-added value are under study to be generated with gluten hydrolyzates (Wang et al., 2007a, 2007b). Similarly, to obtain hypoallergenic wheat flour, bromelain has been used for its ability to break the wheat glutenin IgE epitope Gln-Gln-Gln-Pro-Pro (Feijoo-Siota and Villa, 2011; Tanabe et al., 1996).

The main constituent of bread is starch. As the starch crystallizes, bread becomes hard and unpleasant to eat with age. To avoid wasting bread and to extend shelf life, the addition of lipase and amylase enzymes in bread making reduces the crystallization of starch in the bread. The savings were chiefly determined by avoided grain production and bread transportation (Jegannathan and Nielsen, 2013) (Table 28.3).

TABLE 28.3 Enzyme Classification

Enzyme (Classification)	Substrate in Foods	Reactions	Application in Baked Products
Amylolytic enzymes	Starch	Hydrolysis of linkages	Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products, intensifying bread flavor and color; Decrease of bread crumb firming rate; Antistaling effects.
α-Amylases	Amylose and amylopectin	α(1→4)-D-glycosidic [endo], *liberating α-dextrins*	
β-Amylases		α(1→4)-D-glycosidic [exo], *liberating β-dextrins and β-maltose*	
Glucoamylase or amyloglucosidase		α(1→4)- and α(1→6)-D-glucosidic, *liberating β-glucose*	
Maltogenic α-amylase		α(1→4)-D-glycosidic, liberating maltose	
Maltooligosaccharides forming amylases		Liberation of maltotetraose or maltohexaose	
Pullulanase	Amylopectin	α(1→6)-D-glycosidic	
Isoamylase	Amylopectin	α(1→6)-D-glycosidic	
Transferases, Amylomaltases, Amylosucrases, Ciclodextringlycosyltransferases	Amylose, amylopectin and dextrins	Hydrolysis of α(1→4) glycosidic bonds and transference of a reducing group to a nonreducing acceptor (monosaccharide unit)	

28.7 ENZYMES AS FOOD ADDITIVES

Food additives are substances that are not normally consumed as food itself, but are added intentionally to perform certain technical functions, for example to color, sweeten, or maintain freshness while preventing deterioration. Many food additives naturally occur and some are even essential nutrients (food ingredients). However, it is the technical purpose that classifies them as food additives and makes them subject to safety requirements.

The increasing public awareness of the nutritional characteristics of their diet and, in particular, of the additives have strongly influenced the food industry. It is evident by the high number of industrial products that are low in sodium, fat, caffeine, and cholesterol as well as the preference of aspartame over saccharine and the search for natural alternative antioxidants, preservatives, and colorants (Christen and López-Munguía, 1994).

Enzymes as biocatalysts offer an extensive variety of possibilities for food flavor production:

1. Their specificity enables the production of certain chemicals that are difficult to synthesize;
2. Their stereo selectivity is an important advantage for the food industry where a specific optical conformation may be associated with flavor properties.

Enzymes may also be used directly as food additives to produce or liberate flavor from precursors, and to remove off-flavors caused by specific compounds occurring naturally or produced during processing (Bigelis, 1992) (Table 28.4).

At any stage of the manufacturing, processing, preparation, treatment, packaging, transport, or storage of foods, enzymes are capable of catalyzing a specific biochemical reaction and are thus added to improve the quality of different foods.

The enzymes are increasingly used to drive chemical reactions outside the cell, in particular, the use of the biocatalysts as food additives and in processing raw materials. Enzymes have long been used by the industrial product makers as major tools to transform the raw materials into end products. The food industry is constantly seeking advanced technologies to meet consumer demand. Several enzymes can improve the texture, flavor, digestibility, and nutritional value when purified and added to food preparations. The economic benefit of using technical enzyme preparations lies in lowered process costs, in the reduction of the environmental impact by making use of renewable resources, and often in increasing the quality of the products. Also, preservation makes a significant impact on the quality of food as well of beverages. It is well known, for example, that modern processes convert juices

TABLE 28.4 Enzyme Technology Related to Food Flavor

Biocatalysts in processes for flavor production
Additives to enhance or produce flavor from precursors
Additives in flavor extraction processes from natural raw materials
Inactivation of endogenous enzymes to avoid off-flavor generation
Activation of endogenous enzymes to induce reactions leading to flavor production.
Use of enzymes for the elimination of off-flavors

into concentrates that, except for aroma, can be stored for a long time without loss in quality. Stabilizing flavor and color is also an example of improved preservation. Thus, numerous purified enzymes are now being widely used not only in food processing but also as food additives (Porta et al., 2010).

28.8 CONCLUSION

The beauty and charm of plant-derived enzymes used in the food industries are diverse. In the global market, the aggregate demand for enzymes is projected to rise at a fast pace in the near future. There is a strong growth in the product category led by several markets, including animal feed and the food and beverage industry. In past decades, enzymes were considered too delicate to survive the extreme conditions in real reaction vessels. Thus, many industrial sectors were restrained from embracing enzyme technology. The actualization of enzyme applications in industrial processes requires high-performance enzymes with specific characteristics that will stimulate research to explore new avenues to overcome their weaknesses. Some of the strategies in the field are exploiting novel enzymes from nature, improving existing catalytic properties, broadening specialized enzymes to serve new functions, optimizing the formulation of enzyme preparations, and de novo designing biocatalysts. These approaches have provided valuable candidates for the biocatalytic processes. However, the breakthroughs of enzyme products for biochemical technology should be recruited. Advancement in plant biotechnology offers a constructive position for the development of enzymes utilized in the food industry and will continue to facilitate their applications to provide a sustainable environment for improving the quality of human life.

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CHAPTER

29

Exploiting Microbial Enzymes for Augmenting Crop Production

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29.1 INTRODUCTION

Enzymes, the biocatalysts, are involved in all stages of metabolic and biochemical processes as a key player. Enzymes are present in every living cell, hence in all microorganisms. Every microorganism produces a variety of enzymes performing various metabolic functions such as hydrolysis, oxidation, or reduction. Microbial enzymes are known to be superior enzymes that perform a vital role in crop production. They are actively involved in plant metabolism, nutrient uptake, and protection from various types of stresses. Microbial enzymes were first discovered in the 20th century. The isolation, characterization, and production of these enzymes from bench scale to pilot scale and their application have continuously been upgraded. Microbial enzymes are of great importance for plants as well as for soil health. Microorganisms including bacteria, actinomycetes, and fungi produce a diverse array of microbial enzymes (Fig. 29.1).

The plant and soil microbiome plays a direct and indispensable role in plant growth. A wide array of microorganisms resides in soil rhizosphere, rhizoplane, and plant tissues (Saharan and Nehra, 2011). Microorganisms, colonized in the rhizosphere, are characterized in accordance to their effects on plants and the way they interact with plants. Some trigger beneficial effects whereas others are pathogenic. For the selection and enrichment of the types of microorganisms, plants play a major role on the basis of the constituents of their root exudates. Thus, the microbial population of a certain rhizosphere depends on the type and amount of organic components of exudates, and the microbial activities for using these exudates as an energy source (Saharan and Nehra, 2011).

https://doi.org/10.1016/B978-0-12-813280-7.00029-3

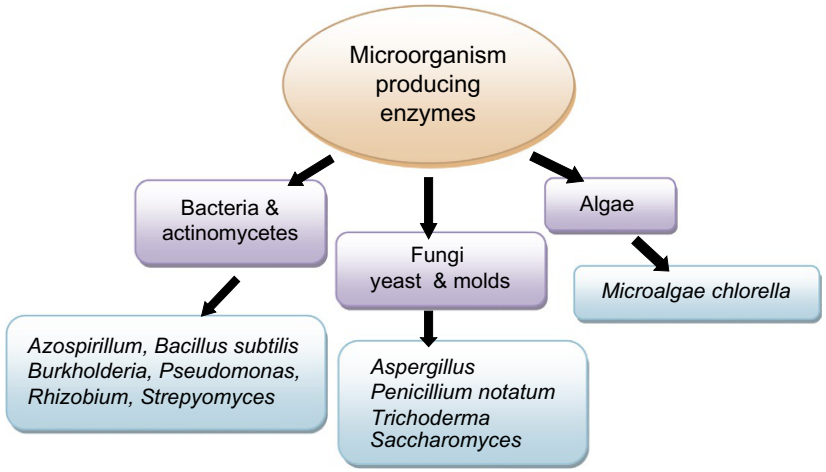

FIG. 29.1 Different types of microorganisms which produces enzymes.

29.2 PLANT GROWTH-PROMOTING MICROORGANISM

Microorganisms inhabiting plant roots have a positive impact directly or indirectly toward the growth and development of plants and are termed as plant growth-promoting microorganisms (PGPMs). PGPMs are a diverse group of microbes present in the rhizosphere and within the root. PGPMs improve the quality of plant growth (Gupta et al., 2000). These microorganisms can directly perform the proliferation of plants by nitrogen fixation, phosphorus solubilization, and by the synthesis of enzymes that can regulate plant growth and development. They have the ability to solubilize and sequester Fe by producing siderophores; thereafter, iron is made available to the plants that can be transported. PGPMs also synthesize several phytohormones that can affect all the stages of plant growth and development. A particular microorganism may affect plant growth and development using any one or more of these mechanisms. Moreover, many plant growth-promoting bacteria possess several activities that enable them to facilitate plant growth and, of these, they may utilize different ones at various times during the life cycle of the plant. However, indirect promotion of plant growth occurs by decreasing or preventing the deleterious effects of phytopathogenic organisms by any one or more of several different mechanisms, including the production of antibiotics or fungal cell wall-degrading enzymes by bacteria. Various bacterial species of genera *Alcaligenes*, *Azospirillum*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* are affiliated with the plant rhizosphere and provide a beneficial effect on plant growth, interacting at the rhizosphere level. Similarly, mycorrhiza formation may assist plant growth. However, the communication between mycorrhiza and other specific microorganisms may cause a depletion of the positive effect on plant growth (Vazquez et al., 2000). Free-living microbial inoculants could fuel the colonization of mycorrhiza (Vosatka et al., 1999). Mycorrhiza formation can affect the microbial population in the rhizosphere by changing the root exudation patterns or through fungal exudates. In this context, alterations of the microbial patterns in the

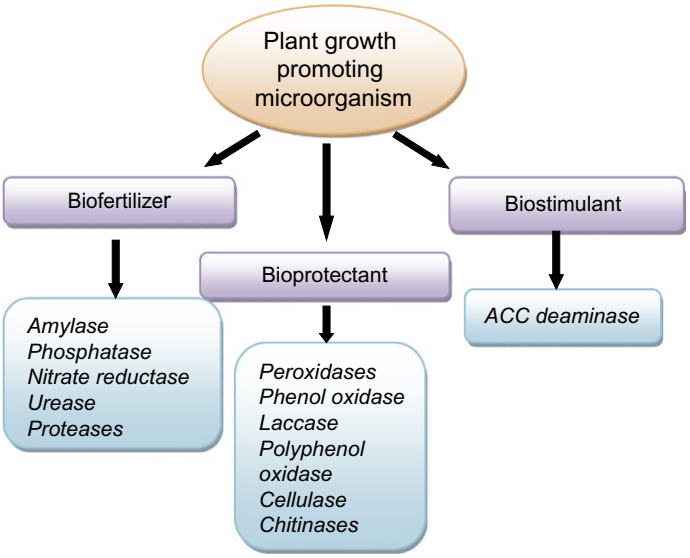

FIG. 29.2 Different types of enzymes produced by plant growth promoting microorganisms.

rhizosphere, due to the formation of arbuscular mycorrhizal, have been assessed through measurements of different soil enzyme activities (Camprubi et al., 1995). Many fungal species including *Trichoderma* and *Gliocladium* can impart a beneficial impact on improving plant growth and development (Harman et al., 2004). The *Trichoderma* species are also known to be used as biological control agents for controlling different species of plant fungus diseases for decades (Harman et al., 2004) as well as for enhancing seed germination under biotic and abiotic stresses (Mastouri et al., 2010). Microorganisms, which promote plant growth, are usually used as inoculants for the increased growth and production of agricultural crops. They also offer an alternative for the pesticides, chemical fertilizers, and supplements, depending on the type and concentration of organic constituents of exudates as well as the corresponding skill of the bacteria to deploy these as sources of energy in synthesizing the appropriate enzyme pattern. On the basis of various traits and the availability of enzymes, PGPMs can be classified into three broad categories: biofertilizers, biostimulants, and bioprotectants (Fig. 29.2).

29.3 MICROBIAL ENZYMES ACT AS BIOFERTILIZERS

Biofertilizer, a term known worldwide, is comprised of most commonly degradable substances and soil microorganisms, which collectively maintain the composition of micro and major nutrients for the development and growth of plants (Vessey, 2003). The biofertilizer is normally applied to the soil, seed, plant surfaces, and rhizospheric region, which promotes the growth by maintaining nutrient availability to the plant. The biological activities of microorganisms (such as biological N_2 fixation and phosphate solublizing) help in the easy uptake

of nutrients to plants. Microbes used as biofertilizer are an arsenal of enzymes that are an integral part of various growth-promoting metabolic activities of the plant. The following microbial enzymes are popularly used as biofertilizers.

29.3.1 Phosphate-Solubilizing Microbial Enzymes

As described previously, the phosphate-solublizing property that promotes plant growth is due to the phosphatase enzymes produced by microorganisms. *Trichoderma*, *Pseudomonas*, *Rhizobium*, *Micrococcus*, and *Erwenia* are some of the most powerful phosphate-solubilizing microbes. There are two main insoluble types of phosphorus available in the soil, that is, mineral and organic, which is an indispensable macronutrient used for growth of the plant (Javaid, 2009). To fulfill the nutritional requirements of plants, phosphorus is normally used as an essential component of chemical fertilizers. Phosphate-solubilizing microorganisms solubilize the inorganic phosphorus into organic acids such as citric and gluconic acid (Rodriguez et al., 2004). The phosphorus binds to the −OH and −COOH groups of organic acids and the soluble phosphorus is released by the soil acidification process. Additionally, the inorganic phosphate content of soil is also solubilized by the microbial production of inorganic acids and chelating chemicals. Furthermore, phosphate-solubilizing microbes also synthesized *exo*-polysaccharides, which bind to free phosphorus and solubilize tricalcium phosphates indirectly, modulating the homeostasis of the phosphorus solubilization (Yi et al., 2008). *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Enterobacter*, and *Streptomyces* are some important genera having the potential ability to solubilize the phosphate. However, the organic phosphorus mineralization occurs via the synthesis and release of enzyme phosphatases, for example, phosphomonoesterase, phosphodiesterase, and phosphotriesterase, which catalyzes the hydrolysis of phosphoric esters (Rodriguez and Fraga, 1999). Examples of some important bacteria that mineralize the organic phosphorus are *Pseudomonas syringae*, *P. cichorii*, *Burkholderia caryophylli*, and *Bacillus megaterium*. In certain cases, both phosphorus solubilization and mineralization activities are present in the same bacterial strain (Tao et al., 2008), which provides soluble phosphorus to the plants. It was observed that the rhizospheric isolates *Pseudomonas* spp. and *Micromonospora endolithica* have the ability to solubilize considerable amounts of phosphorus into organic acids (Poonguzhali et al., 2008). The presence of the phosphatase enzyme is a good indicator of soil fertility (Dick et al., 2000). For example, in the condition of phosphorus deficiency in the soil, plant roots secrete acid phosphatase, which solubilizes and remobilizes the phosphate, thus influencing the ability of the plant to protect itself under phosphorus-stressed conditions. Moreover, these isolates from the rhizhospheric region are involved in other plant growth-promoting activities such as the formation of indole acetic acid, ACC deaminase, and siderophores, which increased root elongation and biomass.

29.3.2 Microbial Enzymes for Nitrogen Assimilation

Nitrogen is an important constituent of crop fertility and productivity. Microorganisms play an important role for nitrogen bioavailability through the assimilation of N_2 into ammonia by nitrogen fixation. Various bacteria and fungi degrade the organic matter and recycle it to fix nitrogen, which can be utilized by other organisms as well as plants. Among all the

microorganisms, the bacteria of the soil environment contribute their role in N_2 fixation by either free-living or symbiotic associations with plants. The microorganisms degrade the organic nitrogen and produce NH^{4+} and NO^{3-}, which are the forms of bioavailable nitrogen to plants (Isobe et al., 2011, 2015). The dynamics of the bioavailable nitrogen in the soil correspond to the microbial activity of a particular environment or ecosystem. The nitrogen-fixing microbes are influenced by environmental factors; hence, the favorable environmental conditions are responsible for better microbial activity as well as the availability of nitrogen to the plants. It was demonstrated that nitrogen transformation via nitrification and denitrification could be described by the presence of nitrifier/denitrifier populations. The free available nitrogen of the atmosphere must be assimilated and transformed into a bioavailable form to be taken up by plants. Lightning strikes fix 10×10^{12} g nitrogen per year; however, most of the environmental nitrogen is fixed by free-living or symbiotic microorganisms known as di-azotrophs. The free-living, nitrogen-fixing bacteria have the nitrogenase enzyme, which transforms the nitrogen into ammonia, which is then transformed by the bacteria into other organic compounds. The majority of the biological nitrogen is fixed by the activity of Mo-nitrogenase, present in some bacteria and Archaea, for example, *Rhizobium* (symbiotic nitrogen fixing) and *Azotobacter* (free-living nitrogen fixing). The symbiotic bacteria establish a mutualistic relationship with the leguminous plant (root nodules), producing ammonia in exchange for carbohydrates. Plants fulfill their nitrogen requirement from the soil by the absorption of amino acids, nitrate, nitrite ions, or ammonium ions through their roots. The available ammonia must be converted into nitrates or nitrites because ammonia is toxic to plants. In the nitrogen-fixation process, nitrification is performed by the *Nitrosomonas* species, which converts ammonia to nitrites (NO^{2-}). On the other hand, *Nitrobacter* is responsible for the oxidation process of nitrites (NO^{2-}) into nitrates (NO^{3-}). The unexploited nitrates are reduced in nitrogen gas by the denitrification process, contributing to the nitrogen cycle. The *Pseudomonas* and *Clostridium* bacterial species are well known for the denitrification process in which these microbes use nitrate as an electron acceptor in the place of oxygen during respiration. Three processes are responsible for denitrification: (i) heterotrophic denitrification, which performs the conversion of nitrate to N_2 using organic matter as a reductant; (ii) anammox, an autotrophic process that converts ammonium to N_2; and (iii) autotrophic denitrification, which converts nitrate to N_2 by utilizing a reduced sulfur species as a reductant. In both heterotrophic and autotrophic denitrification, the reduction of nitrate is performed by the similar metabolic pathway with N_2O as an intermediary product (Sievert et al., 2008). Many species of nitrogenase that produce free-living bacteria (*Azotobacter* and *Azospirillium*), cyanobateria and symbionts (*Rhizobium*), and other N_2-fixing bacteria are commercially available as nitrogenous biofertilizers.

29.3.3 Microbial Ureases

Several bacteria, fungi, yeasts, algae, and even plants produce the enzyme urease (Follmer, 2008). This enzyme hydrolyzes urea fertilizer into NH_3 and CO_2 with a rise in soil pH; free N is then released into the atmosphere through NH_3 volatilization (Fazekasova, 2012). It has been reported that urease synthesis takes place constitutively in some microorganisms (Mobley et al., 1995). However, its synthesis is inhibited when cells grow in the media that have an N source such as NH^{4+} (Geisseler et al., 2010). Furthermore, its production is increased in the

presence of alternative N sources such as urea (Mobley et al., 1995). Soil urease activity has great importance as it modulates the uptake of nitrogen to the plants after the application of urea as fertilizer. The urease enzyme is an ideal candidate that can be widely used to explore the changes in soil fertility, as urease activity increases with the use of organic fertilizers and decreases with soil tillage (Saviozzi et al., 2001). The sources of urease present in the soil are microorganisms and plants, found as both intra- and extracellular enzymes. Alternatively, urease can be extracted from plants or microbes instantly hydrolyzed in the soil by the action of proteolytic enzymes. Urease activity in the soil is affected by various factors such as organic matter content, cropping history of the soil, soil depth, soil amendments, heavy metals, pH, and temperatures (Yang et al., 2006). It is also reported that toxic heavy metals decrease urease activity (Yang et al., 2006). Additionally, urease activity increases with a rise in temperature. As urease has a vital role in the hydrolysis of urea fertilizer, it is important to explore factors that decrease the efficiency of this enzyme in the soil microenvironment.

29.3.4 Microbial Proteases

Proteases are the enzymes that are secreted by microorganisms such as *Aspergillus niger*, *Bacillus subtilis*, etc. Proteases in the soil play a crucial role in N mineralization (Ladd and Jackson, 1982). They are usually affiliated with inorganic and organic colloids (Burns 1982; Nannipieri et al., 1996), thereby regulating the amount of plant-available N and plant growth. Proteases catalyze the hydrolysis of proteins to polypeptides, oligopeptides, and amino acids. As nitrogenous compounds in mineral soils are present in an organic form, their conversion is required to make N free and available to the plants. The extracellular protease is indicative of the biological capacity of soil and plays an important role in the ecology of microorganisms in the ecosystem (Burns, 1982). It is suggested that extracellular proteases play important pathogenic roles in suppressing nematodes in the soil (Siddiqui et al., 2005). Therefore, there is a requirement for the characterization of naturally occurring protease enzyme complexes of the soil ecosystem as they may unveil some unidentified factors that maintain soil health fertility and the crop yield.

29.3.5 Microbial Amylases

Amylase is known as a starch-hydrolyzing enzyme, which is classified into two categories: α-amylase and β-amylase. The abundance of α-amylase is reported in plants, microbes, and soils. It plays a significant role in the conversion of starch into glucose or oligosaccharides and provide the nutrients for the growth of microbes and plants. The production of α-amylase has been reported from various bacteria, actinomycetes, fungi, and genetically modified species of microbes. The bacterial species that have been widely explored for amylase production are *Bacillus spp*., namely, *B. amyloliquefaciens*, *B. licheniformis*, *B. cereus*, and *B. subtilis*. However, the fungal sources of α-amylase are confined mostly to the genera *Aspergillus*, *Penicillium*, and *Rhizopus* (Gopinath et al., 2017). Moreover, plants affect the amylase enzyme activities of soil by direct secretion from their residues, or indirectly by providing substrates for the synthetic activities of microorganisms. For proper management and to maximize the benefits of such enzymes, a good knowledge of understanding the importance of amylases in the soil is required.

29.3.6 Microbial β-Glucosidases

Glucosidase is a commonly present enzyme in soils, and is named on the basis of the type of bond that it hydrolyzes during the process. This enzyme plays an imperative role in soils as it is involved in catalyzing the hydrolysis and biodegradation of various β-glucosidases present in plant debris that is decomposing in the ecosystem (Martinez and Tabatabai, 1997) as well as recycling nutrients. β-glucosidase is typically useful as a soil quality indicator, and it gives an indication of past biological activity and the ability of the soil to stabilize organic matter; it is also used to detect the executive effect on the soils (Ndiaye et al., 2000). β-glucosidase, which is quite sensitive to changes in the pH of soil as well as crop management practices, can be used as a good biochemical indicator for measuring environmental changes happening due to soil acidification in situations involving activities of this enzyme. Hence, a better understanding of the β-glucosidase enzyme activities and the factors affecting them in the ecosystem may contribute to soil health studies as the good health of the soil will result in better crops.

29.3.7 Microbial Peroxidases

Peroxidases are special types of enzymes that contain a heme group in their structure. This enzyme accepts H_2O_2 (hydrogen peroxide) as a final (e^-) electron acceptor in catalyzing various oxidation and hydroxylation reactions (Ralph et al., 2004). The presence of peroxidases has been reported from all types of microorganisms. Peroxide in soil is produced by the degradation of organic matter. The abundance of peroxidases in the soil is responsible for the degradation and assimilation of organic matter through the process of humification and in the xenobiotic remediation (Sinsabaugh, 2005). Extracellular peroxidases are reported from a few genera of basidiomycetes and ascomycetes (Rabinovich et al., 2004). Both lignin and Mn peroxidase contain Fe (heme) as their prosthetic groups. In the light of previous data, it is confirmed that lignin peroxidases directly attacks the phenylpropane units of lignin and oxidize the alpha-beta carbon bonds (C–C). In contrast to lignin peroxidases, Mn (Manganese) peroxidase oxidizes the lignin through an indirect way and generates a diffusable Mn^{3+} ion. This activity of both peroxidases is assisted by aryl alcohol oxidases. Aryl alcohol oxidases oxidize the hydroxyl compounds into carbonyl compounds by generating H_2O_2. Glyoxal oxidase, galactose oxidase, and glucose oxidase are important saccharide-oxidizing enzymes that reduce the oxygen and generates hydrogen peroxide. Therefore, peroxidases play a key role in the removal of toxicants from soil and the decomposition of organic matter, thus providing a positive soil environment for crop production.

29.4 MICROBIAL ENZYMES ACT AS BIOPROTECTANTS

Microorganisms play a key role in the protection and development of plants by producing a variety of enzymatic and nonenzymatic substances (Alabouvette et al., 2006). Microorganisms are associated with plants by various interacting relationships. However, it has now been confirmed that the majority of microorganisms are not pathogenic (Mendes et al., 2013), as most of them are neutral or beneficial for the health and growth of plants. Rhizospheric microorganisms

interacting with a host plant by root exudate and build a strong beneficial relationship (Berendsen et al., 2012; Turner et al., 2013). According to the microbes and host plant association, microbes also produce some primary and secondary metabolites that protect the plant from invading pathogens. (Berendsen et al., 2012; Cameron et al., 2013; Lakshmanan et al., 2014). Plant health is one of the key factors that have to be taken care of for crop growth, development, and yield. In this regard, the protection of the crop and excellent soil health are the areas of concern in organic forming. Therefore, the application of microorganisms that have protective as well as plant growth-stimulation properties will improve plant growth and yield. Various biopreparation products of selected bacterial strains exist in the marketplace. The bio-organic methods are helpful in a significant increase of healthy crop production, which is also part of the public health concern. There are various known *Bacillus* spp. strains that could be utilized as bioprotectants for plants (Liu et al., 2018). Various attempts have been made in the direction of management of plant pathogens, biological control, and plant growth stimulation by the application of microorganisms, which are beneficial in two ways (Borriss, 2015). On the other hand, the native microbial strains are predicted as to be suitable and beneficial bioinoculants because of their greater environmental and ecosystem integration. The application of these bioinoculants could restore and maintain the microbiological equilibrium of soil. PGPBs (plant growth-promoting bacteria) have a biocontrol potential called biocontrol-PGPB, which can produce and secrete various enzymatic substances responsible for the inhibition of plant pathogens with the stimulation of plant growth. There are varieties of microbial enzymes produced that act as bioprotectants; some of them are described below.

29.4.1 Microbial Phenol Oxidases

Microorganisms play an essential role as a bioprotectant for plants by producing various enzymes and bioproducts. Microorganisms and plants both produce all types of phenol oxidases such as intracellular and extracellular. Plants normally utilize phenol oxidases for the synthesis of lignin and other secondary compounds. Phenol oxidases (PO) are very important enzymes because they are known as a bioprotectant that is mainly excreted by microorganisms. POs are much less stable in an extracellular environment; hence they interact very actively with organic substances (such as lignin) and oxidize them by accepting oxygen as the final electron acceptor (Sinsabaugh, 2010). The ascomycete and basidiomycete classes of fungi use intracellular phenol oxidases for the synthesis of protective compounds such as melanin, which are often involved in the spore formation and process of morphogenesis. Additionally, extracellular PO produced by both fungal and bacterial species counter the toxic effects of phenolic compounds and metallic ions, which helps in the development of antimicrobial systems. Phenol oxidases are independent agents that are released in the atmosphere either by discharge or by bursting of the cell, which is able to catalyze imprecise reactions together with Mn^{2+} and Fe^{2+} oxidation that can polymerize or reverse transform (depolymerize) the phenolic molecules. The transformation reactions of the phenol molecule affect the soil microbial composition due to the toxic property of phenols (Gianfreda et al., 2005).

29.4.2 Microbial Laccases

Laccases are the biggest group of POs present in the rhizosphere. Laccase is an oxidoreductase class enzyme that catalyzes the oxidation reaction for various aromatic

compounds (predominantly phenols) and the reduction reaction for oxygen to form water. Laccases are conventionally supposed to be a type of myco-enzyme (Claus, 2003; Baldrian, 2006). However, laccase is generally a multicopper oxidases (MCO), generally disseminated in both bacteria and archaea. They work similar to the enzymes that have various atoms (e.g. Cu, Mn, Zn, or Fe atoms) in their combinations. Apart from higher plants and fungi, laccases are also synthesized by several bacterial strains such as *Azospirillum lipoferum* and *Alteromonas* sp. Laccases have copper atoms containing enzymes and these atoms are mainly responsible for the catalytic mechanisms for lignin degradation and depolymerization (Hoegger et al., 2006). The molecular weight (MW) of the laccase enzyme is so high (70,000) that its deep penetration into wood is impossible. Laccases are of 0.5–0.8 V redox-potential (low), therefore making it incapable of oxidizing the nonphenolic etherified lignin compositions, which are of >1.5 V redox-potential (Galli and Gentili, 2004). Due to these restrictions, laccase can only oxidize the substrate surface phenolic units of lignin. Therefore, to counter this problem, laccase is frequently applied to the nonphenolic lignin with an oxidation mediator molecule (Galli and Gentili, 2004). However, laccase is strongly considered as a lignin-degrading enzyme as well as a bioprotectant from bacterial pathogens.

29.4.3 Microbial Polyphenol Oxidases

The majority of soil polyphenol oxidases are secreted from plant roots and propagation residues. PPO also known as tyrosinases can catalyse hydroxylation of monophenol and biphenol. Further it can also oxidise biphenols and produce quinones. Several bacterial strains (e.g. *Alcaligenes*, *Nocardia*, *Arthrobacter*, *Strepomyces*, and *Pseudomonas*) are known for the secretion of polyphenol oxidases that readily degrade the single ring aromatic substances. However, the laccase enzyme uses free radicals to interact with the highly complex chemical structure of lignin and its degradation to a variety of other compounds.

29.4.4 Microbial Chitinases

Chitinase, also known as chitinolytic enzymes, are major enzymes that catalyze the degradation of chitin by hydrolysis. Chitin is also known as the major structural component of most of the fungal cell walls, which helps in the parasitic action on the plants. Chitinase is an agriculturally essential enzyme that is synthesized by both microorganisms and plants (Deshpande et al., 1986). Several studies have mentioned that chitin hydrolysis occurs due to the enzymatic activity of *Bacillus*, for example, *B. subtilis* or *B. amyloliquefaciens* (Wang et al., 2006; Songsiriritthigul et al., 2010). These enzymes also act as efficient biopesticides for parasitic fungi and insects. Furthermore, the presence of different forms of chitinase in the environment has revealed its importance in controlling soilborne pathogens such as *Rhizoctonia solani* and *Sclerotium rolfsii*. This enzyme attacks the cell walls of the pathogenic fungi and degrades it into smaller subunits, acting as a biocontrol agent. This enzyme can be used to increase plant health and growth due to its environment friendly antipathogenic activity.

29.4.5 Microbial Cellulases

Cellulose is one of the most prominent organic compounds present in the biological atmosphere, contributing almost 50% of the total biomass produced by the fixation of CO_2 in photosynthesis. Primarily, plant residue degradation via fungus and bacteria is responsible for the abundance of cellulose in soils. Moreover, the microbial activity is comparatively maximum in the rhizospheric region due to the release of root exudates (Renella et al., 2007). Microbial survival and growth are important in most soils, which depend upon the carbon source present in the cellulose content of the soils. The cellulose content of plant debris is dissociated into high molecular weight oligo-saccharides (such as glucose and cellobiose) by cellulases enzymes providing carbon as an energy source to soil microorganisms. Cellulases are the important class of enzymes that catalyzes the cellulose degradation. Basically, cellulases comprise three types of enzymes that include endo-1, 4- β-glucanase (randomly attacks on the cellulose); exo-1, 4- β-glucanase (attacks on glucose or cellobiose units from cellulose chains); and β-D-glucosidase (hydrolyses cellobiose and cellodextrins to form glucose). The widely studied fungal species such as *Penicillium*, *Trichoderma*, *Aspergillus*, and *Humicola* have high cellulolytic activity. A few bacterial strains belonging to the genera *Cellumonas*, *Bacilli*, *Actinomucor*, *Streptomycetes*, *Streptomyces,* and *Pseudomonas* are good source of cellulases. Various blends consisting of cellulases, hemicellulases, and pectinases have been investigated to amplify crop growth and also protect from plant diseases. The increase in crop yields, percent seed germination, plant growth, and flowering have been reported by various cellulolytic fungi such as *Geocladium* sp., *Chaetomium* sp., *Trichoderma* sp., and *Penicillium* sp. (Bhat, 2000). β-1,3-glucanase isolated from *T. harzianum* CECT 2413 induces different types of morphological changes in plants such as swelling of the hyphal tip, cytoplasm leakage, and formation septae. It was also reported that this particular enzyme controls plant disease by inhibiting the *Rhizopus solani* and *Fusarium* sp. growth (Gupta et al., 2003). Furthermore, it has also been reported that the synergistic action of *N*-acetyl glucosaminidase and β-1,3-glucanase from *T. harzianum* (P1 strain) inhibited the germination of spores and the elongation of the germ tube of *B. cinerea* (Vos et al., 2015). The growth of pathogenic fungi, namely *Pythium,* on cucumber seedlings was reduced by the hyper-cellulose degrading mutant of *T. longibrachiatum*, which synthesized high levels of β-1, 4-endoglucanase. Therefore the application of cellulase can be proposed as biological control agents that protects seeds as well as plants from plant pathogens. Additionally, the soil quality was also improved by the application of cellulase, which reduces the dependence of chemical fertilizers. The decaying of cellulose in soil is catalyzed by adding exogenous cellulase supplementation. Thus, the application of exogenous cellulases can be a potential way to enhance the decomposition of plant debris and increase soil fertility. It has been reported that the cellulase activities in agricultural fields are influenced by some factors. These factors are mostly the soil pH, water, temperature, and oxygen (abiotic conditions); the chemical organization of organic matter and its abundance in the soil; the quality and quantity of organic substance/plant debris and minerals; and the trace elements from fungicides. It was reported that cellulases present in black soil have a greater stimulatory effect that that present in red soil (Srinivasulu and Rangaswamy, 2006). Several distinguished mechanisms were proposed for the degradation of cellulose. It was suggested that cellulase activity is found significantly in the soils at 50–60°C temperatures; however soil cellulase was degraded at the higher temperatures (60–70°C).

Cellulases enzymes have their own importance for recycling of the most abundant polymers of the globe. The cellulose of the atmosphere can be implemented as a predictive tool for maintaining soil productivity programs.

29.5 MICROBIAL ENZYMES ACT AS BIOSTIMULANTS

Microbial enzymes act as biostimulants by increasing nutrient uptake and by stimulating the plant metabolism and conferring protection against abiotic stress. The application of microorganisms such as *Pseudomonas* and *Azospirillum*, *Acinetobacter* and *Bacillus* application to the soil and plant have been identified in the enhancement of macro and micro nutrients uptake after application. The microbial inoculants have increased the uptake of Zn (Yazdani et al., 2011); Ca, Mg (Giri and Mukerji, 2004); Cu, Mn (Liu et al., 2000); and S (Banerjee et al., 2006). However, the mechanisms involved are still being elucidated. Kohler et al. (2008) reported that the combined inoculation of two different microorganisms (*Pseudomonas mendocina* and the AMF *Glomus intraradices*) significantly boosted the uptake of Ca, Fe, and Mn (Manganese) in *Lactuca sativa* (lettuce). In another study, of the two soil biostimulants (Z93 and W91), one is significantly identified and marketed in the United States for the increase of many crop yields on the application of 0.2–1.1 L/ha (http://www.agspectrum.com). These compounds have been commercialized by Ag Spectrum Co. (DeWitt, Iowa) with solutions comprised of fermented products and trace minerals. Chen et al. (2002) suggested that these compounds are involved in the degradation and mineralization of soil organic substances. These are also reported for the stimulatory and inhibiting properties for the microbial community, which leads to the increase of nitrogen availability in the soil. These biostimulants act by augmenting substrate-induced respiration and dehydrogenase as well as cellulose enzymatic activity in the soil. Many microbial enzymes such as dehydrogenase, catalase, urease, and phosphatase catalyze a wide range of soil biological processes (Nannipieri et al., 2002) catalyzing the transformation and degradation of organic matter. These enzymes help in maintaining soil nutrient cycling (Burns, 1978, 1983) and hence they are also responsible for alteration of microbial community and nutrition level of soil. Microbial inoculants have the ability to alter root and shoot architecture and also promote plant growth by synthesizing and degradating organic matter into the plant hormones (Bhattacharyya and Jha, 2012; Idris et al., 2007) or modifying plant hormone status. These beneficial microorganisms can also be responsible for the synthesis of auxins, cytokinins, gibberellins, and ethylene, which regulate various physiological processes of plants.

29.5.1 Microbial ACC Deaminases

PGPR (plant growth-promoting rhizobacteria) produces a crucial enzyme ACC (1-aminocyclopropane-1-carboxylic acid) deaminase. ACC deaminase regulates the ethylene level by metabolizing I-aminocyclopropane-1-carboxylic acid (ACC) into (α) alpha-ketobutyrate and NH_3 (ammonia) (Shaharoona et al., 2007). Bacterial enzyme 1-aminocyclopropane-1carboxylate deaminase permanently cleaves ACC (a precursor of biosynthesis of ethylene in plants) (Saraf et al., 2010). The ACC deaminase enzyme regulates

the plant growth by cleaving ACC produced by plants and thereby minimizing the ethylene level in the plant, which helps in the development of resistance against various environmental stressors. The bacterial ACC deaminase helps in the reduction of the physiological damage to plants from the extremes of temperature, phytopathogen infection, flooding, high salt, exposure to metals and organic contaminants, insect predation, and drought stress. High levels of ethylene are normally required for the removal of seed dormancy; however, an excess level of ethylene exposure can cause inhibition of root elongation. In both *Pseudomonas* sp. strain ACP and *P. putida* GR12-2, ACC deaminase activity has been induced by ACC at levels as low as 100 nM (Jacobson et al., 1994). After growing on a rich medium, both bacterial strains were then transferred to a minimal medium that contains only ACC as the nitrogen source. Furthermore, the estimation of produced α-ketobutyrate by the hydrolysis of ACC deaminase corresponds to the enzymatic activity (Honma and Shimomura, 1978). ACC deaminase has been characterized from various microbial sources only but still there are no reports on microorganisms with potential for the synthesis of ethylene by utilizing ACC as substrate. However there are strong evidences of production ACC from SAM via ACC synthase pathway in *Pecilillum citrinium*. It is reported that the ACC present in the intracellular spaces of this fungus can stimulate ACC deaminase. These ACC deaminase-producing bacteria are advantageous to plant growth promotion because, in the natural atmosphere, plants are normally focused on ethylene-producing stresses. The plants such as canola, peppers, and tomatoes are more sensitive to ethylene stress. The growth of these plants is regulated by applying ACC deaminase-containing PGPR. This activity of PGPR can be exploited in all the fields of agricultural settings. Microbial inoculants increase the availability of nutrients for the plants and also promote the growth via assimilation of substances that can act as plant hormones as well as bioprotectants and biostimulants. Various enzymes produced by microorganisms are involved in vast activities that promote the growth and development of plants (Table 29.1). Many such commercial formulations with a consortia of these microorganisms are available in the market, but their performance in the fields has yet to be explored. Plants treated with bioinoculants with promising enzymatic activities can be the

TABLE 29.1 Enzymes Produced by Various Microorganisms and Their Agricultural Significance

Enzyme	Microorganisms	Functions	Microbial Activity (Indicator)	Agriculture Significance	References
Phosphatase	*Bacillus*, *Pseudomonas*, *Micrococcus*, *Rhizobium*, *Erwenia*, *Rhizobia meliloti*, etc	Mineralization of organic phosphorous	P-cycling	Synthesis of organic acids by solubilizing inorganic phosphorus	Glick (2012)
Nitrate reductase	*Klebsiella aerogenes*, *Klebsiella oxytoca*, *Bacillus licheniformis*, *Micrococcus denitrificans*, *Clostridium perfringens*	Denitrification	N-cycling	Nitrogen fixation	Sievert et al. (2008)

TABLE 29.1 Enzymes Produced by Various Microorganisms and Their Agricultural Significance—cont'd

Enzyme	Microorganisms	Functions	Microbial Activity (Indicator)	Agriculture Significance	References
Urease	*Citrobacter freundii*, *Pseudomonas chlororaphis*, *Serratia proteamaculans* etc.	Hydrolysis of Urea	N-cycling	Synthesis of NH_3 and CO_2, which help in the rise in soil pH	Fazekasova (2012)
Protease	*Aspergillus niger*, *Bacillus subtilis*, etc.	Hydrolysis of Protein	N-cycling	Hydrolyze proteins into amino acids.	Siddiqui et al. (2005)
α-Amylase	*Bacillus*, *Aspergillus*, *Lactobacillus manihotivorans* etc.	Starch hydrolysis	C-cycling	α-Amylase converts starch into glucose	Gopinath et al. (2017)
β-Amylase	*Bacillus* sp., *Bacillus cereus*, *Streptomyces*, *Rhizopus*, etc.	Starch hydrolysis	C-cycling	β-amylase converts starch to maltose	Gopinath et al. (2017)
Peroxidase (Lignin peroxidase)	*Streptomyces viridosporus*, *Pseudomonas* sp., *Rhodococcus jostii*, *Escherichia coli*, etc	Detoxification	N-cycling	Release H_2O and O_2 from hydrogen peroxide	Ralph et al. (2004)
Phenol Oxidase	*Bacillus amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, etc.	Lignin hydrolysis	C-cycling	Oxidize phenolic compounds in organic matter	Sinsabaugh (2010)
Laccase	*Bacillus subtilis*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhizopus*	Lignin degradation	C-cycling	Oxidation of various phenolic compounds	Sinsabaugh (2010)
Polyphenol oxidase	*Alcaligenes*, *Arthrobacter*, *Nocardia*, *Pseudomonas*, and *Strepomyces*	Lignin degradation	C-cycling	Coverts polyphenols into diphenols, and further into *O*-Quinone	Sinsabaugh (2010)
Chitinase	*Klebsiella aerogenes*, *Micrococcus denitrificans*, *Escherichia coli*, *Enterobacter cloacae*, *Serratia*, *Vibrio*	Hydrolysis of Chitin	C-cycling	degradation and hydrolysis of chitin	Wang et al. (2006)
Cellulase	*Aspergillus niger*, *Trichoderma atroviride*, *Polyporus* sp., *Pleurotus*	Cellulose degradation	C-cycling	Catalyze the degradation of cellulose	Yang et al. (2006)
ACC deaminase	*Azospirillum*, *Bacillus*, *Burkholderia*, *Pseudomonas* and *Rhizobium* etc.	Involve in purine metabolism synthesis	N-cycling	Synthesis of ammonia and α-ketobutyrate	Glick (2012)

most effective approach for growth promotion of seedlings under axenic as well as field conditions. The molecular and biochemical methods involved in PGPR need to be investigated to understand the mechanism of various biochemical applications in agriculture.

29.6 CONCLUSION

The treatment of bioinoculants having promising growth-promoting enzymatic activities is the most valuable mode for growth promotion of plant seedlings. Various formulations comprising a consortia of enzyme-producing PGPR are commercially available. However, their plant growth abilities in field conditions must be characterized. The molecular and biochemical methods involved in PGPR need to be further investigated to understand their exact mechanism of action. Hence, the application of microbial enzymes as agricultural assets for improving and increasing crop yields requires the incorporation of such capable microorganisms for plant growth-promoting attributes. Further, the microbial enzymes can also be overexpressed, optimized, and manipulated for maximum utilization of their performance. Bioinnoculants amended with active enzymes can increase crop production by increasing nutrient availability as well as by maintaining healthy soil conditions. Hence, they can be developed into an effective tool for the maintenance of sustainable agriculture.

Acknowledgments

This manuscript is Integral University Communication No: IU/R&D/2018-MCN000350. We wish to acknowledge financial grant support from UP-CST- **(No. CST/D-3087).**

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CHAPTER

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Plant Growth-Promoting Microbial Enzymes

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30.1 INTRODUCTION

The continuously growing global population has also increased food demand and, consequently, the requirement for cropland, livestock feed, and energy use, which will cause changes in land use. It has been demonstrated that changes in land use for agriculture have modified natural systems, which include the decline of global biodiversity, the loss of organic carbon in soils, the increase in greenhouse gas emissions (Dunn et al., 2013), environmental damage, and pest resistance due to long-term applications of agrochemicals, among others (Jepson et al., 2014). Therefore, there is a growing global concern to move food production to a sustainable path to minimize environmental impact. Plant growth-promoting rhizobacteria (PGPR) have been applied in crops to improve yields as efficiently as the use of agrochemicals, due to their ability to enhance plant tolerance to stressful environments such as drought, salinity, high concentrations of heavy metals, nutrient deficiency, or weed infestation through different mechanisms in an ecofriendly way (Glick et al., 2007; Nadeem et al., 2014). This kind of microorganism lives in the rhizosphere, rhizoplane, root tissue, and/or in a specialized root structure named the nodule (Nadeem et al., 2014). This is where the plant roots exude a variety of organic nutrients, such as amino acids, vitamins, sugars, proteins, organic acids, phytosiderophores, etc., that are used as a source of nutrition for microbial growth as well as signals to attract microbial populations (Drogue et al., 2013). They promote plant growth directly or indirectly by enzymatically improving nutrient availability and uptake, hormonal stimulation, or plant pathogens suppression (Berg, 2009).

The understanding of these beneficial plant-rhizobacteria interactions could lead to the development of biotechnological agricultural supplies. Therefore, this chapter highlights the present knowledge about mechanisms involved in enhancing plant growth as well as

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00030-X

aspects concerning production systems of these enzymes, such as submerged and solid-state fermentation, using different substrates, and their application in agriculture.

30.2 SYNTHESIS OF PHYTOHORMONES

Enhancing plant growth could occur as a result of the production or metabolizing of chemical signaling compounds by PGPRs that indirectly increase the uptake of water and nutrients by plants due to their influence in root elongation, root hair formation, and architecture (Vacheron et al., 2013). They regulate physiological processes through complex networks of crosstalk and feedback (Dodd et al., 2010).

Phytohormone secretion by PGPRs activates biosynthetic signaling pathways (Achard et al., 2006). Auxins, cytokinins, gibberellins, and brassinosteroids promote plant growth while other phytohormones such as ethylene, abscisic acid, and jasmonates control growth activities through mechanisms of defense from inhibitory growth processes, including dormancy, senescence, abscission, etc. (Vacheron et al., 2013). There are different metabolic pathways used by PGPRs to produce phytohormones. For example, many PGPRs use tryptophan from root secretion as precursor for indole-3-acetic acid (IAA) biosynthesis, the best-characterized auxin and one of the most important mechanisms of bacterial plant growth promotion (Naveed et al., 2015), while other bacteria synthesize abscisic acid (ABA) as a product of carotenoid metabolism (Marasco and Schmidt-Dannert, 2008).

On the other hand, many PGPRs also modify the rhizospheric phytohormone concentration because they consume them or their precursors as carbon or nitrogen sources, or else produce secondary metabolites that interfere with plant hormone synthesis, such as 2,4-diacetylphloroglucinol and nitric oxide (Vacheron et al., 2013). Some rhizobacteria of the genus *Arthobacter*, *Alcaligenes*, *Pseudomonas*, and *Bradyrhizobium* can use IAA as a source of carbon (C) and/or nitrogen (N) from plants and many plant-associated microorganisms (Leveau and Gerards, 2008). Nitrite reductases from several PGPR strains, such as *Azospirillum brasilense*, make them capable of producing NO during root colonization, which is involved in the auxin signaling pathway that controls lateral root formation (Molina-Favero et al., 2008). It has also been demonstrated that at low concentrations of 2,4-diacetylphloroglucinol (DAPG), an antimicrobial compound of some rhizobacterial strains could act as a phytohormone to induce systemic resistance (ISR) by the stimulation of root exudation and enhancing root branching (Weller et al., 2007).

Several enzymes are involved in the anabolism and catabolism of phytohormones, such as indolepyruvate decarboxylase that transforms indole-3-pyruvic acid (IPyA) to indole-3-acetaldehyde (Sgroy et al., 2009), or tryptophan-2-monooxygenase that catalyzes the conversion of tryptophan to indole-3-acetamide, both crucial enzymes in IAA biosynthesis. 1-aminocyclopropane-1-carboxylate (ACC) deaminase has been widely studied as a key enzyme in plant growth stimulation in adverse environmental conditions because stress-induced ethylene synthesis inhibits plant growth (Glick, 2014; Sgroy et al., 2009). This enzyme lets bacteria metabolize the amino acid ACC exuded by roots, the direct biosynthetic precursor of ethylene, to produce ammonia and α-ketobutyrate, using ACC as the source of nitrogen (Glick et al., 2007). Adenylate isopentenyl transferases (IPT) are another important rhizobacterial enzyme in the phytohormone metabolism, participating in cytokinins biosynthesis mainly produced by phytopathogens (Hutcheson and Kosuge, 1985).

Phytohormone production by bacteria in batch cultures to formulate agricultural supplies to promote growth in plants has been widely studied (Belimov et al., 2014; Khan et al., 2014). There is also a growing interest in identifying and characterizing genes involved in the phytohormone metabolism as well as inserting them into other bacteria or plants due to their effectiveness in promoting plant growth (Heydarian et al., 2016). However, genetic determinants of rhizobacterial phytohormones production are still insufficient.

30.3 IMPROVEMENT OF NUTRIENTS ACQUISITION

PGPRs promote plant growth by their contribution to essential minerals acquisition through the solubilization of phosphorus (P) and potassium (K) and the uptake of nitrogen (N), which represent their most important effects when they are applied for biological fertilization. The elements N, K, and P are taken up by plants from soil minerals, organic materials, and synthetic fertilizers, and they could act as important limiting factors for crops. Therefore, agrochemicals that provide these minerals have been widely used in agriculture. However, extensive use of these fertilizers could destroy soil structures, decrease the organic matter content in soils, and cause environmental pollution by contaminating underground water (Meena et al., 2015). The application of PGPRs could reduce the amount of chemical fertilizer input by improving the nutrient availability and other plant growth-promoting mechanisms. Their use represents an opportunity to counteract agrochemical disadvantages on human health and the environmental.

Many free-living diazotrophs; associative and endophytes such as *Anabaena, Nostoc, Azospirillum, Azotobacter, Gluconoacetobacter, Azocarus*, etc.; and symbiotic PGPR, such as members of the rhizobiaceae family that live in symbiosis with leguminous plants and nonleguminous trees, can contribute to N inputs through fixation reactions where they transform molecular N_2 and organic forms of N into ammonia by a nitrogenase enzyme complex (Ahemad and Khan, 2012). These processes make N available to plants and represent another important mechanism to promote plant growth by rhizobacteria due to the fact that N is the main yield-limiting factor in crops. Rhizosphere dynamics and root behavior are influenced by the microbial-mediated mineralization of organic N compounds to ammonium and the subsequent nitrification to nitrate (Richardson et al., 2009). Otherwise, biological N fixation represents two-thirds of the nitrogen fixed globally (Ahemad and Kibret, 2014), and the application of this kind of bacteria could be a sustainable and economical alternative to chemical fertilizers.

Nitrogenase is the enzyme responsible for nitrogen fixation, and it has three isozymes that differ in the transition metal present at the active site: the molybdenum (Mo)-nitrogenase, the canonical form of the enzyme, and vanadium (V) and iron (Fe)-nitrogenases, that are referred to as alternative nitrogenases (Bellenger et al., 2014). Mo-nitrogenase has higher specificity than the alternative ones, and it is synthesized when Mo is available in soils. Genes for Mo-nitrogenase are present in all N_2-fixing bacteria, but only some of them have the genes for alternative nitrogenases (Bellenger et al., 2014). Although the role played by V and Fe-nitrogenases in N_2 fixation is still unknown, chemical and molecular studies have been done to define their contribution to this process (Bellenger et al., 2014; Zhang et al., 2016). Another important enzyme in nitrogen fixation is the glutamine synthetase that is involved in N fixation regulation in some bacteria through its positive control in nitrogenase synthesis in diazotrophic microorganisms (Fernandes et al., 2017).

Rizosphere pH is modified by plant-uptake of different forms of N, which influences nutrient acquisition; it mainly affects the availability of P and micronutrients such as Zn, Mn, or Fe. This pH change is related to the influx of protons in the NO_3^- uptake or the net release of protons for NH_4^+ uptake (Richardson et al., 2009), and can alter the quantity or composition of root exudates and, consequently, influence the microbial communities in the rhizosphere (Lee and Gaskins, 1982). It has also been demonstrated that N-fixing bacteria increase nodule number and mass as well as enhance shoot dry weight and growth when they are applied in consortia or by themselves (Lowman et al., 2016). Moreover, there is a growing interest nowadays in developing N-fixing cereals by direct *nif*-gene transfer, the gene for microbial nitrogenase enzyme (Curatti and Rubio, 2014; Geddes et al., 2015), which could revolutionize world agricultural systems. However, there are important barriers to achieve this, such as the sensitivity of nitrogenase to oxygen and the apparent complexity of its biosynthesis.

On the other hand, P is the second most important plant growth-limiting nutrient, but the quantity of free available inorganic P (Pi) in the soil solution is often very low because plants can only absorb P when it is bonded with oxygen as in the monobasic and dibasic forms (Kruse et al., 2015), and the majority of phosphates are commonly accumulated with soil constituents (Lambers et al., 2013). The fixation and precipitation of P in soils is determined by aspects such as pH, moisture content, temperature, and minerals already present in the soil (Walpola, 2012). The low concentration of P makes it necessary to apply fertilizers constantly, but just one part of these phosphates is absorbed by plants and the rest is converted into insoluble forms. In addition, phosphates could run off on agricultural lands by water erosion or filtrate to underground water reservoirs (Sharpley et al., 2001). Phosphate-solubilizing bacteria (PSB) from the rhizosphere represent an option to increase the soluble P in the soil solution due to their ability to hydrolyze organic and inorganic phosphates and make them available for plant uptake. *Azotobacter, Bacillus, Pseudomonas, Rhizobium, Actinomycetes, Enterobacter,* and *Burkholderia*, among other bacterial genus, have been described as effective PSBs (Kang et al., 2014; Karpagam and Nagalakshmi, 2014). The composition of the PSB population in soils is related to chemical and physical properties, organic matter, and P content. For example, semiarid regions present generally small PSB populations because of high temperatures and low organic matter (Gupta et al., 1986). Pi solubilization occurs typically by the release of several organic acids such as oxalic, citric, malonic, succinic, malic, lactic, acetic, gluconic, fumaric, glyconic, 2-ketogluconic acid, etc., that lower the pH in the rhizosphere. Subsequently, the bound forms of phosphate in soils are released and the organic acids compete with the P binding sites (Khan et al., 2010; Marciano Marra et al., 2012). PSB also mobilizes P from poorly soluble phosphates and makes them soluble by the chelation-mediated mechanism (Mohammadi, 2012). Organic acids are produced mostly by oxidative respiration or fermentation of organic carbon sources (Trolove et al., 2003), and the glucose dehydrogenase is a key enzyme in their synthesis (Sashidhar and Podile, 2010).

Otherwise, organic phosphates, which constitute 4%–90% of the total soil P, are mineralized and solubilized by the action of phosphatases and phytases (Maougal et al., 2014; Ponmurugan and Gopi, 2006). Phosphatases use a wide range of organic substrates as sources of P and transform them to Pi at soil pH ranging from acid to neutral values. Acid phosphatases play a major role in this process, although alkaline phosphatases are also present (Rodríguez and Fraga, 1999). Meanwhile, the most abundant organophosphorus compound in soils is the phytin, a salt produced by the chelation of Ca or Mg by phytic acid; therefore, phytase enzymes

are also produced by PSB. Other specific phosphohydrolases are required to solubilize other organic P compounds, such as 3′-nucleotidases, 5′-nucleotidases, and hexose phosphatases (Jonas et al., 2008). It is important to mention that PSB could also immobilize P sometimes, competing with plants to uptake the available P. When environmental stress conditions appear, this stored phosphate is released and plants take it to fulfill their phosphorus requirement (Walpola, 2012). Mineralization and immobilization are influenced by the composition and structure of microbiomes, plant exudates, and soil characteristics (Ponmurugan and Gopi, 2006).

In addition, K is the third most important major essential plant nutrient. It is involved in enzyme activation, protein synthesis, efficiency of water use, and transportation of water and nutrients in plant tissues (Zhang and Kong, 2014). Despite being an abundant element, most of the K in soils is unavailable for plant uptake. Additionally, intensification of agriculture and leaching of K contribute to decreasing the K reserve in soils at a faster rate (Rengel and Damon, 2008), causing K deficiency in acid, sandy, waterlogged, and saline soils, for example (Mengel et al., 2001). Several K-solubilizer rhizobacteria (KSR) have been studied such as *Bacillus edaphicus, Klebsiella variicola, Agrobacterium tumefaciens, Rhizobium pusense,* and *Flavobacterium anhulense* (Zhang and Kong, 2014). As well as in the solubilization of P, KSR excretes organic acids that decrease pH and release K from K-bearing minerals (Meena et al., 2015; Zhang and Kong, 2014) or chelating the primary mineral's silicon ions to solubilizing the K (Meena et al., 2015). Several reports have demonstrated an improvement in seedling height, dry weight, plant growth, and absorption of N and P when KSR is applied to different crops such as tobacco, cotton, pepper, cucumber, eggplant, etc. This happened mainly when KSB bacteria were applied with rock materials (Han et al., 2006; Zhang and Kong, 2014).

Hence, the applying of KSR, PSB, and an N-fixing bacterium as part of biofertilizers to improve plant's nutrient uptake in crops has the potential for practical application, but their impact on plant growth and the rhizosphere microbial community have to be studied deeply.

30.4 ANTIOXIDANT PROTECTION

Saline soils and saline irrigation represent an important production problem due to salinity-suppressed plant growth, mainly in arid and semiarid zones (Parida and Das, 2005). Salt osmotic stress limits water absorption from soils, and the consequently high concentration of Na^+ and Cl^- ions in plant cells causes ion stress that leads to an ionic imbalance in tissues and provokes the inhibition of nutrient uptake (Hasegawa et al., 2000). Reactive oxygen species, such as superoxide ion, hydrogen peroxide, and hydroxyl radicals, are generated as a consequence of the ion stress and could damage the plants (Kohler et al., 2009). Plants have enzymatic and nonenzymatic antioxidant protection against oxidative and salinity stresses (Upadhyay et al., 2012). PGPRs can alleviate the effect of osmotic stress by plant modulation of antioxidant enzymes, such as catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX), nitrate reductase, and peroxidase (POX). Moreover, this kind of rhizobacteria has catabolic versatility, colonizes roots, and produces a wide range of enzymes and metabolites that contributes to plant growth while withstanding biotic and abiotic stress conditions. *Bacillus subtilis* and *Arthrobacter* sp. inoculation on wheat under different salinity regimes generated an increase in dry biomass, total soluble sugars, and

proline content. These bacteria also reduced the activity level of CAT, GR, and APX as compared to controls in wheat leaves (Upadhyay et al., 2012). Similar conditions were found in lettuce inoculated with *Serratia* sp. and *Rhizobium* sp., where a reduction in APX and GR activities was found (Lee et al., 2005). However, lettuce inoculated with *Pseudomonas mendocina* Palleroni increased CAT and POX on lettuce leaves (Kohler et al., 2009). Thus, stress resistance in plants has been related to more effective antioxidant systems (Bor et al., 2003).

Drought stress also limits the growth and productivity of crops, affecting plant water retention in cells and the whole plant while generating specific and unspecific reactions and damage (Sandhya et al., 2010). PGPRs can help plants tolerate drought stress by several mechanisms, such as exopolysaccharide production (Sandhya et al., 2009). A big part of the damage in plants under abiotic stress is due to oxidative injury caused by the imbalance between the generation of reactive oxygen species (ROS) and their detoxification by antioxidant enzymes at the cellular level, whose activities are affected by PGPR. APX, CAT, and GPX activities in inoculated maize seedlings with *Pseudomonas* spp. decreased as compared to uninoculated ones under drought stress, which can be related to the lower stress in seeds with bacteria (Sandhya et al., 2010).

Antioxidant enzymes are also essential in the amelioration of adverse effects of heavy metals. Wheat under Zn stress and inoculated with a resistant *Pseudomonas aeruginosa* enhanced SOD, POD, and CAT activities as well as the concentration of nonenzymatic components such as ascorbic acid and total phenolics, which reduce H_2O_2 and malondialdehyde (MDA) due to the scavenging of ROS (Islam et al., 2014).

Therefore, the microbial inoculation of plants can be a cost-effective alternative to overcome abiotic stress (Bano and Fatima, 2009).

30.5 BIOLOGICAL CONTROL

Beside siderophores production, PGPRs produce other allelochemicals that include metabolites, antibiotics, and lytic enzymes, which reduce the damage of plant pathogens that interfere in plant growth. Environmental conditions such as water, nutrition, bacterial density, soil structure, and texture determinate the allelopathic effect (Barazani and Friedman, 2008).

Lytic enzymes such as chitinases, laminarinase, cellulases, proteases, and lipases have been applied to lyse the cell walls of many phytopathogenic fungi of the genus *Fusarium, Phytophtora, Rhizoctonia, Sclerotium, Aspergillus,* and *Pythium*, among others (Balvantin-Garcia et al., 2011; Medina-de la Rosa et al., 2016).

On the other hand, many rhizobacteria are able to produce low molecular weight compounds with an antibiosis effect over enzymes and the metabolism of other microorganisms, retarding or stopping their growth (Kundan et al., 2015). For example, many strains of the *Pseudomonas* and *Serratia* genus produce several antibiotic metabolites such as pyoluteorin, pyrrolnitrin, phenazine-l-carboxylic acid, DAPG, and biosurfactant antibiotics (Wang et al., 2015). The secretion of these antibiotics is known as the major biocontrol mechanism of soilborne pathogens.

Otherwise, hydrogen cyanide (HCN) is a secondary metabolite produced by many rhizobacteria during the stationary growth phase that confers them a selective advantage over other organisms. This phytotoxic agent inhibits enzymes involved in major metabolic

processes and some cyanide-producing rhizobacteria are host specific, living in symbiosis with their roots. HCN also seems to inhibit the electron transport chain and the energy supply to cells. Moreover, these bacteria can act as biological weed control agents with minimal deleterious effects for the host plant, mainly synthesized by *Pseudomonas* and *Bacillus* species (Kremer and Souissi, 2001).

The production of enzymes can also interfere with pathogen activities, alleviating their adverse effects on plant growth, that is, the degradation of unfavorable pathogenic toxins by detoxification enzymes such as albicidin detoxification by an esterase from *Pantoea dispersa* (Pieterse et al., 2014).

The nutrient competition of PGPRs against pathogens is also present in soils with a nutrient deficiency, where the nutrient solubilization and N_2 fixation abilities of PGPR let them rapidly colonize the surfaces of plants and consequently inhibit the growth of pathogenic microorganisms (Kundan et al., 2015).

Recently, attention has been paid to the possibility of actinomycetes to protect roots by inhibiting the development of fungal pathogens through the enzyme production that degrades the fungal cell wall or the production of antifungal compounds, and for their capacity to carry out symbiotic nitrogen fixation (Dahal et al., 2017; Errakhi et al., 2007). For this reason, Thampi and Suseela Bhai (2017) isolated and characterized rhizospheric actinobacterial strains for exploiting their antagonistic potential against pathogens of black pepper (*Phytophthora capsica* and *Sclerotium rolfsii*). From these strains, three isolates identified as *Streptomyces* by molecular and morphological characterization showed more than 90% inhibition against targeted pathogens. These isolates exhibited productions of hydrolytic enzymes such as amylases, proteases, lipases, and cellulases as well as the production of indole acetic acid and siderophores.

30.6 ACTINOMYCETES AS PGPRS

Global agricultural productivity is limited by soil salinity, one of the major stresses affecting more than 45 million ha of irrigated land worldwide (Munns and Tester, 2008). Moreover, by 2050, it is expected that the salinization of soil will increase with a result of the loss of up to 50% of arable land (Singh et al., 2015). To mitigate stress effects on plants, the application of microorganisms with plant growth-promoting (PGP) traits is considered an attractive and environmentally friendly strategy. These microorganisms can mediate mechanisms such as biosynthesizing phytohormones, raising the soil phosphate solubilization and increasing its absorption in plants while lowering the level of ethylene by the production of 1-aminocyclopropane-1-carboxylic acid (ACC) (Glick, 2014; Qin et al., 2011). Moreover, the production of cytokinin and auxin, antioxidant enzymes such as catalase, and volatile substances were also reported to have been exhibited by PGGR strains to tolerate abiotic stress in plants. Most studies of growth-promoting rhizobacteria and actinobacteria have not been extensively tested as a tool for improving plant growth under stress conditions, especially endophytic actinobacteria (Barnawal et al., 2014). Some authors have reported the isolation of *Streptomyces* strains, which could promote plant growth by the production of indole acetic acid (IAA), siderophore, and ACC deaminase under saline soil conditions (Palaniyandi et al., 2014). Qin et al. (2017) reported the plant growth-promoting traits of *Streptomyces* sp. KLBMP

TABLE 30.1 Beneficial Traits of PGP Actynomycetes

Plant Growth Promoting Mechanisms of Actynomycetes
1. Improving the root development
2. Enhancing leaf photosynthesis
3. Promoting grain filling
4. Improving the ear characteristics and grain yield
5. Improving the leaf inducible enzyme activities

5084 on the halophyte *Limonium sinensis* under salt stress conditions (0, 100, and 250 mM NaCl). Inoculated plants had greater fresh weight, leaf and root length, and total chlorophyll and proline content in both high salinity and normal conditions. These plants also showed more antioxidant enzymes and less accumulation of Na^+.

In another study carried out in maize seeds, the soaking treatment with *Streptomyces roche* D74 and *S. pactum* Act1, and their mixes, resulted in hypocotyls, radical, and fibrous root numbers by 43.4, 26.4, and 100.7 %, respectively. Also, leaf inducible enzyme activities of maize seedlings increased. Compared with the control, several significant effects were observed (Table 30.1) (Ma et al., 2017).

30.7 ENZYMES PRODUCED BY ACTINOMYCETES

Actinomycetes are known to produce cell wall hydrolases (El-Tarabily and Sivasithamparam, 2006) and antibiotics (Baltz, 2016), which suppress soil-borne phytopathogens such as *Fusarium oxysporum, Xanthomonas oryzae, Rhizotocnia solani,* and other plant pathogens (Goudjal et al., 2014; Xu et al., 2015). Li et al. (2017) reported five actinomycetes showing significant inhibitory effects on plant pathogens: *Streptomyces globisporus* subsp. *globisporus, Streptomyces globisporus, S. flavotricini, S. pactum,* and *S. senoensis*. *Sclerotium rolfsii* is a soilborne fungal plant pathogen widely distributed in tropical and subtropical regions that attacks the base of the plant stem and nearby soil. At the final stage of infection, the disruption of water and nutrient transportation by the pathogen leads to plant death (Gao, 2010). Two of the actinomycetes evaluated showed higher inhibitory effects and were subsequently examined for the inhibition of sclerotial germination. Mainly, the antagonic effect was the result of the production of extracellular cell wall-degrading enzymes in the culture conditions such as chitinase, B-1,3-glucosidase, and FP-cellulase as well as the use of Sr as the sole carbon source. Chitinase activity was present in the cell culture supernatant of all five strains, but the highest activity reached 11.2 U in strain Act12. FP-cellulase activity was only detected in strain 25 and glucosidase was not detected in supernatants of both strains Act12 and 420. In a study carried out by Kim and Song (2016), 400 *Streptomyces* strains were isolated from soils and their antifungal activities and involved mechanisms were investigated. Among them, *Streptomyces costaricanus* HR391 inhibited mycelial growth of *Fusarium oxysporum* f. sp. *raphini*, *F. oxysporum* f. sp. niveum, *F. oxysporum* f. sp. *lycopersici*, and *R. solani* by 21–26% in comparison to those of the uninoculated control after a 7-day incubation on a PDB medium. Within mechanisms of

biocontrol, production of siderophore, chitinase, glucanase, rhamnolipid, lipopeptide, iturin A, and surfactine were found.

30.8 SUBSTRATE FOR ENZYME AND PRODUCTION SYSTEMS

There are two production systems in biotechnology industries: submerged fermentation (SmF) and solid-state fermentation (SSF). SmF for production of biologically active secondary metabolites is sometimes preferred because the process associated with the scale up is much simpler compared with SSF. It also allows greater control of pH, heat, and nutrient conditions. However, SSF systems are very useful when large quantities of secondary metabolites are required in short fermentation periods, with minimal cost of media and downstream processing even factor mentioned above are more difficult to control in large-scale SSF (Robinson et al., 2001).

SmF has been used to produce clavulanic acid by *Streptomyces clavuligerus*, evaluating the effect of stirring conditions, oxygen transportation, and dissolved oxygen. This system was also used for the improvement of pectinase production by *Streptomyces* sp. GHBA10 isolated from mangrove samples (Arijit et al., 2013). Ayub et al. (2014) also obtained high yields of amylase using *Thermoactinomyces sacchari* using SmF with 5% inoculum, 150 rpm, 60°C, and pH 7.5. Synthesis of L-glutaminase by *Streptomyces avermitilis* GLU1 also was achieved by this fermentation system, varying physiological parameters (Abdallah et al., 2012). Production of antifungal and insecticidal secondary metabolite (dibutyl phthalate) was also produced by SmF by *Streptomyces atrovirens* SBTA 23.

In the case of SSF, the main advantage is the use of nutrient-rich waste materials as substrate, a high product concentration with a relatively low energy requirement (Yang and Yuan, 1990). The most critical factors in this system are substrate particle size and moisture level (Liu and Tzeng, 1999). For Actinomycetes, SSF is mainly used for antibiotic production: cephamycin C is produced by *Streptomyces cattleya, S. clavuligerus,* and *Nocardia lactamdurans,* tetracycline by *Streptomyces viridifaciens* and *S. clavuligerus,* neomycin by *S. fradiae,* and the antiparasitic avermectin B1b by *S. avermitilis.*

In the case of substrates used in SSF, they can be divided into three groups: (a) starchy substrates, (b) cellulose or lignocellulose, and (c) those with soluble sugar.

For enzyme production, starch 2.5% and glucose 3% were used for amylase under SmF. Sisal waste and sugarcane bagasse have been also reported for production of amylolytic enzymes using *Streptomyces* sp. SLBA-08. Avermectin B1b was obtained by SmF with *S. avermitilis,* using a medium containing soluble cornstarch (Siddique et al., 2013) Sugar-cane bagasse has been also used as a support and phosphatidylcholine as an inductor to produce high phospholipase A (PLA) activity under SSF by *Streptomyces djakartensis* (240 U/gS). Other substrates such as textured soy and PUF/perlite were also used but lower activity was detected (70 and 128 U/gS, respectively) (Sutto-Ortiz et al., 2017).

Li et al. (2017) identified five actinomycetes showing significant inhibitory effects for the biocontrol of *Sclerotium rolfsii*, a soilborne fungal pathogen. These actinomycetes were *Streptomyces globisporus* subsp. *globisporus, S. globisporus, S. pactum, and S. senoensis*. To facilitate soil colonization by these actinomycetes, bioformulations were prepared by solid-state fermentation in a medium containing mainly peat. All five strains synthesized extracellular cell wall-degrading enzymes such as chitinase, β-1,3-glucosidase, and FP-cellulase while

preventing germination of this fungal pathogen. Besides these enzymes, protease was produced extracellularly by *Streptomyces hygroscopicus* B04 by SSF using various combinations of rapeseed meal, wheat bran, vermicompost, and pig manure, with wheat bran and vermicompost showing the best results. Using these substrates, a biofertilizer was developed (BOF-B04) that could be a potential biocontrol agent for controlling strawberry root rot because it decreases the levels of stress-related enzyme activity (Shen et al., 2016).

Protease production (1193.77 U/g) has been also reported by *Brevibacterium luteolum* under SSF using grounded wheat bran and rice flour as an inducer. In this case, the protease production was enhanced in the presence of 25% rice bran along with 75% wheat bran in comparison when using wheat bran as the sole carbon source (740 U/g). The maximum protease activity was obtained after 96 h of incubation with 15% inoculum of actinomycete, developing a mechanism for regulated release protease from cheaper agrowastes (Renganath Rao et al., 2017).

30.9 CONCLUSIONS

Actinomycetes have gained interest in agroindustry as a source of biologically active compounds, biocontrol agents, and PGPRs. Within actinomycetes, *Streptomyces* is the most used. According to Tanaka and Omura (1993), about 60% of the new insecticides and herbicides originated from these microorganisms. This growing interest is due to their low toxicity and environmental friendliness; they have a degradable nature while being highly specific and less toxic to nontarget organisms. However, the identification of novel species as well as the mechanism of action of these bioagents is needed for the development of actinomycete-based products commercially available with a prolonged shelf life.

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CHAPTER

31

New Features and Properties of Microbial Cellulases Required for Bioconversion of Agro-industrial Wastes

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31.1 INTRODUCTION

One of the major social problems present in modern society is hunger and poverty. The Food and Agriculture Organization of the United Nations (FAO) has stated that all people have the right to sufficient and assorted food that meets their nutritional and economic needs (FAO, 2008). The development and implementation of different strategies to overcome hunger and malnutrition in the world, include efforts aimed at increasing and improving the production of agricultural products, which are largely processed to produce products and Derivatives through diverse processing technologies that result in the generation of large quantities of byproducts, creating a secondary problem of pollution that needs integral management at various levels (Yazid et al., 2017). Biotechnology has been one of the most developed areas in recent years due to the inherent possibilities to generate solutions to

https://doi.org/10.1016/B978-0-12-813280-7.00031-1

diverse problems in modern society. Management and valorization of agricultural wastes is a milestone of the implementation of bioprocesses in food and agriculture activities. Biotechnological strategies in this area are focused on the production of energy (or combustibles), biopolymers, and value-added products such as enzymes, spores, or other biological resources (Tsouko et al., 2017).

Agricultural and agroindustrial wastes include complex materials such as bagasse, straws and stems, cobs, fruit peels and husks, skin, bones, fat, and any part of a processed food source (plant or animal) that is not used in the primary process (Sannik et al., 2013; Yazid et al., 2017). Vegetal wastes are generally rich in lignocellulosic materials and polyphenolic compounds, making them a good source for obtaining simple sugars that are capable of being used as a carbon source for the fermentation process (van Beilen and Li, 2002) and of phenolic compounds and their derivatives that present strong antioxidant activity (Mishra et al., 2011). Many of the strategies to manage these wastes are enzyme-based technologies. Beginning their industrial use in the early 19th century with application in detergents to dissolve protein in dirt (Binod et al., 2013) commercial enzymes have evolved and reached a level of very high importance in industrial use. In the issue of agroindustrial waste management, a type of enzyme of major importance is cellulases.

Cellulases, a group of enzymes that comprehends *endo*-glucanases, cellobiohydrolases, and β-glucosidases (Binod et al., 2013), are the most-studied group of enzymes. They also present a major expansion in use due to usefulness in the production of biofuels from lignocellulosic materials (Machado and Pereira, 2010), a crescent area of research where the function is to convert the cellulose from fiber-rich vegetal wastes into simple sugars capable of being fermented. Most agroalimentary byproducts (from vegetal raw materials) are rich in cellulose and lignin, making it necessary to pretreat the material by physical, chemical, or microbial techniques to achieve an efficient process. Cellulose is obtained from microbial cultures; fungal species such as *Penicillium, Trametes, Aspergillus,* and *Pycnoporus,* among others, have been demonstrated be a good source of cellulases (Sánchez, 2009).

On the other hand, solid-state fermentation (SSF) has become an important technological strategy in obtaining several enzymes, including cellulases (Romo et al., 2015). SSF can use a wide variety of solid material such as peels, husks, cobs, and many other alimentary wastes as solid support for the culture in a very limited presence of free water. The environment of SSF is favorable for a great variety of microorganisms (including fungal, yeast, and bacterial strains) making it possible to use cultured media without sterilization or, in some cases, without an inoculums. These characteristics may translate in a reduction of the process costs, making the scaling of the process cheaper due to the lack of unitary operation (Yazid et al., 2017). In this chapter, we will review the function, properties, and other features related to the performance of cellulases in the bioconversion and valorization of agroindustrial wastes.

31.2 CELLULASES: BIOTECHNOLOGY OF FERMENTATION, MICROORGANISM, SUBSTRATE, AND BIOREACTOR

SSF has been historically important for humankind for centuries, mainly for bread and cheese production in many countries around the world. In fact, in the last decade it has still been used to get high-value products such as biomolecules as well as for its application in

many important industries such as pharmaceutical, textile, biochemical, food, and bioenergy (Soccol et al., 2017). SSF refers to the bioprocess where microbial growth and product formation occur in the surface of the solid matrix with the absence or near absence of free water, but the moisture is observed to the solid substrate to promote growth and the microbial metabolism (Fang and Xia, 2015; Pirota et al., 2014). This technique has some important advantages such as using cheap substrates, fewer sterility requirements, and unnecessary complex equipment that result in a low cost of production. It also enables higher enzymatic productivity for many enzymes, hence allowing a higher final concentration of products. Also, it is a friendly process without environmental pollution or water contamination because it allows the use of solid agroindustrial wastes as a carbon source in their natural form and facilitates soil waste management (Soccol et al., 2017; Yoon et al., 2014). SSF is a potential biotechnological technique for the production of fungus lignocellulolytic enzymes such as cellulase (Behera and Ray, 2016). Of the factors to consider during the development of SSF, the most important are the choice of microorganisms and the choice of substrate. Much recent research proves that microorganisms such as yeast, bacteria, and actinomycetes are appropriate for SSF, being filamentous fungi, the most suitable microorganism because this technique mimics the natural habitat (Soccol et al., 2017). Fungi produce an extensive set of enzymes to degrade lignocellulosic plant biomass. Fungal cellulases are among the most widely exploited microbial enzymes for many industries and environmental applications (Shah et al., 2017).

Agroindustrial activities provide a large variety of residues, including sugarcane bagasse, cereals as wheat bran, rice bran, and soybean, coffee pulp and husks, fruit peels, and pomegranate and corn cobs, which are also called wastes without any important application. (Urbaniec and Bakker, 2015). Otherwise, its high accumulation is considered an environmental pollution factor. Those wastes can be used as a substrate for SSF (Delabona et al., 2012; Elegbede and Lateef, 2017; Urbaniec and Bakker, 2015), basically composed of cellulose, hemicellulose, lignin, starch pectin, and other fibers (Kawee-Ai et al., 2016; Zhang et al., 2016a). Usually these agroresidues are not only a solid support for nutrient absorption and biomass growth, but they are also a source of carbon and nutrients. Cellulase production by fungi is mainly regulated at the transcriptional level. Substrates such as cellulose, lactose, and sophorose act as inductors while glucose normally acts as a gene repressor. The presence of low-level constitutive enzymes is required to initiate cellulose degradation, which generates soluble inducers that can enter the cell and activate cellulase gene transcription (Shah et al., 2017).

Cellulase is known to be produced not only by fungi but also by bacteria, protozoa, plants, and some members of the animal kingdom. A cellulase system from aerobic and anaerobic bacteria and fungi has been extensively studied during the past two decades. A cellulolytic enzyme system can be complexed or noncomplexed. Noncomplexed cellulases are found in aerobic fungi, bacteria, and actinomycetes. These organisms secrete cellulases as free enzymes in the exterior while in anaerobic bacteria and fungi, these enzymes are organized as high-molecular weight complexes called cellulosomes. (Shah et al., 2017). Cellulases are one of the most important microorganisms used in industry, and they mainly consist of cellobiohydrolases (CBH. EC 3.2.1.91), endoglucanases (EG: EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21). The cellobiohydrolases were found to be the most important components in the cellulose mixture for a complete enzymatic hydrolysis of a lignocellulosic biomass (Fang and Xia, 2015). Endoglucanases act randomly on the

cellulose chain to produce cello-oligosaccharides while cellobiohydrolases act on exposed chain ends by splitting off cellobiose. Cellobiose is subsequently hydrolyzed by cellobiase to form glucose. This hypothesis is, however, more applicable and acceptable worldwide for the decomposition of cellulosic biomass (Kuhad et al., 2016; Lynd et al., 2002). Currently, cellulolytic enzymes from fungi are being produced on a large scale for various applications such as textile processing, laundry and detergents, animal feed improvement, food processing,and biofuel production, as reported by Kuhad et al. (2011) (Table 31.1). Enzyme-based lignocellulosic biomass bioconversion into a variety of valuable products can be the most efficient and ecofriendly approach (Shah et al., 2017).

TABLE 31.1 Application of Cellulases in Various Industries (Kuhad et al., 2011)

Industry	Applications
Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids, single cell proteins, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder
Detergents	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antiredeposition of ink particles
Fermentation	Improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines; improved primary fermentation and quality of beer; improved viscosity and filterability of wort; improved must clarification in wine production; improved filtration rate and wine stability
Food	Release of antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits
Pulp and paper	Coadditive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energy requirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freeness and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, and sanitary paper
Textile	Biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of color brightness
Others	Improved carotenoids extraction; improved oxidation and color stability of carotenoids; improved olive oil extraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste; production of hybrid molecules; production of designer cellulosomes

31.3 BIOCONVERSION OF RESIDUES RICH IN CELLULOSE TO PRODUCTS OF ADDED VALUE

Within the methodology for the conversion of biomass rich in cellulose to products of high added value such as bioethanol is the pretreatment of the biomass. Currently, three basic methods are used: physical (pyrolysis), chemical (use of strong acids and/or alkali), and biological (microorganisms). Table 31.2 shows some physical and chemical pretreatments, the type of biomass, and yields. In this section we will focus on the biological method and

TABLE 31.2 Summary of Some Typed of Pretreatment to Covert Biomass Rich in Cellulose

Pretreatment	Conditions	Biomass	Product Yield	Reference
Ammonia fiber explosion (AFEX)	Ammonia loading NH_3 to dry biomass loading (w/w) (1:1–1:2), moisture content (db%) (40–60), time (min) (15–30), 100°C	Corn stover, prairie cord grass and switchgrass	Biooil until 49% Biochar until 25% Syngas until 31%	Sundaram et al. (2017)
Milox pulping	Formic acid at 10:1 solvent to biomass weight ratio. H_2O_2 was added at 1%–3%, temperatures ranges of 25–90°C	Beech wood	Pulp 44%–79%. Lignin 2%–13% Cellulose 51%–84% Hemicellulose 11%–23%	Kalogiannis et al. (2015)
Fast pyrolysis	Moisture content 65 ± 10% and smaller size; temperature of 450°C using a fluidized bed reactor 36 wt%	Oil palm	4.46 kg CO_2/kg of biooil	Chan et al. (2016)
Diluted acid	Sulfuric acid 0.05 wt%, type flowthrough and batch, temperature of 140–180°C and time 12–192 min	Poplar chips	30%–35% lignin	Bhagia et al. (2016)
Alkali-assisted ultrasonication	NaOH 1% for 15–60 min at 25 kHz	*Cynodon ductylon* and *Elusine indicus*	Reducing sugars 5.4%–59%	Gabhane et al. (2014)
Diluted acid	Temperature of 110–130°C; acid (% *w/v*) 1–2; time 30–60 min	Crop of *P. aquatic*	Glucose 54%–65%	Karapatsia et al. (2017)
Steam explosion	Sugarcane bagasse mass 40 g; volume 800 mL of water, citric acid 0.01 M, NaOH 0.1 M; temperature of 180°C, pressure 8.8 kgf/cm, time 5 min	Sugarcane bagasse	Water explosion bagasse 89%, acid explosion bagasse 76%, NaOH explosion bagasse 71%	Silva et al. (2017)

Continued

TABLE 31.2 Summary of Some Typed of Pretreatment to Covert Biomass Rich in Cellulose—cont'd

Pretreatment	Conditions	Biomass	Product Yield	Reference
Dilute acid	Liquid-ratio of 1:20 (*w*/*v*), sulfuric concentration 0.03–0.3 M, time of 86–140 min, temperatures of 86–140°C	Sorghum	Glucose 3.92 g/L; Xylose 22.4 g/L	Deshavath et al. (2017)
Alkali fractionation	NaOH 2%; temperature of 90°C for 2.5 h, solid-liquid ratio 1:30 g/mL	*Eucalyptus urophylla*	Lignin 8.14–12.22 kg	Sun et al. (2014)
CaO	Temperature of 50°C for 4 h	*M. aeruginosa*	Reduced sugars Glc 16 mM	Khan et al. (2017)

will explain some examples for the bioconversion of cellulose-rich biomass from filamentous fungi. The ability of micromycetes *Trichoderma viride* and *Aspergillus terreus* to decompose the cellulose-containing substrates was studied. The *A. terreus* inoculum was added to a liquid Czapek-Dox medium as a spore suspension. After *A. terreus* cultivation for 10 days, the fungus biomass and the resulting solid precipitate and culture liquid were used as a substrate for biogas production. *T. viride* was used to study the influence of the substrate on the effectiveness of the biodegradation of cellulose-containing substrates. Under these conditions, the use of fungi is a promising means of pretreating lignin-containing paper wastes and plant cosubstrates (Prokudina et al., 2016). In other research, the potential of two lignocellulosic wastewaters, namely corncob waste liquor, paper mill effluent, and a cellulosic waste as feedstock were evaluated for microbial oil production by *Aspergillus awamori* (MTCC 11639). Lipid production was evaluated using different types of wastewaters as carbon sources using *A. awamori*. All the experiments were performed in individual batch reactors (volume of 500/250 mL) under batch mode at ambient temperature (29 ± 2°C; pH 5.5; 100 rpm; incubation time 72 h) by keeping in a rotary shaker at 150 rpm. They concluded that the fuel production from lignocellulosic compounds using *A. awamori* is a good alternative that is cost-effective and can serve as a platform to address the impending energy crisis in the context of environmental biorefinery (Subhash and Mohan, 2015).

On the other hand, a formulated biocontrol agent with *T. viride* and an indigenous *Trichoderma* spp. were used to evaluate the efficiency of the cellulolytic property for the conversion of cellulosic wastes such as vegetable waste and sugarcane bagasse to simple sugar. The best results were for the production rate estimated in carboxy methyl cellulose, vegetable waste, and sugarcane bagasse of 70% and 100%, respectively by *T. viride* where it was 54.6%, 66.3%, and 83.3% in the fermented broths of *Trichoderma* spp. KT. They concluded that the cellulosic waste can be very well used as a resource for bioethanol production (Juneius and Kavitha, 2017). Further, they analyzed the fungi for their capacity to bioconvert *Miscanthus* cell walls but also their ability to secrete total proteins; to secrete enzymes with the activities of xylanases, exocellulases, endocellulases, and beta-glucosidases; and to remove specific parts of *Miscanthus* cell walls, that is, glucan, xylan, arabinan, and lignin. One of their conclusions regarding unsampled enzyme activity was that the four types of enzymes analyzed here,

endocellulase, exocellulase, beta-glucosidase, and xylanase, explained just one-quarter of the biomass loss. Clearly, other enzymes and processes are playing important roles in biomass conversion (Shrestha et al., 2015). In contrast, the aim of the other study was to efficiently convert oil palm empty fruit bunch fiber into both cellulase and bioethanol. The SSF was carried out at a temperature of 30 ± 1°C for 7 days, and the initial pH was 5.0. In this part of the process the results were for enzyme activity FPase 20.2, CMCase 132.5, β-glucosidase 101.1, xylanase 70.2, β-xylosidase 16.2 IU/g using *T. reesei* RUT-C30. They concluded that oil palm empty fruit bunch fiber is a potential feedstock for both fuel ethanol and cellulase production. In all processes, 8.8 g of ethanol could be produced from 0.1 kg of oil palm empty fruit bunch fiber without any usage of commercial cellulase (Zhu et al., 2014). In another study, mushroom cultivation waste material was utilized as a lignocellulosic source for cellulolytic enzyme production by *Aspergillus tubingensis* HS1–5 and bioethanol production. Substrates formulated with mushroom cultivation waste material were subjected to SSF by the fungal strain. The material was enriched with the Mandels mineral salts medium with the initial moisture content adjusted to 65% (*w*/w). After cooling, the substrates were inoculated with 10 mL of the fungal spore suspension and incubated at 30°C for 10 days. The results obtained were that the cellulolytic enzymes CMCase and β-glucosidase produced from mushroom cultivation waste material by *A. tubingensis* HS1–5 under a moisture content of 65% at pH 5.0 and at a temperature of 30°C exhibited great activity of 1789.17 ± 108.48 and 45.03 ± 4.70 IU/gds, respectively. They concluded that this waste material showed a candidate for a low-cost raw material to produce cellulolytic enzymes as well as bioethanol production (Sotthisawad et al., 2017). Finally, fungi have the potential for lignocellulose degradation and can be used for the in situ decomposition of crop residues. In this case, lignocellulose degrading fungal spp. were isolated and evaluated for the activity of lignocellulolytic enzymes. The fermentation process was carried out with 5.0 g of rice and a wheat straw mixture (4:1), then moistened with 15.0 mL of Reese's mineral medium in a 250 mL flask with inoculum. Seven days after incubation, it was at 30°C. Later, the enzyme was extracted using a citrate buffer (0.05 M, pH 4.8) and was put through centrifugation at 9000 rpm for 10 min at 4°C. The results obtained showed that fungal isolates from rice-wheat fields can enhance the degradation of rice and wheat residues with no adverse effect on wheat growth (Choudhary et al., 2016). Some progress has been made in the use of microorganisms for the bioconversion of cellulose-rich waste to obtain high added value products with wide applications in the chemical, biotechnology, and food industries.

31.4 GENERAL ASPECTS IN THE IMMOBILIZATION OF CELLULASE

Climate change is affecting our world, and as a result, every responsible industry is making efforts to reduce its greenhouse gas emissions. Therefore, the advance of new technologies that help to reduce the use of toxic products for the environment has been of research interest. Lately, remarkable attention has been given to the use of biocatalyst (enzymes) immobilization to preserve its stability and facilitate its reusability. A global trend in the use of cellulase has arisen due to its applicability in the food pharmaceutical and agriculture industry as well as bioenergetics industries, in the latter to degrade lignocellulosic material

to produce biofuels (Mubarak et al., 2014; Podrepšek et al., 2012; Zhang et al., 2016b). An immobilized enzyme is defined as the enzyme physically confined to a certain defined region while retaining its most catalytic activity, allowing the flow of substrate and products. In others words, an interaction of the active biocatalyst with the mechanic and chemistry stability of the support (Jegannathan et al., 2008; Sánchez et al., 2014). Some different materials have been explored in the literature to immobilize cellulase enzymes, including various polymers, inorganic materials (Viet et al., 2013; Wang et al., 2015; Zhang et al., 2016b), carbon nanotubes (Mubarak et al., 2014), magnetic particles (Bohara et al., 2016; Khoshnevisan et al., 2011; Li et al., 2015; Mishra and Sardar, 2015; Song et al., 2016; Zang et al., 2014; Zhang et al., 2015), and microspheres (Li et al., 2013).

31.5 TECHNIQUES FOR IMMOBILIZATION OF CELLULASE

Different techniques have been developed for cellulase enzyme immobilization and have been reported in published articles. These techniques are fundamentally based on two principles: physical retention and chemical bonds (Sánchez et al., 2014). They can be classified in three groups depending on how the enzyme attaches to the matrix: carrier bonding, cross-linking, and entrapment (Datta et al., 2013; Zhang et al., 2012; Zhao et al., 2015) (Fig. 31.1). Depending on the type of interactions between enzyme and carriers, these techniques can be further classified into irreversible and reversible immobilization techniques (Brena and Batista, 2006). When applying irreversible techniques, the enzyme cannot be detached from the matrix with-

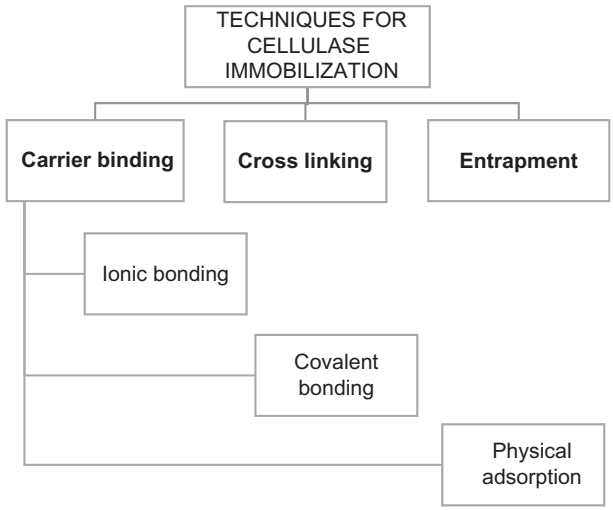

FIG. 31.1 Various techniques for cellulase enzyme immobilization. *Adapted from Mohamad, N.R., Marzuki, N.H.C., Buang, N.A., Huyop, F., Wahab, R.A., 2015. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol. Equip. 29, 205–220; Zhang, B., Weng, Y., Xu, H., Mao, Z., 2012. Enzyme immobilization for biodiesel production. Appl. Microbiol. Biotechnol. 93, 61–70; Zhao, X., Qi, F., Yuan, C., Du, W., Liu, D., 2015. Lipase-catalyzed process for biodiesel production: enzyme immobilization, process simulation and optimization. Renew. Sust. Energ. Rev. 44, 182–197.*

out altering the enzyme's biological activity or the matrix characteristics. On the other hand, when applying reversible techniques, the enzyme can be gently detached from the matrix (Zhao et al., 2015). Covalent bonding, entrapment, and cross-linking are the most commonly used procedures for the irreversible immobilization of cellulase. Physical adsorption and various noncovalent bindings, such as ionic bonding, are well-known reversible immobilization procedures. Each immobilization technique has its own advantages and disadvantages for cellulase immobilization. The aim of the studies performed in the immobilization of cellulase and other enzymes until now is based on the determination of optimal conditions used to maintain enzymatic activity with the highest possible biocatalyst reuse.

31.5.1 Immobilization of Cellulase by Physical Adsorption

Adsorption is a method commonly used for enzyme immobilization. This process is the result of noncovalent interactions in which the enzyme is adsorbed by nonspecific forces such as Vander Waals forces, hydrogen bonds, electrostatic interaction (also ionic binding), and hydrophobic interactions (Datta et al., 2013; Jegannathan et al., 2008). This method is widely employed due to its commercial advantages, such as: (1) easy operation and preparation; (2) low cost of carrier materials and immobilization procedure; (3) no chemical additives are employed; (4) easy regeneration of carriers for recycling; and (5) high activity (Sánchez et al., 2014; Zhang et al., 2012; Zhao et al., 2015). The cellulase Onozuka enzyme from *T. viride* was immobilized in functionalized multiwalled carbon nanotubes (MWCNTs) via the physical adsorption method to yield a stable enzyme with easy separation. The cellulase retains up 52% its original activity (97.58%) after six cycles of consecutive hydrolysis reaction with carboxymethyl cellulose (CMC), starting with 97.58% in initial activity (Mubarak et al., 2014). Khoshnevisan et al. (2011) reported that at the immobilization of the cellulase (endoglucanase) enzyme at commercial superparamagnetic nanoparticles (MNPs), the enzyme was bound via physical adsorption (ionic bond). This study showed that the binding onto MNPs can allow the enzyme to obtain the conformational and structural arrangement for a better activity and stability, providing a greater resistance to a medium with higher alkalinity in comparison with the free enzyme (Khoshnevisan et al., 2011). Taha (2017) reported the use of two different carriers, the first an anion exchanger AM-21-A, and the second a cation exchange fiber VION KN-1, to immobilize the cellulase enzyme. The result obtained via physical adsorption showed low activity retention 35% than other techniques probed (Taha, 2017).

31.5.2 Immobilization of Cellulase by Covalent Bonding

Immobilization by covalent bindings refers to a chemical reaction between the active amino acid residues outside the catalytic activity and the binding site of the enzyme toward the active groups of the carrier (Zhao et al., 2015). This technique provides a more stable attachment, minimizing enzyme leakage from the support, compared to the adsorption process (Alftrén and Hobley, 2014). In Table 31.3, some recent progress in the immobilization of cellulase using covalent binding is summarized. Various carriers have been employed for this technique, including polymers, inorganic material, and magnetic nanoparticles. Cellulases immobilized on nanostructured carriers with magnetic properties offer the advantages of magnetic separation and repeated use for a continuous hydrolysis process. Song

TABLE 31.3 Some Supports Used at Cellulase Enzyme Immobilization Process

Supports/Carriers	Composition	Immobilization Techniques	References
Magnetic particles	Silica [a]MNP	Covalent binding	Song et al. (2016) and Alftrén and Hobley (2014)
	AF-$CoFe_2O_4$	Covalent binding	Bohara et al. (2016)
	Fe_3O_4	Physical adsorption	Khoshnevisan et al. (2011)
	[b]GNP—[a]MNP	Covalent binding	Gokhale et al. (2013)
	Polyvinyl alcohol/Fe_2O_3	Covalent binding	Liao et al. (2010)
Carbon nanotubes	Multiwalled carbon nanotubes (MWCNTs)	Physical adsorption	Mubarak et al. (2014)
Polymer naturals—inorganic materials and synthetic polymers	Anion exchanger Cation exchange fiber	Physical adsorption	Taha (2017)
	glutaraldehyde/Modified glutaraldehyde	Covalent binding	Taha (2017)
	Silica gel—(3 aminopropyl) triethoxy-silane—glutaraldehyde	Cross-linking	Zhang et al. (2016b)
	Sodium alginate—calcium chloride—gluteraldehyde	Cross-linking	Wang et al. (2015)
	Alginate calcium	Entrapment	Viet et al. (2013)

[a] *MNP: magnetic nanoparticles.*
[b] *GNP: grapheme nanoplatelet.*

et al. (2016) demonstrated simultaneous immobilization of two types of cellulases (cellobiohydrolase D, β-glucosidase A) from *Aspergillus nidulans* pEXPYR at the same carrier by covalent binding. This study showed that enzymes immobilized on silica MNP achieved 100% immobilization efficiency while retaining about 85% and 43% of their initial activities after three and 10 reuses, respectively (Song et al., 2016). This is compared with the study realized by Bohara et al. (2016) using cobalt ferrite nanoparticles to immobilize cellulase enzyme from *Trichoderma reesei* ATCC 26921, where its activity retained was more than 64% even after six cycles of reuse. Alftrén and Hobley (2014) studied the immobilization of whole cellulase commercial mixtures on nonporous silica magnetic microparticles to enable enzyme reuse as well as the effect of surfactant addition on the enzymatic hydrolysis process. The result suggested that the hydrolysis yield increased with the addition of surfactants such as Tween 80 or bovine serum albumin (BSA), making possible the reuse of the enzyme with a real lignocellulosic substrate (Alftrén and Hobley, 2014). A novel magnetoresponsive biocompatible graphene support was reported by Gokhale et al. (2013). When using this carrier, magnetic nanoparticles were incorporated at graphene nanoplatelets for immobilization of a commercial cellulase. The immobilization process combines the use of quenched and annealed polyelectrolytes, which are used for efficient electrostatic binding of anionic iron oxide nanoparticles. Polyacrylic acid, a weak polyelectrolyte, makes the support surface be biocompatible. The immobilized retained about 55% of the original specific activity even

after four cycles of reuse (Gokhale et al., 2013). The use of a polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle was evaluated by Liao et al. (2010), using commercial cellulase (R10). The immobilization process consisted of frozen and thawed methods so as to form a microemulsion system with small size and excellent specific activity. The biocatalyst maintained 40% activity even after four cycles of reuse in a wet milling treatment for the efficient conversion of microcrystalline cellulose (Liao et al., 2010).

31.5.3 Immobilization of Cellulase by Entrapment

Immobilization by entrapment refers to the capture of an enzyme inside a polymer or microcapsules of polymers without alteration between the exchanger of substrate and products through these polymers (Sánchez et al., 2014; Zhao et al., 2015). In his recent work, Viet et al. (2013) showed the used of sodium alginate to capture the cellulase enzyme from *T. reesei* at different beads sizes, enzymes concentrations, temperatures, and pH conditions, retaining high enzymatic activity and permitting the reuse of the immobilized until eight cycles, where it reduced the correlation of activity to 20% (Viet et al., 2013). Andriani et al. (2012) immobilized the cellulase enzyme produced by *Bacillus subtilis* TD6 using calcium alginate beads. The immobilized enzyme can be reused up to four times, where the activity retained is less than 10%. The storage stability of entrapped cellulase at 4°C was found to be up to 12 days, while at 30°C, the enzyme lost its activity within 3 days (Andriani et al., 2012).

31.5.4 Immobilization of Cellulase by Cross-Linking

Immobilization by cross-linking refers to the process to immobilize the enzyme due to the formation of intermolecular cross-linkages by the addition of bifunctional or multifunctional crosslinking reagents such as gluteraldehyde (Zhang et al., 2016b) and alginate-glutaraldehyde (Wang et al., 2015) that form an irreversible intermolecular linkage among molecules of the enzyme (a three-dimensional structure). This method offers big stability of the enzyme due to the rigidity of the structure formed, which is capable of resisting extreme conditions of pH and temperature but has a loss of enzymatic activity. Among the work carried out using this methodology, we can cite the study performed by Zhang et al. (2016b), where a commercial cellulase enzyme was immobilized onto pretreated silica gel surfaces and glutaraldehyde was used as a cross-linker. Significant activity over multiple reuses was observed; after 15 cycles of reuse the activity retained was 31%. The enzyme immobilized retained 48% of its initial activity after 4 days and 22% even after 14 days (Zhang et al., 2016b). In another study, Wang et al. (2015) reported the immobilization of a cold-active cellulase from psychrophilic bacterium (*Pseudoalteromonas* sp. NJ64) obtained from the Antarctic for ethanolic fermentation of the kelp cellulose. The enzymatic activity of this cold-active cellulase was 49.7 U/mL. Under optimal immobilization conditions, its activity recovery rate was 51.58%, and the relative enzymatic activity of the immobilized was 58.37% after seven recovery cycles (Wang et al., 2015). Podrepšek et al. (2012) reported the stability of immobilized commercial cellulase enzymes under different pressure conditions exposed to carbon dioxide. The result showed a lower enzymatic activity of 0.1 unit/mL than a free enzyme at the same conditions (Podrepšek et al., 2012). Another study of interest used nanofibrous poly

vinyl alcohol membranes to immobilize a cellulase commercial enzyme by the electrospinning and casting method. The enzymatic activity was more than 65% higher than the free enzyme and nanofibers were superior compared with casting film (Wu et al., 2005).

31.6 BENEFITS OF IMMOBILIZATION

Similar to other biocatalyst immobilizationa, cellulase enzyme immobilization showed many advantages over free cellulase, such as easy recovery and reuse, higher adaptability for continuous operations, fewer effluent problems, pH and thermal stability, and a higher tolerance to reactants and products (Khoshnevisan et al., 2011; Netto et al., 2013; Sánchez et al., 2014; Zhang et al., 2016b). The high cost of the process of immobilization is a disadvantage. However, it can be disregarded if it is considered the value of the recovered enzyme from the reaction medium in a continuous operation (Sánchez et al., 2014). The challenge of its application at the industrial level presents some drawbacks such as a loss of enzymatic activity during immobilization and the high cost of the carriers (Zhao et al., 2015).

31.7 GENERAL PERSPECTIVES OF CELLULASE PRODUCTION

The food industry represents one of the economic sectors where microbial metabolites have found a wide variety of applications. This is the case of some enzymes, particularly cellulases, which have played a very important role as food additives. Most cellulases have been produced by submerged cultures at an industrial level. Many works in the literature present detailed aspects involved with those enzymes and their importance in the food industry. However, the production and application studies of those enzymes produced by SSF are minimal in comparison with submerged fermentation. Microbial cellulases are widely used as an aid in processing in food industries. However, the fields of new industrial and analytical applications have been extended in recent years, making it necessary to study more deeply this kind of enzyme. Erroneously, it has been considered that enzymes reviewed in this work have been extensively studied and do not represent a good model for additional studies. Several reports on the influence of cultural conditions on the synthesis of microbial enzymes have been made but there is not yet a comprehensive set of models of enzyme regulation. The effect of different carbon sources has been studied. The optimal concentration of constituents in a medium for the enhanced production of enzymes has been determined. The effect of initial pH, temperature, and air flow rate on enzyme production was also studied.

Many applications in detergents and drugs as well as the feed and food industries have been proposed. Although a fair amount of work has been done on novel reactors and processes for the production of food enzymes, extensive work has yet to be carried out on several topics related to food enzyme production. For example, intensive work on kinetic studies in bioreactors remains to be done. Extensive studies are needed for the optimization of the food grade cellulose production in SSF, considering that this kind of process provides higher productivity due to high levels of enzyme yields and shorter fermentation times. In submerged cultures, the enzyme activity decreases by the increased the supplementation of the carbon source, but not in SSF. This fact suggests that regulatory phenomenon such

as induction-repression or activation–inhibition is different in both cultures. On the other hand, it is necessary to carry out efforts to understand the importance of enzymes related to the degradation or modification of their substrates and those food enzymes considered as accessories.

According to the previous section, the production of cellulases related to the degradation of different complex substrates requires more study. Also, the optimization of physical parameters such as pH, aeration, and agitation in bioreactors should be done. In this sense, to produce cellulases, extensive work on bioreactors—including kinetics—should be done to analyze the whole process. On the other hand, high concentrations of carbon sources inhibit enzyme synthesis in submerged fermentation. This observation strongly supports the need to produce cellulases by feed batch fermentation with the corresponding optimization work. It should be stressed that the literature on high cell density cultivation for food enzymes is not available. Continuous culture for production of these enzymes should be studied to develop this mode of operation at an industrial level. The fact that cellulase production by SSF is not strongly repressed by carbon sources makes this field an attractive way to produce food enzymes. It should be stressed that the literature on high cell density cultivation for food enzyme production is not available. Further studies on the design of bioreactors for the production of food enzymes can be attempted. An interesting opportunity is the production by SSF of these enzymes using genetically modified organisms acting on the new kind of biomass for their valorization.

31.8 CONCLUSION

This chapter revealed the importance of the bioconversion of agroindustrial waste in recent years. Due to the great development of biotechnology, especially in the field of fermentation technology, it is now easier to take advantage of natural resources than in previous years. We are always looking for simple methodologies to obtain value-added products from waste material, which is now going to boost novel approaches for obtaining new products.

Acknowledgments

Authors thank National Council of Science and Technology of Mexico for the financial support through the program—National Fellowships-CONACYT-2016.

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CHAPTER

32

Oxylipins and Green Leaf Volatiles: Application of Enzymes From Plant Origin to Produce Flavors and Antifungal Aldehydes

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ABBREVIATIONS

GLV	green leaf volatile
LOX	lipoxygenase
AOS	allene oxide synthase
DES	divinyl ether synthase
HPL	hydroperoxide lyase
ADH	alcohol dehydrogenase
POX	peroxygenase
GL	galactolipase
MGDG	monogalactosyldiacylglycerol
HPO	hydroperoxy-acids
9-HPOD	(10*E*,12*E*)-9-hydroperoxy-10,12-octadecadienoic acid
13-HPOD	(9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid
9-HPOT	(10*E*,12*E*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid
13-HPOT	(9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid

32.1 INTRODUCTION

Enzymes carry over specific biochemical reactions. In the application of microbial and plant enzymes to the food, laundry, and cosmetics industries, recombinant proteins, nanotechnologies, and gene engineering have made great advancements for the benefit of the consumer. Plant enzymes play an important role as biocatalysts in the production of useful compounds,

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00032-3

flavors, antimicrobials, antifeedants, and insecticides, brining a positive impact to various industries and applications. Enzymes are sought to possess desired activities under industrial conditions. The sequences of plant enzymes have been achieved by searching genomic libraries for homologues, or through protein engineering or mutagenesis to optimize process conditions. The use of mutated enzymes results in positive benefits on catalysis, selective and stereospecific products, etc. The use of recombinant enzymes may positively impact the quality of products and decrease the costs, obtaining the aims of waste reduction and avoiding the use of plant material that can be destined for food consumption. Lipoxygenase (LOX), the enzyme that starts the oxylipin synthesis pathway, catalyzes the site-specific oxygenation of linoleic (C18:2) and linolenic acids (C18:3), leading to 9- or 13-hydroperoxide derivatives. 9- and 13-hydroperoxides are converted into a vast array of products through the involvement of enzymes belonging to different branches of the oxylipins pathway downstream to LOX (Casey, 1999; Feussner and Wasternack, 2002).

LOXs catalyze the production of volatiles and flavors, pleasant or unpleasant, thus influencing the organoleptic properties of food products. LOXs perform cooxidation reactions, resulting in the bleaching of carotenoids and the oxidation of polyunsaturated fatty acids (PUFA) in aged nuts (Ciarmiello et al., 2013; Hughes et al., 2009).

Vegetable oils rich in linoleic and linolenic acids and soybean flour rich in the LOX enzyme have been used to produce the substrates for the synthesis of C6 and C9 aldehydes and alcohols as natural food additives (Casey and Hughes, 2004). The volatile aldehydes may reconstitute the flavor of food products that have been sterilized. On the other side, some C6 aldehydes are unpleasant. This is the case of the soybean, in which the *n*-hexanal (one of the C6 aldehydes produced by hydroperoxide lyase (HPL) catalysis) is the cause of the "grassy beany" unpleasant flavor. Green leaf volatiles (GLVs), with various aldeydes and alcohols, constitute the "fresh green" odor in fruits and vegetables. The exploitation of oxylipins as food additives has been contained because the enzymatic synthesis leads to low amounts and GLVs are unstable during storage. Industrially, only a few 6-carbon aldehyde and alcohols, that is, trans-2-hexenal, (*Z*)-Hex-3-enal, and cis-3-hexenol, are produced nonenzymatically for flavoring (Ashurst, 1991). The applicative uses of the synthesis of green leaf/fresh flavor producing short (C6, C9) aldehydes have led to the establishment of companies exploiting several patented methods (Arterburn et al., 2008, 2011; Brash et al., 2001; Brunerie, 1990; Christensen et al., 2008; Dangi, 2011; Goers et al., 1987; Holtz et al., 2001; Kanisawa and Itoh, 1988, Muller et al., 1996;) and protocols derived from LOX studies (Casey and Hughes, 2004; Fukushige and Hildebrand, 2005; Heshof et al., 2016; Ibdah et al., 2010). LOXs have been applied to bread making and flavor production (Whitehead et al., 1995); these enzymes may have negative effects such as decolorization, production of off-flavors, and changes in the antioxidant status of foods and nuts (Casey et al., 1996; Ciarmiello et al., 2013). In food applications, the use crude plant materials leads to the presence of different enzymes that may consume PUFA and the hydroperoxide products (Whitehead et al., 1995); the understanding of the LOX pathway allowed the understanding of the activities and properties of plant enzymes and the selective removal of specific downstream enzymes from plant extracts in the LOX pathway.

32.2 OXYLIPIN BIOSYNTHESIS PATHWAYS

Oxylipins are synthesized from α-linolenic acid (α-LeA) and linoleic acid (LA) by oxidative processes that are initiated by LOX leading to (10E,12E,15Z)-9-hydroperoxy-10,12,

15-octadecatrienoic acid (9-HPOT) or (9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) from linolenic acid (α-LeA), and (10*E*,12*E*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) or to (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD) from linoleic acid. (9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) produced from α-LeA is then metabolized to form (*Z*)-3-hexenal and 12-oxo-(*Z*)-9-dodecenoic acid (leading to the synthesis of traumatin) (Grechkin, 1998) (Figs. 32.1 and 32.2). The LOX pathway metabolism is complex in the leaves. First, triglycerides are hydrolyzed to free fatty acids (FFA) by lipases. Subsequently, LOX stereospecifically oxidizes α-LeA and linoleic acid. The products of these reactions can be isomerized, enzymatically or not, to form 12-oxo-(*E*)-9-dodecenoic acid and (*E*)-2-hexenal, hexanal, and nonenal isomers, transformed by alcohol dehydrogenase (ADH) to form the corresponding C_6- or C_9-alcohols.

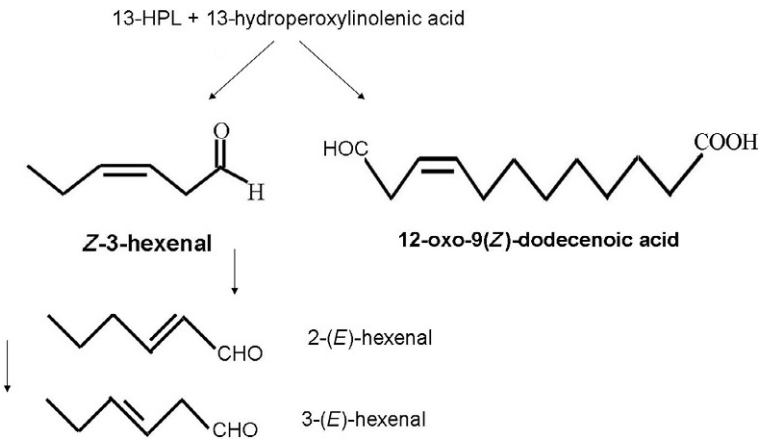

FIG. 32.1 Chemical structure of the products of 13-HPL, 12-oxo-9(*Z*)-dodecenoic acid and 3-(*Z*)-hexenal, and the isomers formed in downstream pathways.

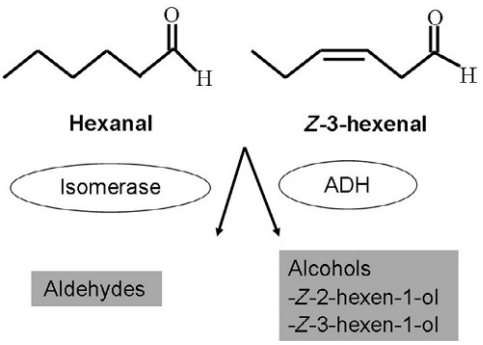

FIG. 32.2 Structure of products formed from Z-3-hexenal and from hexanal through alcohol dehydrogenase and isomerase activity.

32.2.1 Galactolipase-Mediated Release of C18 Polyunsaturated Fatty Acids

PUFA are substrates for LOXs. In cells, phospholipases release fatty acids from triacylglycerols. In potato "*deficient in anther dehiscence 1*" (*dad1*) mutants, a phospholipases A was identified as a flower-specific PLA1 phospholipase A (Bonaventure et al., 2011; Ellinger et al., 2010). Patatins are other lipases that may release fatty acids from triglycerides (Spelbrink et al., 2015). The release of α-LeA from galactolipids by phospholipase A1 (PLA_1), which has *sn-1* specificity, provides the substrate for the LOX pathway.

32.2.2 The Lipoxygenase Biosynthetic Pathway

LOX is an enzyme greatly studied in animals and plants. Plant LOX are key enzymes of the oxylipin synthesis pathway. LOXs (linoleate:O_2 oxidoreductase, EC 1.13.11) are nonheme, iron-containing, fatty acid dioxygenases, ubiquitous in animals, plants, fungi, and bacteria. LOX catalyzes the bioxygenation of PUFA, containing a cis, cis-1,4-pentadiene ring, leading to conjugated hydroperoxydienoic acids (Baysal and Demirdoven, 2007). The oxygenation of α-linolenic acid (α-LeA) and linoleic acid (LA) at carbon atom 9 or at C-13 leads to two derivatives, the (9*S*)-hydroperoxy- and the (13*S*)-hydroperoxy derivatives of C18 PUFA. Plant lipoxigenases have been classified as 9-LOX or 13-LOX, depending on the position and specificity of the oxygenation of C18 PUFA. 13-LOX, 9-LOX, and mixed type 9/13-LOX are at the base of the different pathways leading to the different oxylipins (Feussner et al., 1995a, 2001; Veldink et al., 1998; Wasternack and Hause, 2013).

The compartmentalization of LOX is also used for the differentiation in function. A first cellular localization is in lipid bodies (LBs) and associated with the microsomal fraction. LBs are rich in triglycerides, and the external phospholipid monolayer supports the LOX insertion in the membranes, favoring its activity. Plastidial LOX is localized in chloroplasts, where the products 13-HPOT and 13-HPOD may be directed to jasmonic acid production or to the synthesis of keto acids (Santino et al., 2010). The 13-LOX product 13-H(P)O(D/T)E is the S isomer. The autooxidation of PUFA can give rise nonenzymatically to the R enantiomer of hydroperoxy fatty acids. In water-stressed chickpea roots, an increase in the 13(S) hydroperoxy fatty acid (13-HPOD) was detected: the S-13-HPOD is the specific substrate for 13-allene oxide synthase (13-AOS) while the R enantiomer is produced by auto oxidation. In parallel, the 9-HPOD S enantiomer, the specific substrate for 9-HPL, was found to accumulate in the same 2-h period of stress treatment (Poltronieri et al., 2014). The fate of 9- or 13-hydroperoxy PUFAs is complex, as these compounds are metabolized by enzymes that in a few cases compete for the same substrate (Santino et al., 2010; Weichert et al., 2002). In the divergent pathways, the activity of LOX leads to the appearance of small C5, C6, and C9 volatiles that may characterize the crop or fruit, as in the tomato with the 13-LOX TomloxC (Shen et al., 2014). In the next subchapter, the divergent pathways involved in the synthesis of jasmonic acid (JA) and of short chain aldehydes and alcohols will be discussed.

32.3 PRODUCTION OF RECOMBINANT LOX ENZYMES AND MUTANTS WITH MODIFIED ACTIVITY

The oxygenation of α-linolenic acid (α-LeA) and linoleic acid (LA) at carbon atom 9 or at C-13 leads to two derivatives, the (9*S*)-hydroperoxy- and the (13*S*)-hydroperoxy derivatives

of C18 PUFA. Plant lipoxigenases have been classified as 9-LOX or 13-LOX, depending on the position and specificity of the oxygenation of C18 PUFA. Therefore Lox enzymes are classified as 13-LOX, 9-LOX, and mixed type 9/13-LOX. Hazelnut LOX1 and LOX2 form a mixture of 9- and 13-hydroperoxides in a 2:1 ratio; the same was found for LOX-3 in pea seed extracted from LBs and the microsome fraction (Hughes et al., 1998; Santino et al., 2003). High-performance liquid chromatography (HPLC) has been applied to the separation of the oxygenated products of LOX enzymatic activity. Peak absorbance at 234 nm allows the identification of the hydroxy-10,12-octadienoic acid (HODE). LOX enzymes have been overexpressed in *Escherichia coli* (Santino et al., 2003, 2005b) and in yeasts (Huang and Schwab, 2011) such as *Pichia* and *Saccharomyces cerevisiae* (Buchhaupt et al., 2012). Because thermal inactivation may affect the stability of the enzyme, various attempts to engineer the LOX have been performed (Anese and Sovrano, 2004). Single amino acid mutations have been tested to identify the residues affecting LOX activity (Hughes et al., 2001; Sloane et al., 1991; Sloane, 1996). In these studies, a 13-LOX was modified to catalyze the 9-LOX reaction by a single amino acid substitution (Hornung et al., 1999, 2000). Among plant LOX enzymes, only a potato tuber LOX was shown to convert arachidonic acid (AA), a C20 PUFA, to a mixture of 11-, 8- and 5-HETE in a ratio of 1:1:2. Feussner and Kuhn performed a single amino acid mutation based on the Borngraber determinant of the cucumber LB LOX ($V_{542}F$) (Borngraber et al., 1996, 1999). By site-directed mutagenesis, Feussner and Kuhn created the $V_{576}F$ LOX mutant (by alignment with F_{353} of the rabbit 15-LOX), resulting in a highly specific pattern of oxygenated products deriving from AA (Feussner and Kűhn, 2000). The presence of bulky phenylalanine in concert with H_{608} led to a $V_{542}F$ mutant enzyme with a new positional specificity, converting α-LeA in (6*S*,7*E*,9*Z*,12*Z*)-6-hydroperoxy-7,9,12-octadecatrienoic acid.

A scheme of the amino acids presumably involved in LOX specificity is presented in Table 32.1.

TABLE 32.1 Amino Acids Supposed to Determine Positional Specificity of LOX in Plants

	Accession Number	Based on Sloane et al. (1991) and Sloane (1996)	Based on Borngraber et al. (1996)
13-LOX			
Cucumber lipid body LOX	X92890	$Thr_{607}His_{608}$	Val_{542}
Soybean seed LOX-1	P08170	$Thr_{556}Phe_{557}$	Ser_{491}
Potato LOX-H1	X96405	$Ser_{614}Phe_{615}$	Ser_{551}
Arabidopsis LOX-2	P38418	$Cys_{611}Phe_{612}$	Ala_{548}
9-LOX			
Potato tuber LOX	P37831	$Thr_{579}Val_{580}$	Val_{514}
Tobacco elicitor-ind. LOX	X84040	$Thr_{580}Val_{581}$	Ser_{515}
Arabidopsis LOX-1	Q06327	$Thr_{577}Val_{578}$	Ser_{522}
Barley grain LOX-A	L35931	$Thr_{574}Val_{575}$	Ser_{511}

Because LOX substrates are hydrophobic, methods to prepare a reaction mixture consist of emulsifiers as well as high molecular structures, uni- and/or multilaminar micelles, or liposomes (Kermasha et al., 2002). Various detergents have been used for LOX assays. By altering the physicochemical state of the fatty acid substrate, researchers showed a change of the positional specificity of the soybean LOX-1 (Began et al., 1999). Using Tween 20-solubilized LA, LOX-1 produced the (13*S*)-hydroperoxy derivative at alkaline pH (Gardner, 1989, 1997). Using polyenoic fatty acids included into phosphatidylcholine micelles with the hydrophobic tail groups facing to the inside, scientists showed that the fatty acids were optimum substrates for soybean LOX-1. When LA was inserted into phosphatidylcholine micelles with the hydrophobic tail groups facing the inside, LOX-1 synthesized exclusively the (9*S*)-hydroperoxy- or (5*S*)-hydroperoxy derivative. Thus, LOX-1 activity was dependent on the orientation of the LA inserted in the micelle. The enzyme eliminates stereospecifically a hydrogen atom from the methylene ring in PUFA in a reaction in concert with the reduction of iron to Fe(II). The enzyme-alkyl radical complex is thus oxidized by O_2 to form an enzyme-peroxy radical complex, followed by the transfer of electrons from the ferrous atom to the peroxy group. Protonation and dissociation from the enzyme are the steps leading to the formation of the hydroperoxide (Baysal and Demirdoven, 2007; Liavonchanka and Feussner, 2006). LOX has been extensively characterized in pea seeds (Szymanowska et al., 2009), melon (Tang et al., 2015), and cucumber (Weichert et al., 2002). LOX activity is regiospecific on PUFA substrates possessing a 1,4-pentadiene motif, such as linoleic or linolenic acids. The amino acid determinants of LOX specificity have been identified, leading to the conversion of a cucumber 13-LOX in a 9-LOX by a single amino acid substitution (Hornung et al., 1999). In the 13-LOX, the positions aligning with histidine 608 of LOX are occupied by phenylalanine or histidine. In contrast, valine is located at this position in all plant 9-LOXs. The H_{608} was found to be the primary determinant of positional specificity in cucumber 13-LOX. The substitution of this amino acid by valine, a less bulky amino acid, allowed the change of the positional specificity of the 13-LOX into a 9-LOX. The $H_{608}V$ substitution probably unmasked a positively charged arginine, R_{758}, altering the substrate configuration and favoring the formation of the 9-HPODE. The $H_{608}V$ substitution allowed an inverse orientation head-to-tail of the PUFA. Trilinolein, devoid of free carboxylic groups, is oxygenated by the wild-type enzyme and by the $H_{608}V$ mutant to the same (13*S*)-hydro(pero)xy derivative. Hughes et al. (2001) produced various mutated LOX proteins to study the linoleate-binding binding to the enzyme. They pointed to a possible role of these amino acids in pea 9/13-LOX. The mutation of pea 9/13-LOX in the residue equivalent to that of cucumber 13/9-LOX with dual positional specificity resulted in a negative effect: they concluded that the mechanisms controlling positional specificity in pea 9/13-LOX were different. They studied also the modeling of pea 13/9-LOX. Dual positional specificity in pea LOXs is probably determined by the degree of penetration of the methyl terminus of linoleate and by the volume of the linoleate-binding pocket, while in the cucumber the substrate orientation is the principal determinant (Heshof et al., 2016).

32.4 THE DIVERGENCE OF PATHWAYS LEADING TO VARIOUS OXYLIPINS

The hydroperoxy fatty acids are metabolized via several pathways (Feussner and Wasternack, 2002). The three main pathways are directed through three enzymes: AOS, HPL, and divinyl ether synthase (DES). Hydroperoxides are converted by DES to produce antifungal divinyl ethers such as colneleic and colnelenic acid.

The DES, AOS, and HPL enzymes are structurally related, heme-containing proteins. These enzymes form an atypical group of cytochrome P450 enzymes (CYP74) in a reaction that does not require O_2 or NAD(P)H. These enzymes use a carbocation intermediate, leading to different final products (Hughes et al., 2009).

32.4.1 JA Synthesis Pathway

Oxylipins synthesis is dependent on the specificities and compartmentalization of the enzymes in the pathway. The first part of the jasmonate (JA) pathway, for instance, which requires the sequential action of LOXs, AOSs, and allene oxide cyclases (AOCs), occurs in the plastids (Feussner et al., 1995b), where LOX-13, AOS, AOC, and 12-oxo-phytodienoic acid (OPDA) reductase (OPR) are involved in Jasmonic acid production. OPDA is finally converted into the biologically active jasmonate in a separate subcellular compartment—the peroxisome (Hughes et al., 2009; Wasternack and Hause, 2013). The AOS pathway leads to the formation of unstable allene oxide intermediates that are either hydrolyzed nonenzymatically to yield α- or γ-ketols, or metabolized to jasmonic acid (JA) (Brash, 2009; Stumpe et al., 2006; Wasternack and Hause, 2013), with a role in plant defense and in response to abiotic stress (Santino et al., 2010).

32.4.2 HPL Branch of the LOX Pathway in Plastids

13-HPL CYP74B and 9/13-HPL CYP74C enzymes contribute to two different pathways, the first in the production of colneleic/colnelenic acids and the second in the synthesis of six and nine carbon aldehydes and GLVs, respectively (Matsui et al., 2000; Matsui, 2006). LOXs, AOS, and HPL show a close association at the level of the grana thylakoids, where the enzymes required for OPDA biosynthesis appear to be closely connected. The 13-HPL thus competes with AOS for the substrate 13-PODE. 13-Hydroperoxide availability is an important control point in the biosynthesis of different classes of oxylipins. Many HPLs are membrane-bound enzymes, except the watermelon HPL (Fukushige and Hildebrand, 2005), especially the 13-HPLs, located in chloroplast membranes (Hughes et al., 2009; Santino et al., 2010). HPL is constituted by several monomers of 55 kDa. HPLs are very susceptible to freezing and to detergents that denature the binding to the heme; they also need cryoprotectants for conservation, such as cysteine, glycerol, and salts (Gigot et al., 2010; Rabetafika et al., 2008; Rasool and Mohamed, 2016; Rodrigo et al., 2007). The pH at which HPL exerts its activity is higher than 6 and the working temperature is around 30°C. Biochemical characterization of recombinant HPLs revealed that, as in the case of AOSs, some HPLs show a strict specificity for 13-hydroperoxides (CYP74B subgroup) with consequent production of (Z)-hex-3-enal and (Z)-12-oxododec-9-enoic acid from 13-HPOT.

32.4.3 9/13-HPL (CYP74C) in the Synthesis of Green Leaf Volatiles

Some HPLs metabolize both 9- and 13-hydroperoxides. The CYP74C subgroup of CYP74 enzymes is represented by 9/13-HPLs of numerous plant species. These HPLs produce short-chain aldehydes (C6 or C9) and the corresponding C12 or C9 oxoacids. The volatile aldehydes confer the aroma to flowers and fruits. 9/13-HPLs have been identified in many crops

relevant to human consumption, such as cucumbers (Hornostaj and Robinson, 1999; Wan et al., 2013), tea (Ono et al., 2016), grapes (Qian et al., 2016; Zhu et al., 2012), and apples (Espino-Díaz et al., 2016).

9/13-HPLs forming C_9-aldehydes, (3*Z*)-nonenal, and (3*Z*,6*Z*)-nonadienal, were found in the *Cucurbitaceae* family, but they occur in plant species such as *Medicago* spp., rice, and almonds. The 9/13-HPLs use as a substrate both 9- and 13-hydroperoxides. Starting from 9-HPOT, (*E*,*Z*)-nona-2,6-dienal and C_9-oxo-acids are produced. Differently from 13-HPLs that are expressed in leaves and green tissues, 9/13-HPLs are also expressed in roots, as in rice (OsHPL1) and in *Medicago truncatula* (MtHPLF) (De Domenico et al., 2007). The 9/13-HPL co-localizes with 9-LOX in the microsomal fraction. 9/13-HPLs produce short-chain aldehydes and keto fatty acids (C12- or C9-ω keto compounds). Even though localization in microsomes was established for 9-LOXs, different compartmentalization was also shown, such as the association with LBs (De Domenico et al., 2007). The colocalization of 9-LOXs and of 9/13-HPL was shown for the first time for a 9-HPL from almonds (Mita et al., 2005): the 9-HPL was present in the LBs fraction when expressed in tobacco protoplasts (De Domenico et al., 2007). Also the cucumber 9/13-HPL was found localized in LBs (unpublished data), as well as *M. truncatula* 9/13-HPL. The compartmentalization of 9-HPLs to LBs, similarly to 9-LOXs, ensures insertion into the phospholipid bilayer and the availability of glycerophospholipids to provide linoleic and linolenic acid substrates and their derivatives. For studies of the subcellular localization, plant 9/13-HPLs were YFP-tagged. *M. truncatula* 9/13-HPL (HPLF) was detected in microsomes and lipid droplets (LD) (De Domenico et al., 2007). The association with LD or LBs resulted in an active enzyme. The activation of CYP74C3 associated with LBs was found comparable to that obtained with synthetic detergent micelles (Hughes et al., 2006). The localization of LOX and HPL in this compartment therefore has biological relevance. The association of a CYP74 monomer with membrane phospholipids in an active conformation means that the active enzyme is physically bound to a membrane anchor. However, the physicochemical characteristics of protein binding to membrane phospholipids and to a detergent micelle are slightly different (Gigot et al., 2010). The surface hydrophobic nature of CYP74 enzymes plays a role in the positioning of protein, providing the exact orientation for substrate binding. It may also serve to localize CYP74 enzymes through membrane attachment to a specific cellular compartment, where substrate triacylglycerols rich in PUFA are concentrated.

32.4.4 Engineering of HPLs

Purified active HPLs were analyzed for studying the secondary structure, using differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy. HPLs were shown to possess α-helix, 13%, and β-sheet structure, 29%, random coil, 53%, and 5% turns (Santiago-Gómez et al., 2010). Recombinant HLPs were produced in various systems, such as *E. coli* and yeasts (*S. cerevisiae*, *Yarrowia lipolytica*, *Pichia* spp.) (Atwal et al., 2005; Bourel et al., 2004; Buchhaupt et al., 2012; Huang and Schwab, 2011, 2012; Jacopini et al., 2016a, b; Noordermeer et al., 2000, 2002; Santiago-Gómez et al., 2010; Tijet et al., 2000, 2001). Several HPL mutants have been characterized. Two active site residues were identified that can switch AOS to HPL catalytic activity (Grechkin, 1998). The first one, Phe_{137}, is aligned with the conjugated diene of the hydroperoxy fatty acid. Site-directed mutagenesis of Leu_{137} allowed the transformation

of AOS into HPL; the HPL activity led to the formation of GLV aldehydes (Toporkova et al., 2008). Bioinformatics analyses allowed the identification of determinants of activity of CYP74 family enzymes. Two mutants, $F_{295}I$ and $S_{297}A$ of the tomato allene oxide synthase (LeAOS3), were obtained by site-directed mutagenesis. The mutants showed enzyme activity of HPL using the natural 9*S*-hydroperoxy-linoleic acid substrate; the original AOS activity was not detectable ($F_{295}I$) or highly reduced ($S_{297}A$). Both sites 295 and 297 localized within the "I-helix central domain" ("oxygen binding domain") were shown to determine the CYP74 type of catalysis (Brash, 2009). These amino acids are located on Asn residue (Asn_{296} in the tomato AOS enzyme). The $Phe_{295}I$ and $Ser_{297}A$ mutations both switched AOS to HPL catalytic activity (Toporkova et al., 2008). A 13-HPL from guava was studied by gene shuffling and directed evolution. The random mutagenesis process improved the functionality of the recombinant enzyme expressed in *E. coli*, obtaining a 15-fold increase in yield (Brühlmann et al., 2013). The product yield factor is due to a higher total turnover number of the enzyme, to higher solubility, and to an increase in heme content. Mutated amino acids were found on the surface of the protein, not in the active site. Finally, recently researchers have found that CYP74 enzymes are present also in brown algae, with epoxyalcohol synthase activity (Toporkova et al., 2017), pointing to a general role of CYP74 enzymes in plants in the synthesis of oxylipins.

32.4.5 Applications of Volatile Short-Chain Green Leaf Volatiles in the Food Industry

GLVs are commonly used as flavors to confer a fresh green odor to vegetable or fruit food products. This mixture of volatile compounds leads to characteristic plant flavors called the green notes (Ibdah et al., 2010). These molecules confer freshness and authenticity to food products, thus attracting the interest of the world market of flavors and the food industry. Several groups have developed biocatalytic production protocols and methods to produce GLVs and the requests for natural flavors. Vegetable oils rich in PUFA can be converted by plant LOX and HPL into natural GLVs. Using plant enzymes, the HPL step of bioconversion is the bottleneck of the reaction because HPLs are unstable and can be inhibited by their substrates (Gigot et al., 2010). Therefore, there is a need to find suitable hosts, yeasts (*Pichia pastoris*, *P. anomala*, *Y. lipolytica*), and fungi (Hall et al., 2007) for the production and subsequent storage in an active form of recombinant HPLs; these goals have been reached by several researchers. The combined action of the enzymes lipase, LOX, and HPL convert PUFA substrates such as C18:2 and C18:3 fatty acids into short-chain volatiles. The reaction produces the volatile aliphatic 6- to 9 carbon including cis-3-hexenol (6C) (6C, leaf alcohol) and trans-2-hexanal (6C, leaf aldehyde). The C6 or C9- alcohols are produced by yeast alcohol dehydrogenase ADH, adding an enzyme to the reaction mix (Gargouri et al., 2004). The aldehydes play a role of defense against insects and pathogens, either as signal molecules or due to their antiinsect and antifungal activity. C6 and C9 aldehydes are synthesized from 13- and 9-hydroperoxides. (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal are obtained from 9-hydroperoxy derivatives of linoleic and linolenic acids, whereas hexanal and (2*E*)-hexenal originate from 13-hydroperoxy derivatives. Both C6 and C9 aldehydes can be further metabolized in vivo by alcohol dehydrogenase to form the corresponding alcohols. Volatile aldehydes contributing to the characteristic flavors of vegetables and fruits are used as food additives in the agrofood industry to restore food flavor after industrial processing of foods (Casey and Hughes, 2004;

Prost et al., 2005; Santino et al., 2005a, b). Some C6 aldehydes are perceived as unpleasant. In soybeans and other legumes, LOX and HPL activity produces *n*-hexanal, responsible for the unpleasant "grassy beany" flavor (Lei and Boatright, 2008; Mellor et al., 2010). Biotech applications of CYP74 enzymes are in constant evolution and development. A factor that has limited CYP74 enzyme biotechnological applications is the difficulty in making available the enzymes at an industrial level. This is important in particular for HPLs. CYP74 enzymes are present in plants at extremely low levels. Therefore, recombinant enzymes produced in expression systems (such as in *Pichia*) have been recently developed to produce recombinant CYP74 enzymes. CYP74 enzymes are unstable after purification, as are their products (oxylipins). However, (*Z*)-Hex-3-enal production is produced and marketed by companies through chemical synthesis, a product that needs to be labeled as "synthetic." The specific isomers enzymatically produced are therefore a rich source of structural diversity for the flavor industry (Kunishima et al., 2016). Starting from vegetable oils rich in linoleic and linolenic acids, the action of LOX and CYP74C3 enzymes converts the hydroperoxides into a vast array of C6 and C9 compounds, having flavors of "fatty green fruity," "leafy green fruity," and "freshly mowed grass." Other volatile products produced by the HPL reaction are similar to the smell of old leather and cucumber, with aromas of watermelons and apples. These natural compounds are of high commercial value. HPLs offer the potential to catalyze and produce a vast array of flavors in combination with other naturally occurring enzymatic processes or by directed evolution of the enzyme sequence.

32.4.6 Stable, Dried CYP74 Enzymes

Present recombinant HPL preparations, although being improved, still incur stability problems. Often fresh crude extracts have been used, and this translated into problems in production, stability, and storage. HPL production methods have been optimized to the production of potentially gram quantities of purified recombinant CYP74 enzymes (Casey and Hughes, 2004). Extensive stability and enzyme activity tests have been carried out to identify conditions for storage and stabilization in a dried, detergent-free state at an optimized state. These studies have been focused either on an HPL from *M. truncatula* and on CYP74D4, AOS from *A. thaliana*. Ultraviolet/visible spectra of detergent-free preparations of CYP74C3, CYP74D4 and CYP74B1 (pepper 13-HPL) have been acquired. The studies were performed on enzymes before and after freeze drying (Casey and Hughes, 2004). Measurements of activity allowed the quantification of the loss of active proteins due to freeze drying (Heshof et al., 2016; Ibdah et al., 2010). Engineered bacteria and other production systems have been grown in a medium supplemented with 10% PUFA rich oil to produce green note compounds. Bacteria transformed with the *NX6125* gene degraded linoleic- and linolenic acid into leaf aldehydes and alcohols. In particular, Ibdah et al. (2010) described a new method for the processing and recovery of GLVs that were purified using an Amberlite XAD-4 adsorbent resin, followed by elution with ethanol. New solutions to provide HPL during the entire year have been developed and implemented. The techniques for enzyme extraction and storage require optimization to prevent and reduce to a minimum the loss in enzyme activity. HPLs were stabilized by the addition of chemical compounds, including salt, sugars, and polyols. Several authors have already used chemicals such as dithiothreitol or glycerol to prevent HPL degradation during extraction and storage. KCl prevents HPL denaturation

during long-term storage or lyophilization, and glycine is one of the best additives to keep high enzyme activity and improve stability. These studies have shown that the use of detergents has to be avoided; detergent-free freeze drying improved the storage and stability of the HPL proteins, and this result has been related to the preservation of the heme binding. Detergent-free HPL extracts maintain the heme group and preserve the enzyme stability, even for weeks or months (Gigot et al., 2010).

32.4.7 Encapsulation of Enzymes and Their Use in the Industry

Enzymes are catalytic proteins and are highly liable to physiological conditions such as pH, temperature, and chemicals such as denaturation agents. Due to their sensitivity, enzymes have been modified and encapsulated or immobilized. Industrial processes use these preparations (lipases, proteases, LOXs) as food supplements, pharmaceutical products, hygiene products, etc.

The methods for immobilization of enzymes for food application are under continuous development. Immobilization involves various techniques designed to protect the enzymes using a physical support. Physical (adsorption, entrapment, or membrane confinement) and chemical methods (covalent bonds or cross-linking) are possible opportunities and technologies to immobilize the enzymes for industrial applications. Various methodologies have been applied to make enzymes more stable and active during the time of use, such as immobilization, encapsulation, spray drying, and granulation (fluid bed coating, fluid bed agglomeration, alone or in combination with spray drying and gel entrapment (Mateo et al., 2007; Prado Barragàn et al., 2015).

32.4.8 Emulsions and Double Emulsions

Emulsions are systems to solubilize proteins containing hydrophobic domains offering the advantage of spray drying using encapsulants (lactose, caseinate) for long-term storage. Protein and polysaccharide-based hydrophilic emulsifiers, such as whey proteins, have been used as emulsifiers. Protein-based emulsifiers have shown the ability to increase emulsion stability, especially if used with low molecular weight emulsifiers (De Lorenzis et al., 2008; Lamba et al., 2015; O'Regan and Mulvihill, 2010). Among the techniques to prepare emulsions are "one-step" and "two-step" techniques. The first one involves heating of a nonionic emulsifier in a mixture of different emulsifiers, causing phase inversion that leads to the formation of double emulsion (De Lorenzis et al., 2008). In the "two-step" emulsification process, the primary emulsion is based on a low hydrophilic-lipophilic balance (HLB) surfactant in the case of water/oil/water (W/O/W) emulsion and a high HLB surfactant in the case of oil/water/oil (O/W/O) emulsion. A stable emulsion is reached when no observable changes over time can be observed in the size distribution of droplets, the state of aggregation, and the spatial arrangement. It was defined in terms of encapsulation efficiency, encapsulation stability, and sedimentation stability. Flavor extracts from plants are labile and highly sensitive to degradation by environment factors such as oxygen or light. Orange oil encapsulated in O/W/O's inner oil phase was shown to be more stable, with a reduced release of volatiles and protection from atmospheric conditions (Cho and Park, 2003).

32.4.9 Liposomes and Reconstituted Lipid Bodies

Liposomes are used to encapsulate molecules and enzymes (Qu et al., 2010). The encapsulation efficiency of enzymes relies on the preparation method. Still, there are bottlenecks to be solved, such as the difficulty of entrapment of large enzymes and the reduction of protein interaction and aggregation. Liposomes are relatively stable in liquid systems. However, a partial loss and leakage from liposomes reduce the enzyme content during the product's shelf life. Recently, Bonsegna et al. (2011) showed that plant LBs can be assembled in the laboratory to carry and deliver hydrophobic compounds and lipophilic bioactives. The natural LOX and HPL compartmentalization within LBs (De Domenico et al., 2007) and the possibility to reconstitute in vitro the LB structure (De Domenico et al., 2011) with a defined composition of proteins and triglycerides make these delivery systems highly attractive as factories for the production of GLVs. LBs reconstituted with the LOX/HPL complex are suitable for the production of GLVs and fresh fruit flavors. Furthermore, in the filed of natural antifungine compounds, antifeedants, and preserving agents, these systems may be used to produce hexanal, *n*-decyl aldehydes, hexenyl acetate with antifungal activity (Santino et al., 2005a), and (2*E*)-hexenal, (2*E*,6*Z*) nonadienal, and (2*E*)-nonenal as antimite compounds (Hubert et al., 2008).

32.5 CONCLUSIONS

The oxylipins biosynthesis pathways are a complex system that allows plant to produce a large set of bioactive compounds, either in whole plants or in reconstituted systems. In the coming years, C6 and C9 aldehydes will be more and more applied for their nature of natural products in the food industry and for the preservation of foods. The application of up-to-date biotechnology is providing tools and methods for enzyme improvement and the development of new, specific mutants adapted to the lipophilic environment such as encapsulation into LBs and liposomes.

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CHAPTER

33

Role of Soil Enzymes in Sustainable Crop Production

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33.1 INTRODUCTION

Soil quality is a key factor for the growth of crop plants and the deciding factor for the availability of plant nutrients. There is a need to enhance the food grain production by 280 million tons to fulfill the food demands of the growing population by 2020 (Singh et al., 2016). The use of chemical fertilizers since the green revolution has enhanced food grain production with a quantum jump (Dotaniya, 2013; Dotaniya et al., 2014; Dotaniya and Kushwah, 2013). However, this pace of grain production cannot be sustained for long due to overmining nutrients from the soil other than those supplied through fertilizers, the depletion of soil organic matter, and the changing climatic factors (Meena et al., 2016a, b). The partial factor productivity is declining with time accompanied by an increase in the fertilizer requirement for the same production level (Dotaniya et al., 2013c). Farmers are often misled with the myth that the application of overdoses of fertilizers and pesticides enhance crop yields proportionally, thereby further decreasing the soil biological quality. The decline rate of soil organic matter reduces the nutrient transformation rate and the availability of plant nutrients in the soil solution (Angers and Eriksen-Hamel, 2008; Dotaniya, 2012; Ghosh and Bhardwaj, 2002; Jha et al., 2012; Kushwah et al., 2014; Lakaria et al., 2012; Omar et al., 2017; Varadachari and Ghosh, 1984). In sandy soils, volatilization and the leaching loss of nitrogenous fertilizers has increased and needs a higher amount of N fertilizers or a split application (Mohanty and Singh, 2002; Saka et al., 2017). The contamination of groundwater with pesticide and fertilizer residue may also be a problem in many parts of the world. Sometimes, overapplication of fertilizers such as phosphatic accumulates in the fields and is not taken up by the

https://doi.org/10.1016/B978-0-12-813280-7.00033-5

plant through physiological mechanisms (Dotaniya et al., 2013d). In all cases, modifications in the application rate, time, and climatic conditions vis-a-vis plant root exudates in the soil for sustainable crop production is a prime need for today's agriculture. Plant nutrient availability is affected by the physical, chemical, and biological properties of soil (Dotaniya et al., 2013a,b,c; Dotaniya and Meena, 2013a,b; Verma et al., 2017a,b). The nutrients in the soil may be present in many chemical forms but the plants can take up only selective forms of the nutrients, which are termed as available forms. For example, nitrogen is taken up by the plants predominantly as nitrates whereas ammonical nitrogen may complement in cases of nitrogen deficiency. Phosphorus is taken up in the form of orthophosphates, the availability of which is greatly influenced by the tendency to get converted to insoluble forms. Soil organic matter acts as a reservoir of plant nutrients that should be released or transformed to available forms for plant uptake. Microflora are the key drivers of nutrient transformations in soil as they mineralize the nutrients as the result of their growth and activity, which can be used by the plants. The mineralization and transformation of nutrients are brought about by the microbial enzymes, which may be extracellular secreted enzymes or endogenous enzymes of microbial cells. The extracellular enzymes are secreted into the soil environment where they either decompose or transform the organic and inorganic nutrient forms to yield available forms (Dotaniya et al., 2017; Pankhurst et al., 1995). These enzymes are collectively called soil enzymes and are vital for maintaining soil fertility and soil health as well as protecting the environment by degrading pollutant molecules (Gianfreda and Rao, 2014; Stirling et al., 2017). The composition and quantity of active soil enzymes and the microbial community itself, at a given point in time, dictate the nutrient availability and thus the health of soil.

Soil quality and soil health are the two terms used to describe the functional state of soil. They describe the soil's capability to sustain crop productivity, maintain environmental quality, and support good plant, animal, and human health. The term "biological soil quality" may be used to indicate the biological function of soil with respect to plant growth and crop productivity (Doran and Zeiss, 2000). "Soil health" is used in an ecological sense and portrays soil as a living and dynamic system whose functions are mediated by living organisms and their interactions with one another, such as a symbiosis or disease. Usage of the term "soil health" emphasizes that the soil ecosystem requires maintenance and conservation to sustain the biodiversity and biological activity present within it (Allen et al., 2011; Doran and Zeiss, 2000). The Soil Science Society of America defined soil quality as, "The capacity of a specific kind of soil to function within natural or managed ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health" (Herrick, 2000). It is sensitive to management practices and thus can be improved by carefully designing the management strategies (Dotaniya and Datta, 2014; Gianfreda and Rao, 2014; Moebius et al., 2007).

33.2 ROLE OF SOIL ENZYMES IN NUTRIENT AVAILABILITY

The major plant nutrients that make up the structural and functional molecules of the plant body are C, H, O, N, P, and K. Secondary nutrients such as Ca, Mg, and S and micronutrients such as B, Cl, Cu, Fe, Mn, Mo, and Zn are also important for plant growth, although in smaller amounts. Among these, C, H, and O are derived from CO_2 and H_2O through photosynthesis and soil uptake, respectively. Other nutrients are acquired in mineral form from the soil. Soil

enzymes play a vital role in initiating and maintaining the biogeochemical cycles of these nutrients and provide the basic support of fertility for the healthy development of plants (Fang et al., 2010). Soil enzymes increase the rate of organic crop residue decomposition by facilitating the contact area of the reactants, for example, the substrate and water during hydrolysis, and release the plant nutrients into the soil solution (Dotaniya et al., 2017; Pankhurst et al., 1995). Rhizosphere, the soil zone in contact with the root and influenced by the root activities or root exudates, is a unique environment where the microorganisms are selectively enriched. As a result, the enzyme activities are more dominant in the rhizosphere zone compared to the bulk soil mediating the biogeochemistry of minerals, and they nourish the soil-plant ecosystems in a better way (Gobran et al., 1998). The important soil enzymes include those involved in C, N, P, and S cycling. The enzymes involved in C cycling may not directly provide nutrients for the plant growth but are necessary for the proliferation of soil microorganisms that promote plant growth by other means. They degrade complex organic carbon compounds to release simple utilizable C compounds such as sugars, organic acids, etc. The enzymes of N, P, and S cycles mineralize inorganic compounds of respective nutrients from the soil organic compounds, which can be utilized by both microorganisms and plants. Some of the various soil enzymes involved in nutrient transformation are presented in Table 33.1.

The composition of enzymes, their activities, and their persistence in soil are influenced by the physical properties, that is, pH, EC, soil texture, and structure as these factors that control the availability of nutrients for microorganisms. Thereby, their activity is reflected in microbial respiration (Fang et al., 2010; Sinsabaugh et al., 2005) and microbial diversity. Sandy soils are more aerated than clay soils, but the low organic matter content limits the growth of microorganisms. Such soils are characterized by low microbial population density and diversity. In saline and alkaline, high pH soils (Tabatabai, 1994), the production of soil enzymes from microorganisms involved in the utilization of plant residues (Saiya-Cork et al., 2002) and other substrates (Castellano and Dick, 1991; Das and Verma, 2011; Fang et al., 2010; Gianfreda and Rao, 2014; Sinsabaugh et al., 2005) is greatly reduced. Similarly, soils degraded due to extensive chemical crop management practices are also poor in soil fertility and have reduced crop production potential (Dotaniya et al., 2016). The addition of FYM, organic manure, crop residue, biofertilizers in the soil, and the reclamation of degraded soil with particular amendments, that is, gypsum in alkaline soils, lime in acidic soil, and FYM in heavy metal-contaminated soils, enhance the microbial count and soil fertility (Gianfreda and Rao, 2014).

33.3 MECHANISM OF ENZYME ACTION IN SOIL

Crop residues incorporated into soil are mineralized by the soil biota to form plant nutrients required for plant growth that can be easily taken up by the plants (Dotaniya et al., 2015; Meena et al., 2016b). Soil microorganisms accelerate the decomposition rate by producing enzymes and influencing the plant nutrient kinetics in soil. Root rhizosphere provides large amounts of low molecular weight organic acids, which act as a carbon source for microbes and show a priming effect on enzyme production, leading to an accelerated release of plant nutrients in the form of inorganic ions (Dotaniya and Meena, 2013a, b; Dotaniya et al., 2014; Meena et al., 2017). The released inorganic ions act as a chelating agent and make a temporary complex with other plant nutrients as well as enzymes (Dotaniya et al., 2013a,b,d). The complex then breaks down and releases the enzymes and available forms of plant nutrients in the

TABLE 33.1 Soil Enzymes Involved in Nutrient Dynamics

Enzyme	Organic Matter Substances Acted On	End Product	Biological Significance	Predictor of Soil Function
Enzyme for H transfer to a variety of substrates				
Dehydrogenase	H donating and accepting species (CO_2, organic acids, alcohols, etc.)	Oxidized or reduced products	Microbial electron transport system, proton release	General index of microbial activity
Enzyme for decomposition of C substrates				
Amylase			Release of carbon compounds required for growth of microbes	
α-Amylase	Starch	Dextrins		Organic matter (OM) decomposition
β-Amylase	Starch	Maltose		OM decomposition
Maltase	Maltose	Glucose		OM decomposition
Cellulase				
Endocellulase	Cellulose	Cellodextrins		OM decomposition
Exocellulase	Cellulose	Cellobiose, cellotriose		OM decomposition
β-Glucosidase	Cellobiose, cellotriose	Glucose		General index of microbial activity
Pectinase	Pectin	Oligosaccharides		OM decomposition
Lignases				
Lignin peroxidase	Lignin	Partially depolymerized lignin		OM decomposition
Manganese peroxidase	Lignin	Partially depolymerized lignin		OM decomposition
Laccase	Lignin	Mixture of aliphatic and aromatic polymers		OM decomposition
Enzyme for decomposition of N substrates				
Protease	Protein	Amino acids	N source for soil microbes	Nutrient cycling
Deaminase or ammonia lyase	Amino acids	Ammonia + organic acids	N nutrition of plants and soil microbes	
Nitrate reductase	Nitrates	Ammonia		
Amidase	N-C bonds of nonproteinaceous organic N compounds	Ammonium (NH_4)		
Urease	Urea	Ammonia and carbon dioxide		

TABLE 33.1 Soil Enzymes Involved in Nutrient Dynamics—cont'd

Enzyme	Organic Matter Substances Acted On	End Product	Biological Significance	Predictor of Soil Function
Enzyme for decomposition of P substrates				
Acid phosphomonoesterase	Organic phosphorus compounds	Orthophosphate	P source for plants and soil microbes	Nutrient cycling
Alkaline phosphomonoesterase	Organic phosphorus compounds	Orthophosphate		
Phosphodiesterases	Nucleic acids and other organic P compounds with phosphate interlinks	Orthophosphate		
Phytases	Phytin	Orthophosphate		
Enzyme for decomposition of S substrates				
Sulfatase	Organic S compounds like S containing amino acids	Sulfate	S source for plants and soil microbes	Nutrient cycling

Adopted from Das, S.K., Verma, A., 2011. Role of soil enzymes in maintaining soil health. In: Shukla, G., Verma, A. (Eds.), Soil Enzymology, vol. 1, Springer International, doi: 10.1007/978-3-642-14225-3_2; Gianfreda, L., Rao, M.A., 2014. Enzymes in Agricultural Sciences. OMICS Group eBooks, USA.

root zone (Fig. 33.1). Some of the soil enzymes degrade the toxic compounds into nontoxic compounds or reduce the uptake of toxic metals by plant roots by chelating them into complexes (Gianfreda and Bollag, 1996; Gianfreda and Rao, 2014; Kizilkaya and Bayrakli, 2005).

The compounds on which soil enzymes act are known as their substrates (Das and Verma, 2011). For example, soil β-glucosidase cleaves the glucose molecule from glucose oligosaccharides (commonly di and trisaccharides) with β (1→4) glycosidic bonds such as cellobioses, cellotrioses, cellodextrins, etc. that are present in the plant residues. Every enzyme is specific to a particular substrate or group of similar substrates that fit into the active reaction site of the enzyme under defined optimum reaction conditions, forming an enzyme-substrate complex (Das and Verma, 2011; Gianfreda and Rao, 2014; Michaelis and Menten, 1913). In the complex, the enzyme catalyzes the reaction and dissociates from the products in the soil solution. The enzyme is then free to bind with another substrate molecule and catalyze the reaction to form products. During the course of reaction, from the initial complex to the final release of product, the enzyme undergoes a series of conformational changes form product enzyme in the native state. The enzymes get adsorbed onto the clay surfaces and remain active for extended periods as they are protected from environmental factors, for example, protection photo-degradation (Tietjen and Wetzel, 2003).

where S is the substrate, E is the soil enzyme, ES is the intermediate enzyme-substrate unstable complex, P is the product of the reaction, and k_1, k_2 and k_3 the reaction rate constant.

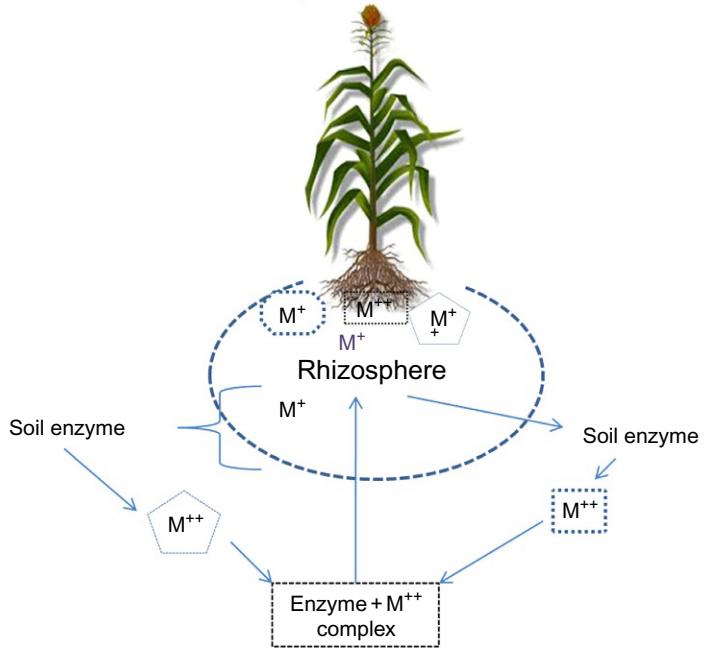

FIG. 33.1 Role of soil enzyme in plant nutrient dynamics.

33.4 SOIL ENZYMES AND THEIR FUNCTIONS

The important soil enzymes belong to the enzyme classes oxidoreductases, hydrolases, and lyases. The oxidoreductases include dehydrogenases specific for different substrates, laccases, and all enzymes involved in the oxidation of different substrates (e.g., those involved in oxidative degradation of toxic organic pollutants). The dehydrogenases and other enzymes involved in oxidation are cell-bound and do not exist as extracellular enzymes. Laccases are shown to exist extracellularly. The dominant class of extracellular enzymes found in the soils is hydrolases. They are the hydrolytic enzymes involved in the breakdown of macromolecules to yield smaller utilizable forms, the removal of inorganic groups or ions releasing the inorganic available forms, etc. They include amylases, cellulases, glucosidases, phosphoesterases, sulfatases, amidases, urease, etc. Lyases are enzymes that remove certain chemical species breaking covalent bonds without hydrolysis. Decarboxylases, dehydratases, ammonia lyases, etc., belong to this class of enzymes, with ammonia lyases being the most important ones. Ammonia lyases deaminate amino acids without hydrolysis; examples include phenylalanine lyase and tyrosine lyase. Enzymes representing other classes are very few in the soil environment and less important in the ecological point of view.

33.4.1 Dehydrogenase Activity (DHA)

This is one of the important soil enzymes to assess the biological activities in the soil. It is categorized under the oxidoreductase class of soil enzymes. Lenhard (1956) first mentioned

the measurement of microorganism metabolic activity through DHA. Dehydrogenases are found in all the live microorganisms and are not extracellular, thus reflecting the microbial activity in soils. They are important transporters of hydrogen ions (protons) and electrons, mediating the oxidation reduction reactions of the cells. Most important are the dehydrogenases that transfer H to generate reductant by oxidation of a variety of substrates and are integral participants of respiration and electron transport chains. These dehydrogenases transfer H^+ ions on either the nicotinamide adenine dinucleotide or the nicotinamide adenine dinucleotide phosphate (Gianfreda and Rao, 2014). The DHA of soil reflects the total oxidative metabolic activities of soil microbes, therefore it is considered a good indicator of biological activities in soil (Gu et al., 2009). The dehydrogenases mediate the oxidation of soil organic matter by transferring protons and electrons from the organic matter to suitable inorganic acceptors. The overall DHA of soils is affected by soil type, moisture content, and the redox state (aeration) in the soil (Brzezinska et al., 1998; Glinski and Stepniewski, 1985; Kandeler, 1996). Increasing the water content from the field capacity to flooded conditions promotes a sharp shift in the activity of aerobic to anaerobic. It is used for a better indicator of microbial oxidative activity (Gu et al., 2009; Tabatabai, 1982; Trevors, 1984).

33.4.2 Amylase

This is a group of enzymes involved in starch hydrolysis and constitutes at least three enzymes. Starch is a polymer of glucose linked by α (1→4) glycosidic bonds. α-Amylase hydrolyzes the α (1→4) glycosidic bonds within the starch molecule, yielding short-length polysaccharides called dextrins. β-Amylase hydrolyzes starch by removing glucose disaccharide moieties from the nonreducing end, yielding maltose as the end product (Thoma et al., 1971). The α-glucosidase or maltase further hydrolyzes maltose to yield glucose units. Both the amylases may be derived from different sources of origin. Although amylases are produced by both microorganisms and plants, any given organism rarely produces both α and β amylases. As mentioned by Ross (1975), amylases are the group of enzymes that influence the effect of agronomic practices, the type of vegetation, etc. Although these enzymes play a role in carbon cycling, their substrate starch is not a major constituent of soil organic inputs, making amylase less important as a soil enzyme.

33.4.3 Cellulase

Similar to amylase, cellulase is also a group of enzymes involved in the decomposition of cellulose, a glucose polymer having β (1→4) glycosidic bonds. The endocellulase hydrolyzes internal glycosidic bonds, producing cellodextrins and exocellulase hydrolyzes removing cellobioses and cellotrioses from the nonreducing terminal of cellulose.

33.4.4 β-Glucosidase

This is a more common and predominant soil enzyme (Eivazi and Tabatabai 1988). β-Glucosidase hydrolyzes the β (1→4) glycosidic bond of the cellobioses and cellotrioses to yield glucose. The organic matter inputs to the soil in the form of plant residues and litter possess high amounts of cellulose that has to be decomposed to be utilized by the soil

microbial community. Cellulases and β-glucosidase are thus important soil enzymes, with the β-glucosidase playing a crucial role in the breakdown of low molecular weight carbohydrates of soil organic matter (Ajwa and Tabatabai, 1994; Tabatabai, 1994). The β-glucosidase enzyme is produced by the majority of the soil microbes and thus is gaining importance as a general index of microbial activity similar to the DHA of soil. It differs from dehydrogenase in that it is an extracellular enzyme. Also, β-glucosidase releases glucose during the decomposition of organic matter and influences the growth of soil microorganisms as glucose is the preferred carbon source for a majority of them (Acosta-Martinez et al., 2011; Gianfreda and Rao, 2014; Tabatabai, 1994). It is stable and less variable with respect to seasonal changes (Turner et al., 2000) and is much influenced by the soil health status, physicochemical properties such as soil pH, and crop management practices, that is, the application and type of fertilizers, organic residues, etc. (Acosta-Martinez and Tabatabai, 2000; Bandick and Dick, 1999). The addition of soil amendments affects the soil pH and therefore, the β-glucosidase activities (Perucci, 1992). Thus for evaluating the effect of crop management practices on soil health, soil β-glucosidase activity serves as one of the best predictors for the improvement and measurement of ecological changes.

33.4.5 Phosphatases

Phosphorus is one of the most important growth-limiting nutrient elements for which the availability to plants is limited, with lower use efficiency not more than 15%–20% although abundant sources are present in soil. Even in the cases of application of P chemical fertilizers, more than 80% gets fixed as immobile stock in soil and is not easily available to plants during crop growth. It is present in the ores in a bound form that can only be liberated in acidic conditions. Some microbes and plant roots solubilize small amounts of phosphates in their vicinities by releasing organic acids into the environment. But more acidic conditions also inhibit the growth of organisms. Thus organic P becomes a more important source to acquire P and enzymes are required to break the phospho-ester bonds in the organic matter. Phosphatases are the extracellular enzymes that catalyze the hydrolysis of phospho-ester bonds in organic P-containing substrates releasing inorganic P in the form of orthophosphates that can be used by soil biota and plants. The phosphatases are categorized into many types based on the number of bonds hydrolyzed to release the orthophosphate and the optimum pH for activity. These enzymes are inductive adaptive (Nannipieri, 1994) enzymes and thus the available soil phosphatase concentration is influenced by the concentration of the available orthophosphates in soil. Increasing the concentration of orthophosphates in the soil solution reduces the activity of phosphatases (Amador et al., 1997; Sarapatka, 2003; Sinsabaugh et al., 1993). Plant roots also produce acid phosphatase during P deficiency and mobilize the in situ immobilized P from the soil. Fungi also produce phosphatases, predominantly acid phosphatases. Bacteria produce mostly alkaline phosphatases, although many can produce both. Phosphodiesterases can release orthophosphates linked by two bonds to the organic matter (e.g., nucleic acids).

33.4.6 Protease

Nitrogen is one of the important plant nutrients, and is taken up from the soil as ammonia and nitrate. Increasing the concentration of N in the soil enhances the plant growth

profusely. Protease is a soil enzyme important in the mineralization of organic N in soil (Ladd and Jackson, 1982). It is linked with organic and inorganic colloidal substances in soil (Burns, 1982; Nannipieri et al., 1996). It is extracellular in nature and plays a vital role in maintaining the ecosystem function and N nutrition for plant growth (Burns, 1982). Protease hydrolyzes the peptide bonds of protein and degrades protein to release short peptides, which are further degraded to constituent amino acids. The amino acids act as nitrogen sources for the soil microbes. They may either be taken up by the microbes or may be further mineralized to release ammonia. The latter case contributes to meeting the plant N requirements. Protease plays an important role in nitrogen mineralization in soils with organic crop residue inputs during crop production.

33.4.7 Urease

To ensure good crop productivity, nitrogen input to the soil is very essential. The crop residues used as organic inputs are usually deficient in nitrogen due to high C:N ratios. In chemical fertilization practices, nitrogen is supplied in the form of urea ($CO(NH_2)_2$), but plants cannot take up urea. It has to be degraded to release ammonia or ammonium ions to be used by plants (Zantua and Bremner, 1977). Urease enzymes released by the soil microbiota, hydrolyze urea into ammonium and carbon dioxide (Andrews et al., 1989; Byrnes and Amberger, 1989; Fazekasova, 2012; Pettit et al., 1976). Urease is produced by almost all the soil microbial groups, that is, fungi, bacteria, yeast, and algae as well as by the roots of some plants (Follmer, 2008). This soil enzyme occurs as both extra and intracellular forms in the microorganisms (Burns, 1986; Mobley and Hausinger, 1989). The nitrogen dynamics in soil are influenced by the urease enzyme as it increases the ammonical nitrogen concentration. The release of ammonium increases the soil pH when fertilized with urea. The nitrogen released is prone to loss by volatilization of ammonia; this also exerts a negative impact on the atmosphere (Simpson and Freney, 1988). It mainly reduces the efficiency of applied N fertilizers. Research on the regulation of the urease activities during crop growth periods is in focus (Das and Verma, 2011; Rotini, 1935). Slo- release urea such as polymer-coated urea reduces the effect of soil urease.

33.4.8 Arylsulfatase

Sulfur is an essential nutrient of plants and other soil organisms. It is an integral part of proteins in the form of S-containing amino acids such as cystein and methionine as well as other compounds such as sulfated carbohydrates; vitamins such as thiamine and biotin; alkaloids of plants such as alicin; plant and microbial defense-related compounds such as mercapto; and functional molecules such as glutathione. Deficiency of S slows down amino acid synthesis, reduces crop growth, and ultimately reduces crop yield. S is taken up by the plants in the form of sulfates; the presence of sulfates is limited by leaching loss and loss into the atmosphere due to microbial sulfate respiration. Organic matter is the primary source of sulfur in soil. The organic sulfur is mineralized into inorganic sulfate by microbes. Soil enzymes that are responsible for the release of inorganic S from the organic substrate into the soil solution are called sulfatases. They are widespread in nature and play a crucial role in the hydrolysis of S esters in soil (Ganeshamurthy et al., 1995; Gupta et al., 1993; Kertesz and Mirleau, 2004).

S-limited or low S conditions in soils induce the synthesis of sulfatases from the bacterial population (McGill and Colle, 1981). The populations of soil bacteria are the main factor affecting the concentration of arylsulfatase in soil and also affecting the application of external application of S sources (Vong et al., 2003). The hydrolysis of aromatic sulfate esters ($R\text{-}O\text{-}SO_3^-$) into phenols (R-OH) and sulfate sulfur (SO_4^{2-}) by splitting the oxygen-sulfur (O-S) linkage and the conversion of inorganic S to plant uptake is mediated only by the enzyme aryl sulfatase (Tabatabai, 1994). The activity of this enzyme is affected by the concentration of inorganic S by soil, especially during the active crop growth period.

33.5 DETERMINATION OF ENZYMATIC ACTIVITIES

A few important enzymes frequently estimated for their activities are discussed below.

33.5.1 Dehydrogenase Activity

Dehydrogenases are respiratory enzymes that transfer two hydrogen atoms from organic compounds to electron acceptors, thereby oxidizing the organic compounds and generating energy. These enzymes are present in all the microorganisms in the soil and so serve as a generalized comparative index of microbiological activity of soils. These enzymes do not occur in a free form and so represent only the activity of live intact cells (Nannipieri et al., 1990). For the assay, as per the method described by Casida et al. (1964), 4 g of air-dried soil samples were placed in centrifuge tubes. Seventy milligrams of calcium carbonate was added to the tubes, followed by 1 mL of 3% solution of triphenyltetrazolium chloride. Four milliliters of distilled water was added to the mixture and the contents were mixed thoroughly using a vortex mixer, then incubated at 37°C for 24 h in the dark. After incubation, the triphenylformazan was extracted using methanol and the final volume was made up of 25 mL. A standard graph was plotted using different concentrations of triphenylformazan. The red color developed was read at 485 nm wave length. This method is simple and easy to perform as compared to other quantitative methods and enables the quantification of DHA of the intact cells of the soil.

33.5.2 Fluorescein Diacetate Hydrolysis

Fluorescein diacetate hydrolysis is used as a measure of total microbial activity as it can be hydrolyzed by both hydrolytic exoenzymes and cell membrane-bound hydrolytic enzymes. It is used for measuring the amount of fungi and bacteria located on acetyl esterases in living protist cells (Brunlus, 1980; Lundgren, 1981; Schnurer and Rosswall, 1982). Fluorescein diacetate is a colorless compound that hydrolyzes to give a yellow-colored compound fluorescein that can be measured colorimetrically (Adam and Duncan, 2001). Two grams of soil were placed in a 50 mL conical flask and 15 mL of 60 mM potassium phosphate buffer pH 7.6 was added. 0.2 mL of FDA (1000 $\mu g\,mL^{-1}$) was added and the flasks were stoppered. Blanks were prepared without soils and controls were kept with soil but without adding FDA. The flasks were incubated for 20 min with shaking at 100 rpm. After incubation, the reaction was terminated using 15 mL of chloroform/methanol (2:1) ratio. The contents were shaken vigorously

and then filtered. The filtrates were read in a spectrophotometer at 490 nm. A standard curve was prepared using the same procedure. The standards used were fluorescein solutions of 0–5 $\mu g\,mL^{-1}$ prepared from a stock solution of 20 $\mu g\,mL^{-1}$. The absorbance values obtained for soil samples were plotted into the standard curve to calculate the hydrolytic activity of soils.

33.5.3 Acid Phosphatase

Phosphatases or phosphomonoesterases are the hydrolytic enzymes that cleave the ester bond between the phosphate group and the organic residue of the organic phosphates. Based on the optimum pH for the activity, phosphatases are of two kinds: acid and alkaline. Acid phosphatases show maximum activity at acidic pH around 6 whereas alkaline phosphatases show maximum activity at alkaline pH around 11. The p-nitrophenyl phosphate acts as a substrate for both acid and alkaline phosphatases. On hydrolysis by either of the enzymes, it yields p-nitrophenol, the color of which is read spectrophotometrically. The method described by Tabatabai (1994) was used in which 1 g of soil was taken in a 50 mL Erlenmeyer flask and 0.2 mL of toluene was added to arrest the microbial activity during the assay. Four milliliters of modified universal buffer (MUB) of pH 6.5 was added, followed by 1 mL 0.05 M p-nitrophenyl phosphate. The flasks were swirled, stoppered, and placed in an incubator at 37°C for 1 h. At the completion of incubation, the flasks were opened and swirled to mix the contents. The end product extraction was done by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH. The contents were mixed properly and filtered. The color intensity was read at 440 nm using a spectrophotometer. A standard curve was prepared using the same procedure but with the standard p-nitrophenol solutions in place of soil samples. The standards were prepared in such a way so as to obtain concentrations of 0, 10, 20, 30, 40, and 50 $\mu g\,mL^{-1}$ of p-nitrophenol. The concentration of p-nitrophenol hydrolyzed by the sample was calculated from the standard curve.

33.5.4 Alkaline Phosphatase

The alkaline phosphatase activity of the soil was assayed similarly to the method used for acid phosphatase activity, except that a buffer of pH 11 was used instead of pH 6.5. The transmittance or absorbance and the yellow color intensity of the filtrate can be read with a spectrophotometer at 440 nm wavelength.

33.5.5 Phosphodiesterases

These are the enzymes that mineralize the phosphates bound to two organic groups. The assay protocol was described by Tabatabai (1994) with Bis-p-nitrophenyl phosphate. The nitrophenol released can be quantified spectrophotometrically.

33.5.6 β-D-Glucosidase

β-D-Glucosidases are the enzymes that cleave glycosidic bonds between glucose units in the dimers and oligomers (yielded from the action of amylases and cellulases) to liberate monosaccharides. For the assay of β-glucosidases, p-nitrophenyl-β-D-glucoside was used,

which on hydrolysis produces p-nitrophenol. The assay was similar to that of phosphatases with a difference in extractant used. One milliliter of 0.5 M $CaCl_2$ and 4 mL of 0.1 M Tris(hydroxymethyl) aminomethane (THAM) buffer were added instead of the $CaCl_2$-NaOH extractant used for the phosphatase assay (Tabatabai, 1994).

33.5.7 Urease

Urease is another important enzyme that is frequently measured in soil microbiological analyses. The method from Tabatabai (1994) is as follows: To 5 g of a soil sample, add 5 mL of urea solution (2 mg/mL), stopper the bottle, and incubate at 37°C for 5 h. Add 50 mL of 2 M KCl-phenyl mercuric acetate (5 mg PMA in 1 L of 2 M KCl) solution and shake for 1 h. Filter under suction with Whatman #42 filter paper. To 1 mL of sample, add 9 mL of KCl-PMA solution and 30 mL of color reagent (25 mL of 2.5% diacetylmonoxime + 10 mL of 0.25% of thiosemicarbazide). Mix and incubate in a boiling water bath for 30 min. Cool and make up the volume to 50 mL, then read the red color with a colorimeter using a #54 green filter.

33.6 KINETICS OF SOIL ENZYME AND SUBTRACT

The rate of reaction catalyzed by the enzyme is affected by the amount and appropriateness of the substrate (Fig. 33.2). In soils, enzymes follow either zero order (reaction rate not dependent on substrate) or first order (reaction rate at any time proportionate to the concentration of substrate) kinetic reactions. In a zero order reaction, the substrate does not have any significant effect on the soil enzyme. This happens when the substrate concentration is high enough to saturate the active sites of all enzyme molecules in the soil. In all the other cases, the enzyme activity occurs as a first-order reaction (Segel, 1975).

Zero-order reaction

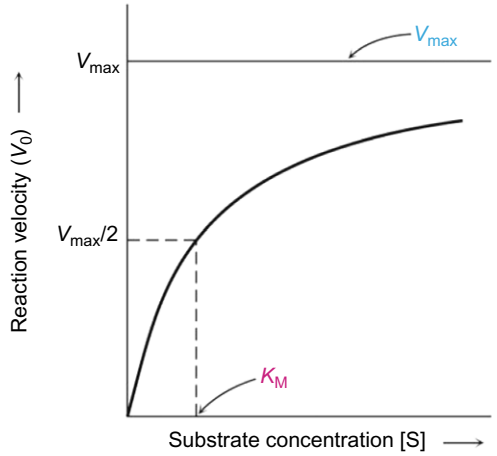

FIG. 33.2 Relationship between reaction rate (V_0) and substrate (S).

$$\frac{dx}{dt} = k \tag{33.1}$$

$$\frac{x}{t} = k \tag{33.2}$$

First-order kinetics

$$\frac{dx}{dt} = k(a - x) \tag{33.3}$$

$$k = \frac{1}{t} \text{In} \frac{a}{a - x} = \frac{2.303}{t} \log \frac{a}{a - x} \tag{33.4}$$

In both reactions, x represents the concentration of converted substrate; t is the time duration in minutes; and k is the proportional constant. In most of the cases, the enzyme-catalyzed reaction follows the rectangular hyperbola path during the enzyme kinetics process. The concentration of substrate determines the order of reactions. At the lower substrate concentrations, the velocity of the reaction follows the first-order kinetics. However, increase the concentration of substrate and the maximum velocity is obtained independent of further additions of substrate; then the reaction follows the zero-order kinetics. The kinetics of enzyme action can be calculated by using the Michaelis-Menten equations.

33.7 FACTORS AFFECTING ENZYMATIC ACTIVITIES IN THE SOIL

The government of China launched a "Grain for Green Project" for enhancing the forest cover in which degraded land may be chemically, physically, or biologically converted into a forest area. For this, the government provided a grain subsidy to farmers. This project highlighted and promoted the poor quality soil into a green forest and reduced the soil erosion (Fang et al., 2007). The various management practices, including crop diversification, crop residue management, optimum tillage operations, and the use of chemicals and fertilizers have had an immense effect on soil microbial biomass, diversity, and the amount of enzymes (Fang et al., 2010; Song et al., 2007).

33.7.1 Soil pH

Each soil enzyme has a definite range of pH for optimum activity. A change in the concentration of H^+ ions in the soil influences enzyme dynamics, substrate degradation, and cofactor alteration by ionization and solubility properties (Tabatabai, 1994). At optimum pH, enzymes are more stable. However, at extreme higher or lower pH, irreversible denaturation occurs and the soil enzymes deteriorate. As the pH deviates from the optimum toward higher or lower pH, the activity reduces and then stops beyond a threshold. Although this is a generalized trend, the influence of pH varies for different enzymes.

33.7.2 Temperature

The enzyme activities increase with increasing temperature, doubling the reaction rate about every 10°C. Beyond the threshold, the enzyme activity sharply decreases and seizes

at high temperatures, causing inactivation. The temperature-based rate constant can be computed by the Arrhenius equation:

$$k = A\exp\left(-E_a / RT\right) \tag{33.5}$$

$$\log k = \left(-E_a / 2.303RT\right) + \log A \tag{33.6}$$

where A is the preexponential factor, E_a represents the energy of activation; R means gas constant; and T is temperature in degrees Kelvin.

Again, the thermal stability of enzymes varies with enzyme type and source. The thermotolerant organisms produce enzymes that can operate at a wide temperature range. Thermophiles produce enzymes that are especially active at high temperatures and show low activities at low temperatures.

33.7.3 Soil Organic Matter

This is a key component of soil fertility and is responsible for mediating the plant nutrient soil toward plant roots. It is the source of the substrates for the diverse soil enzymes produced by the soil microbial population to satisfy their nutritional needs. It directly influences root exudation into the soil and the plant biomass. Increasing the rate of soil organic matter enhances the soil enzyme activity; the increase stops when the organic matter content reaches very high concentrations and further increases do not cause any change in soil enzyme activity.

33.7.4 Cropping Systems

Increasing plant biomass also increases the amount of low molecular weight organic acids in soil via the root exudation (Kuzyakov, 2002). More than 60% of the photosynthate is allocated to the root exudates into soil by the crop plants, which modulate and improve the soil microbiome (Hutsch et al., 2002). These acids enhance the soil microbial population and diversity and thus indirectly affect soil enzymatic activities (Bertin et al., 2003). The nutrient dynamics in the rhizosphere are governed by these organic acids by altering the concentration of available forms of different elements. Plant roots stimulate the soil enzymatic activities by creating favorable conditions for microbial activities (Castellano and Dick, 1991). Profuse plant growth or biomass that is higher above ground highly correlates to greater soil enzyme activity (Fig. 33.3). This effect of plant growth on soil enzyme activities is more prominent in the case of legume crops, due to the greater proliferation of soil microbes due to the increase in available nitrogen excreted by the legumes. An enhancement in the urease and protease-BBA activities during N-fixation was reported by Roldan et al. (2003). The maximum activities of arylsulfatase were observed in cruciferous crops due to high demand for S (Knauff et al., 2003). The enzymatic activities of soil are greatly influenced by the nature of vegetation above ground.

33.7.5 Crop Management Practices

33.7.5.1 Application of Inorganic Fertilizers

The applications of inorganic sources of mineral nutrition influence the diversity and magnitude of soil enzymes in the soil; some of the enzymes increase with an increasing

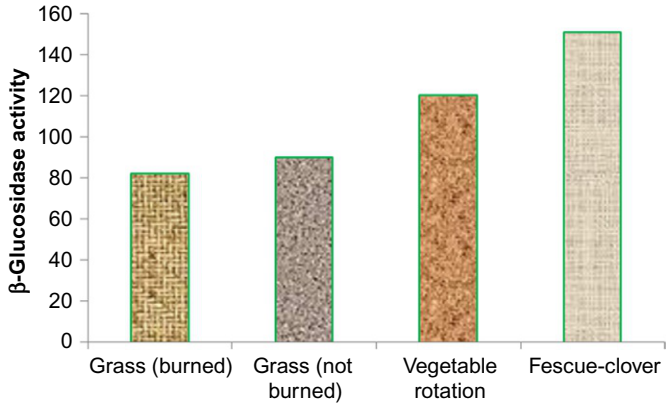

FIG. 33.3 Effect of cropping systems on β-glucosidage soil enzyme. *Modified from Dick, R.P., 1994. Soil enzyme activity as an indicator of soil quality. In: Doran, J.W., et al. (Eds) , Defining Soil Quality for a Sustainable Environment, Madison, WI, pp. 107–124.*

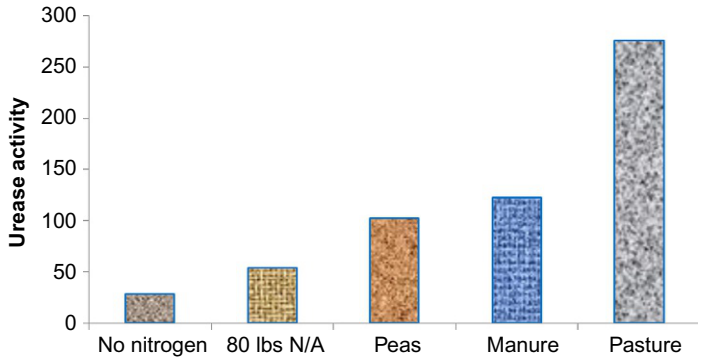

FIG. 33.4 Effect of different crop management practices on urease activity. *Modified from Bandick, A.K., Dick, R.P., 1999. Field management effects on enzyme activities. Soil Biol. Biochem. 31, 1471–1479.*

concentration of inorganic plant nutrients (Fig. 33.4). These are those enzymes, and their source of origin is soil microbes. Under chemical fertilization, the inorganic nutrients added act as an immediate food source for microbes, increasing their numbers, which further produces enzymes to sustain their growth thereby causing an enhancement in total enzyme activity. Also, enzymes are released by the microbes to acquire those elements that are deficient in the fertilizers added. Increasing the N fertilizers accelerates the activity of some C, N, and P cycling enzymes, that is, cellulose (Sinsabaugh et al., 2005), urease (Saiya-Cork et al., 2002), and phosphatases (Guo et al., 2011). However, other studies showed that increasing N fertilizers reduced the activity of urease (Burket and Dick, 1998), cellulases (Sinsabaugh et al., 2005), peroxidase (DeForest et al., 2004), and proteases (Giacometti et al., 2013). It also suggested that some enzyme activities are not affected by the application of inorganic N application (Klose et al., 1999; Klose and Tabatabai, 2000). The amount of fertilizers added determines the response observed. Fertilizers added in recommended doses positively influence

the microbial activity and increase the enzyme activity, whereas using excessive fertilizer deteriorates the microbial diversity, causing a negative effect in the long run.

33.7.5.2 Application of Organic Manures and Composts

Organic fertilizers such as manures and composts improve the soil organic matter content and also stimulate the growth of soil microbes as well as plants. The effects of soil organic matter on enzyme activities are discussed in Section 33.7.3. These effects cause a stable increase in enzymatic activities.

33.7.5.3 Tillage

Tillage alters the physicochemical properties of soil by mixing the upper fertile profile with the lower profile rich in leachates (Rahman et al., 2008) and affecting the soil enzymes. It also influences the soil organic carbon and distribution of water and aeration in the soil profile (Curci et al., 1997). The mineralization rate speeds up due to tillage and a portion of the organic matter is lost into the environment as CO_2. A decrease in the C content in soil reduces the soil enzymatic activities (Madejon et al., 2007). Thus, due to tillage, an initial increase in enzymatic activity may be observed but it is not sustained for a long duration and subsequent decreases occur. Some of the researchers reported that tillage did not affect the activities of some enzymes, that is, dehydrogenase, phosphatases, and urease in soil (Corchran et al., 1989).

33.7.6 Cofactors and Ionic Environment

Many enzymes are not catalytically active by themselves except when combined with specific nonprotein cofactors. In general, cofactors are heat stable, dialyzable substances of low molecular weight. The cofactors may be small organic molecules (e.g., vitamins) or metal ions. Thus, the availability of these metal ions influences the enzyme activity in soils. Sometimes other metal ions may compete with the cofactors and interfere with the complex formation capacity of an enzyme, reducing the performance of a soil enzyme. Dick et al. (1983) conducted an experiment and measured the soil enzyme activities of pyrophosphatase in soil, in which soluble metal was removed with 1 M neutral normal ammonium acetate. This greatly reduced the enzyme activity, but the addition of metal ions again restored the activities of pyrophosphatese in soil.

33.8 FUTURE LINE OF WORK

(1) Soil enzymes act as indicators of microbial activity and reflect the influence of climatic conditions on the soil microbiome. Therefore, studies should be designed to track the kinetics of enzyme activities to understand the effect of climate on soil productivity and sustainability.

(2) As the soil enzymes are sensitive to management practices, appropriate soil enzymes should be identified and devised as indicators for periodic monitoring of the soil health of farmers' fields at regional soil-testing laboratories.

(3) Practices can be designed to target the improvement of specific enzymes to alleviate the deficiency of a particular nutrient.

(4) Research should be extended in exploiting the electron transfer and ionic transformation potential of soil enzymes in designing biofuel cells that can be used to generate electricity and replace batteries.

(5) Research should be taken up in using soil as a source of enzymes in biosensors used to detect the concentration of specific substrates. The use of a soil enzyme pool will prove to be more economical compared to pure enzymes.

33.9 CONCLUSION

Keeping in view the increasing costs of crop production and sustainable crop production as well as combating the decline of productivity are of prime concern to farmers and policy makers. The partial factors of productivity also go down, and the benefit–cost ratio will narrow in the coming years. On the other side, the production of a higher amount of foodstuffs from limited land is a global challenge in front of researchers and policy makers. Increasing use of chemical fertilizers for enhanced production is one side of the coin, but another side is that it reduces the soil health in physical, chemical, and biological terms. Decreasing soil biodiversity and environmental quality force a rational use of chemicals in agriculture. To mitigate the adverse effects of chemical agriculture, organic inputs, organic farming, or integrated nutrient management should be promoted at a global level. Soil enzymes play a crucial role in plant nutrient dynamics and enhance the nutrient use efficiency. The decomposition of soil organic matter and nutrient mineralization processes are also mediated by the soil enzymatic activities. They are sensitive indicators of impacts of management practices and climate change. Different enzymatic activities should be monitored before adapting a management practice as a sustainable crop production practice in a region.

Acknowledgments

The authors are grateful to Dr. U.S. Meena, Department of Agriculture and Animal Husbandry, PSS Central Institute of Vocational Education, Bhopal, India, for the motivation and incorporated suggestions during the execution of this book chapter.

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CHAPTER

34

Enzymes in Pharmaceutical Industry

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34.1 INTRODUCTION

In the biosphere, there is a significant and extensive biological exchange among the various prokaryotic microbes, animals, human beings, and plants. Mostly, the interactions are mutually beneficial to each other. Still, there are many more microorganisms that are pathogenic and that produce disease in plants, humans, and animals. The mechanisms that these pathogenic organisms use to cause infections and diseases are extremely different, affecting virtually all types of tissues. There are two key mechanisms for protection against these pathogenic microorganisms: (i) prevention through vaccines that provide sufficient immunity to fight such infections and harmful microbes and (ii) antibiotics that have created a new line of defense against these harmful organisms. Antibiotics, a very specialized drug or molecule obtained naturally or prepared synthetically, are active against all these pathogenic microbes, fungi, protozoa, and yeasts with a well-defined mechanism and target of action. In recent years, the uncontrolled use of antibiotics produced natural selection pressure and genetic changes within these pathogenic organisms resulted in resistance to antibiotics. Antibiotic-resistant pathogenic bacteria have created life-threatening conditions in animals and humans and in the bacteria itself as they are able to escape the feat of the antibiotic molecules (Allen et al., 2010; Davies and Davies, 2010). Antimicrobial resistance is natural but not a one-way process; several factors contribute to the incidence of antibiotic resistance among susceptible microorganisms. Indiscriminate use and abuse of antibiotics in the clinical environment is the most important factor as it repeatedly exposes deadly infectious pathogens to an ever-increasing concentration of different antibiotic molecules. The increase in antibiotic concentration in turn increases the selection pressure, which later develops resistance against these antibiotic molecules. Pathogenic bacteria are also exposed to the antibiotics through livestock feed and

https://doi.org/10.1016/B978-0-12-813280-7.00034-7

agriculture, where they are used as feed additives. They are simultaneously used for human treatment resulting in the development of resistant bacterial strains that are transferred very easily from one to another.

34.2 WHAT COULD BE A BETTER ALTERNATIVE TO ANTIBIOTICS?

Natural or herbal products have been approached as a better alternative to antibiotics. Though the chances of resistance with these products are less, great efforts are still needed regarding their mechanisms of action, chemical structure, and biosynthesis. The natural products are hard to be done in the laboratories and this is attributed to their complex nature of synthesis. There are still hundreds of unexplored natural products thought to be derived from marine *actinomycetes*, bacterial symbionts, and unculturable microorganisms. Antimicrobial peptides (AMPs) also hold an important place as a chief source of possible new enzybiotic agents (e.g., Magainins, Cathelicidins, Lactoferrins, and Defensins), particularly against multidrug-resistant strains. Bacteriophage/phage therapy initially discovered in the 20th century was considered as a potential therapeutic agent. It was granted the status of "Generally Recognized as Safe" (GRAS) in biocontrol in agriculture and veterinary medicine for use in all food products in 2007 and has become an important tool in the control of many bacterial diseases (Gálvez et al., 2007).

34.3 WHAT ARE ENZYBIOTICS?

The term enzybiotic is formed with the combination of "enzyme" and "antibiotic." It refers to those lytic enzymes that are naturally present in viruses, bacteria, and in the physiological body fluids such as tears, saliva, and mucous; these contain antibacterial or antifungal properties. They are frequently used as preservatives and food additives. Initially, all the bacteriophage enzymes capable of bacterial cell wall-degradation used as antibacterial agents were given the term enzybiotics. Later, enzymes with antifungal activity were also included as enzybiotics (Veiga-Crespo et al., 2007).

34.4 WHY ENZYMES AS THERAPEUTICS?

Even before the inception of biotechnology, enzymes and proteins were used as drugs. Enzymes bind with their targets very specifically and with high affinity. They convert multiple target molecules to the desired products by catalytic action, increasing the rate of chemical interconversions and metabolic processes. This property differentiates them from all other types of drugs. Biochemical diversity and the ease with which the enzyme concentration may be increased by environmental and genetic manipulation give the advantage of using enzymes of microbial origin. Enzymes that are used therapeutically have the great advantage of being economically viable and reliable. Due to high yields, they are easy for product modification and optimization. Digestive and metabolic enzymes can be used either alone or in

permutation with other therapies for the treatment of a number of diseases such as leukemia, skin ulcers, cardiovascular diseases, Parkinson's disease, inflammation, digestive disorders, pancreatic disorders, etc. They are also in use in the diagnosis, biochemical investigation, and monitoring of many alarming diseases (Prajakta and Vidya, 2015).

When enzymes as drugs are added to the cells of gram-positive bacteria, they demolish the cell wall completely. In gram-negative bacteria, due to the presence of the outer membrane, their activity is limited. The target action of enzymes is selective without affecting normal microflora. The major groups of enzymes used for therapeutic purposes are described below.

34.4.1 Peptidoglycan Hydrolases

Peptidoglycan is an important constituent of the bacterial cell wall in gram-positive as well as gram-negative bacteria. N acetyl muramic acid (NAM) and N acetyl glutamic acid (NAG) are its main gears, cross-linked by a peptide chain that forms a strong meshwork in the bacterial structure (Vollmer et al., 2008). Most of the bacteria have a varied group of enzymes known as peptidoglycan hydrolases. By and large, these are implicated in the regulation of the expansion of the cell wall, the peptidoglycan yield, and the separation of progeny cells during cell division and cell lysis. They are also capable of breaking the covalent bonds of the polymeric peptidoglycans. Bacteriophages digest the bacterial cell wall for phage progeny release at the end of its lytic cycle due to the activity of hydrolases enzymes (Fischetti, 2008).

34.4.1.1 Structure of Peptidoglycan Hydrolase

Peptidoglycan hydrolase contain two domain structures with N and C terminal ends. The N terminal is a catalytic domain that is responsible for cell wall cleavage and the C terminal is the cell wall binding domain (CWD). Binding this domain to its cell wall substrate is needed for efficient cleavage of the bacterial cell wall. The cell wall binding domain does not recognize the peptidoglycan itself because its structure is highly conserved. Efficient breakdown requires the binding of the binding domain to its cell wall substrate. Specific receptors in the peptidoglycan layer help in the binding of the enzyme molecule to the sensitive bacteria. The target binding ability of the enzymes also gives them high specificity as these receptors are found only in enzyme-sensitive bacteria.

34.4.1.2 Applications of Peptidoglycan Hydrolases

These enzymes are used for the preparation of the spheroplast required for cell transformation through the extraction of intracellular substances from bacterial cells. It destroys and kill the bacterial cell and releases the progeny virions. (Young and Wang, 2000). Peptidoglycan hydrolases with their bacteriolytic activity and narrow target spectrum have turned up as a miracle drug in the infections of *Enterococci*, which is an important cause of nosocomial infections that are often difficult to treat by even newer antibiotics due to resistance (Fernandez-Gacio et al., 2003). These enzymes in the purified form, natural form, or in a recombinant state can be used externally in plants as well as animals. They are even overexpressed endogenously in transgenic plant or animal organisms to increase their resistance against infectious organisms. (Yazawa et al., 2006). Hydrolases enzymes are also found to be useful in the production of cosmetic and pharmaceutical preparations such as

ointment and cream for skin and soft tissues. (Murashova et al., 1975). Peptidoglycan hydrolases include the following enzymes.

(a) Lysins.
(b) Phage-encoded peptidoglycan hydrolases (PGH)—Endolysins.
(c) Bacteriocins.
(d) Autolysins.
(e) Lysozymes.

(a) Lysin

Lysins are murein hydrolases/double stranded DNA bacteriophage fixed enzymes, alkaline in nature, with a positive charge at the pH of the medium less than their isoelectric point. It damages the bacterial cell wall to release progeny bacteriophages. When supplemented from outside in their pure form, these enzymes generate the total death of the susceptible gram-positive bacteria within a few seconds by acting on the covalent bonds of the peptidoglycan chain, resulting in a unique antimicrobial strategy. Based on their enzymatic specificities, lysins fall into five major classes: *N*-acetylmuramyl-L-alanine amidases, Endopeptidases, *N*-acetylmuramidases (lysozymes), Endo-β-*N*-acetylglucosaminidases, and Lytic trans glycosylases.

Applications of Lysin

The exogenous application of lysins in the gram-positive and gram-negative cells causes lysis with or without the help of an osmotic permeabilizer. A salmonella phage lysin designated SPN1S had a 30-fold increase in lytic activity over the chicken egg white lysozyme and the nonphage-derived cell wall-degrading enzyme. The *Acinetobacter baumannii* phage lysin LysAB2 was capable of effectively lysing seven different bacterial genera, including *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus sanguis*, *Acinetobacter baumannii*, *Escherichia coli*, *Citrobacter freundii,* and *Salmonella* spps. Lysin's peptidoglycan hydrolytic action or its cell wall-binding domain binding function is utilized in food preservation as well as pathogen detection. Phage lysins also function as a preservative agent in the control of foodborne pathogens posing a major threat to the health and well-being of individuals, especially the immunocompromised and old animals. Lysin LysZ5 of the *Listeria monocytogenes* phage controls the bacteria to undetectable levels in soy milk, even at the refrigeration temperature (Ajuebor et al., 2016). Staphylococcal lysin LysH5 eliminates *S. aureus* in milk. The enzyme also exhibited synergy with the bacteriocin, which together form a potential food preservative in the control of food pathogens at low concentrations. Streptococcal lysin PlyC provides protection against *Streptococcus pyogenes*, an infectious bacterial pathogen residing in the nasal cavity (Loeffler et al., 2003, Daniel et al., 2007, and Rashel et al., 2007). In intrapartum prophylaxis during neonatal infections caused by *Streptococcus agalactiae* residing in the genital tract, enzymes have shown useful effects (Pritchard et al., 2004; Cheng et al., 2005). In vivo applications of lysins in the control of infectious bacteria *S. aureus* induced endophthalmitis while a single intravitreal injection of the enzyme after a few hours of infection drastically reduced the bacterial load in mice eyes. This also provided a protective effect on the retina at the tissue level. (Singh et al., 2014). An intraperitoneal injection of *Enterococcus faecalis* in the mouse showed that the lysin IME-EF1 can provide better protection against infectious *E. faecalis* compared to its producing phage. A topical skin application of chimeric lysin ClyS87 was found to be effective for bacteria from the infected skin of mice. The lysin ointment was

better than mupirocin (Pastagia et al., 2011). In one study by Doehn et al. (2013), aerosolized pneumococcal lysins Cpl-1 used against pneumococcal lung infections significantly reduced the bacterial load in the lung, thus protecting the mice from pneumococcal bacteraemia. The engineered pneumococcal lysins Cpl-7s improved the survival rate of the zebrafish embryo. Lysins possess the potential to eliminate biofilms, an important feature of many pathogenic bacteria that results in their tolerance to many antimicrobial agents (Otto, 2008; Son et al., 2010). The most frequently recognized causative agents of biofilm-associated infections are the staphylococci94. SAL-296, CHAPk97, SAL-140, PlyGRCS57 Lysins phi11 Ply187, and the LysH5 phage-encoded lysine can eliminate staphylococcal biofilms. Lysins also have a potential application in the detection and quantification of bacterial pathogens in food materials as well as narrow-spectrum disinfectants, as per Hoopes et al. (2009). The streptococcal lysin PlyC was reported as the first protein-based, narrow-spectrum disinfectant against *Streptococcus equi*. Lysins also control lactic acid bacterial (LAB) contaminations in fuel ethanol fermentation. The streptococcal lysin λSa2 showed lytic activity against the majority of LABs tested. This enzyme also reduces L. *fermentum* in a mock fermentation of corn fiber hydrolysate (Roach et al., 2013).

(b) Phage-encoded peptidoglycan hydrolases (PGH)—Endolysins

Endolysins damage cell walls by degrading peptidoglycan and releasing newly formed virus particles, which causes the host cell to burst. Some phages also encode a second PGH type, namely the virion-associated peptidoglycan hydrolase (VAPGH). Endolysin imitative from phages that infect gram-positive bacteria typically holds a modular domain structure composed of an N terminal enzymatically active domain (EAD), and a C-terminal cell wall-binding domain (CBD) connected by a short linker region.

Application of phage-encoded peptidoglycan hydrolases (PGH)—Endolysins

In contrast to endolysins, VAPGHs degrade the localized peptidoglycan during infection, that is, the initial cell wall penetration. When purified, both PGH types can be bacteriolytic when applied exogenously to cells. Phages also encode polysaccharide depolymerases that degrade the macromolecule carbohydrates of the bacterial cell wall envelope. Extracellular polysaccharides can protect the bacterium from desiccation, antimicrobials, and host immune systems.

(c) Bacteriocins

Bacteriocins are proteinaceous extracellular substances produced by the ribosomes of both gram-positive and gram-negative species. These peptides are either produced spontaneously or undergo certain chemical modifications by Mitomycin C to inhibit the growth of other bacteria. Their lethal activity involves adsorption to the specific receptors on the exterior of specific bacteria, followed by metabolic, biological, and morphological changes resulting in the killing of bacteria. The first bacteriocin was isolated from *Escherichia coli* and was named colisins. Until today, these are one of the most diverse group of antibacterial peptides showing a bactericidal effect through inhibition of cell wall synthesis, permeabilization of the target cell membrane, or inhibition of RNase or DNase activity. On the basis of genetic and biochemical characteristics, bacteriocins are classified into three classes.

Class I bacteriocins

These are heat stable, small-sized (<10 kDa) peptides with an amphiphilic helical form, also known as lantibiotics. For example, Nisin. These bacteriocins shows a broad-range spectrum antimicrobial effect on various pathogens and LAB species, including *L. monocytogenes*,

S. aureus, and *Bacillus cereus*. Being positively charged, bacteriocins bind to phosphate groups on cell membranes, which are negatively charged, and thus insert into the membrane, resulting in pore formation and cell death.

Class II bacteriocins

The peptide chain-like lantothionine or beta lantothionine is absent in this class of bacteriocins, giving a simpler structure than class I bacteriocins. This class includes the subclass II-A (pediocin, enterocin, and sakacin), subclass II-B (lactococcin G, plantaricin, and lactacin F) and subclass II-C (AS-48 from *E. faecalis*).

Class III bacteriocins

This class includes Helveticin J and Millericin B, etc.

Applications of bacteriocins

1. Lantibiotics: It is the only bacteriocin permitted for food submissions that is acknowledged as safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO), which did so in 1969. Moreover, it is also accepted as a biopreservative ingredient in European Union countries and is assigned the number E234. These bacteriocins are highly effective for human health application mainly due to their low toxicity aspect therefore very useful for the treatment of blood pressure, inflammation, allergy, skin infections, mastitis, herpes infection, dental caries, and peptic ulcer.
2. Colicins: These are mostly preferred for hemolytic uremic syndrome, urogenital infections, and hemorrhagic colitis.
3. Microcins: This is also used as an antimicrobial agent, mainly for salmonella infections in animals and humans.
4. Bacteriocins produced by LAB are nontoxic, broad-spectrum, and pH-tolerant, which increases the shelf life of food and is therefore considered safe for food preservation.

(d) Autolysins

Autolysins are enzymes programmed by bacteria that are tangled in cell growth and division, cell wall formation, bacterial protein secretion, and peptidoglycan formation in a bacterial cell. (Vollmer et al., 2008). Among the various others, the LytA amidase of *Streptococcus pneumoniae* was the first autolysin tested as a probable antibacterial agent. Its antibacterial activity was compared with that of Cpl-1 lysin and cefotaxime (Rodriguez-Cerrato et al., 2007). The actions of both LytA amidase and Cpl-1 lysin were much more developed than that of cefotaxime. LytA was basically the most active of the other studied agents with respect to decreasing bacterial titres in peritoneal fluid and blood. (Lothar et al., 1987).

Application of autolysins

Autolysin E (AtlE) from *Staphylococcus epidermidis*, the homologous Autolysin S (Aas) protein from *Staphylococcus saprophyticus*, and Autolysin C (AtlC) from *Staphylococcus caprae* surface-associated proteins have both enzymatic (amidase and glucosaminidase) and adhesive functions. AutolysinE (AtlE) is involved in the initial attachment of the cells to a polymer surface and in biofilm formation. The *atlE* mutant strain was significantly less virulent than the wild type in an intravascular catheter-associated infection model in rats. Autolysin S (Aas) and Autolysin C (AtlC) bind to fibrinogen, and Autolysin S (Aas) also agglutinates sheep erythrocytes. Aae from *S. epidermidis* has bacteriolytic activity, and is involved in the adherence of *S. aureus* to surface-adsorbed fibrinogen. Autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin.

(e) Lysozymes

Lysozyme (or *N*-acetylmuramidase or muramidase) a hydrolytic enzyme, first revealed in the mucus secretion of the nasal area and later purified from various plant, animal, and microbial (bacteria, virus and fungi) materials (Masschalck and Michiels, 2003; Xue et al., 2004; Parisien et al., 2008). These enzymes catalyzes the breakdown of peptidoglycan polymers of the bacterial cell wall at the β1–4 glycosidic bond between *N*-acetylmuramic (NAM) acid and *N*-acetylglucosamine (NAG) residues, thereby lysing sensitive bacteria. The egg white from a chicken and the milk of mammals are the chief (and most unlikely) sources for lysozyme manufacture at an industrial level (concentration 3400 and 5840 mg/L) (Halper et al., 1971). Glutamic acid and aspartic acid residues are directly involved in the breakdown of the glycosidic bond between NAG and NAM, and their presence in the catalytic center is therefore crucial for the hydrolytic activity of the enzyme (Agamy, 2000; Maroni and Cuccuri, 2001; Masschalck and Michiels, 2003; Parisien et al., 2008).

Different classes/types of lysozyme:

(1) c lysozyme: Conventional or chicken-type that is derived from the egg white of domestic chickens (*Gallus gallus*) and from various tissues and secretions of mammals including milk, saliva, tears, urine, and respiratory and cervical secretions.
(2) g-type lysozyme derived from the egg white of the domestic goose (*Anser anser*) (Meyer et al., 1946).
(3) h-type lysozyme obtained from plants (Prager and Wilson, 1974).
(4) i-type lysozyme source obtained from invertebrates (Sinnott, 1990).
(5) b-type lysozymes obtained from bacteria (*Bacillus*) (Heinz et al., 1992).
(6) V-type lysozyme obtained from viruses (Bachali et al., 2004; Xue et al., 2004).

Applications of lysozyme

Lysozyme was first used experimentally in pigs in the mid-2000s. A human lysozyme derived from transgenic goat milk was shown to change metabolite profiles, intestinal microflora, and intestinal morphology. However, enhancements in growth recital due to the lysozyme were not visible. Along with antibiotics, lysozymes were found to be useful in the prophylaxis and treatment of different bacterial infections such as pharyngitis, tonsillitis, dysentery, and wound infections (Sava, 1996). They were used as a carrier for the specific delivery of antibiotic molecules to bacterial cells (Hoq et al., 2008). When enzymes were formulated as a gel for the topical treatment of wounds and acne, different formulations showed utility in the prophylaxis of infections due to skin piercing. Lysozymes have also been used as components of mouthwashes due to their capacity to kill different oral bacteria as well as being an aerosolized lysozyme for respiratory infections. Lysozymes possess RNase A and urinary RNase U, which selectively degrade viral RNA activity against HIV; in the future, they could be used for HIV infections (Glynn, 1968).

34.4.2 Other Therapeutic Enzymes

34.4.2.1 Activase/Alteplase

Activase was the first recombinant enzyme obtained from the human tissue plasminogen activator. The enzyme was approved for therapeutic use by the Food and Drug Administration (FDA) in 1987. Due to its property related to clot destruction, it is also known as a "clot-buster"

enzyme that is preferred for heart problems related to blocked arteries. Xylarinase a fibrinolytic metalloprotease isolated from the fungi xylaria curta, reteplase, tenecteplase, etc. Other enzymes also fall in the ateplase category.

34.4.2.2 Asparginase

Asparaginase is an important enzyme used in the therapeutic and food-manufacturing industries. *E. coli* is the main source of asparaginase, among which spectrila is a new recombinant form of enzyme. Tumor cells are unable to form the aspartate-ammonia ligase, and the nonessential amino acid L-asparagine is not produced as it is prone to weakening in the free exogenous concentration of asparginase, which causes death in the tumor cells. The ability of normal cells to synthesize L-asparagine for their own needs remains unaffected. Due to this property, asparginase remains useful for acute lymphocytic leukemia. The enzyme can be specified intravenously and is effective only when the asparagine levels within the bloodstream are extremely low (Gurung et al., 2013).

34.4.2.3 Chitinase

The sources of these enzymes are bacteriophages, and they are used for various infectious conditions and are even effective against drug-resistant bacterial strains. Chitinase are proteolytic in nature with anti-inflammatory activities. Most of these proteolytic enzymes of bacterial origin are also used in the removal of dead skin from burns (Gurung et al., 2013).

34.4.2.4 Collagenase

The collagenase enzyme is obtained from Clostridium histolyticum, which destroys the collagen layer. It helps to discontinue and remove dead skin and tissue and thus helps in the repair of skin during the healing of burns and skin ulcers. (Ostlie et al., 2012).

34.4.2.5 Lipase

Candida rugosa is the source of these enzymes. Lovastatin, a drug that has the ability to lower the serum level of cholesterol, is prepared from lipase. It is also used in the treatment of malignant tumors as they have the ability to activate the tumor necrosis factor. Dyspepsia, gastrointestinal disturbances, and skin indices of digestive allergies are the other abnormalities in which lipase therapy is used. It is a widely used coronary vasodilator and is synthesized from *S. marcescens* lipase (Matsumae et al., 1993).

34.4.2.6 Nattokinase

A serine proteinase attained from *B. subtilis*, nattokinase affects some factors of blood clotting such as fibrinogen, factor VII, and factor VIII as well as lipids that are related to an augmented risk for cardiovascular disease. Oral administration of nattokinase could be considered as a neutraceutical for cardiac disorders. Nattokinase shows an extended action of preventing blood coagulation and dissolving a prevailing thrombus (Milner, 2008).

34.4.2.7 Serratiopeptidase

It breaks down fibrin, dilutes the fluids shaped from inflammation and injury, and smooths their drainage, which increases the speed of tissue repair. It prevents the proclamation of bradykinin and diminishes pain and inflammation.

34.4.2.8 Sacrosidase

Congenital sucrase-isomaltase deficiency (CSID) can be cured with this enzyme (b-fructofuranoside fructohydrolase). It is obtained from *Saccharomyces cerevisiae*, and can be taken orally.

34.4.2.9 Pegadamase Bovine

It is a modified enzyme used in enzyme replacement therapy for the treatment of diseases associated with a deficiency of adenosine deaminase. The condition is known as severe combined immunodeficiency disease (SCID). It was used in the first successful application of enzyme therapy for a congenital disease. The enzyme adenosine deaminase slashes the extra adenosine present in the circulation of patients and reduces the toxicity to the immune system of the raised adenosine levels.

34.4.2.10 PEGylated Arginine Deaminase

It is an arginine-degrading enzyme that impedes human melanoma and hepatocellular carcinomas. In these conditions, the body is unable to synthesize arginine due to a lack of arginosuccinate synthetase movement.

34.4.2.11 L-Amino Acid-Ligase

Empedobacter brevis is the main source of this enzyme. It catalyzes the ligation of two amino acids, L-alanine and L-glutamine, which ARE easily digested in the human body; hence, it is used in nutritional therapy. It also prevents muscle wasting and increases the synthesis of protein in muscles. In *B. subtilis*, a novel enzyme coded by a gene YwfE was identified, which catalyzed this dipeptide formation from unprotected amino acids in an ATP-dependent manner.

34.4.2.12 Streptokinase, Urokinase

Streptokinase is an enzyme used as a therapeutic due to its thrombolytic activity. It is inactive as such but on combining with the circulating plasminogen, it forms an activator complex that then results in partial proteolysis of other plasminogen molecules to plasmin. Being antigenic, in nature it can even cause hypersensitivity reactions and anaphylaxis. Urokinase unswervingly activates plasminogen and is nonantigenic (Banerjee et al., 2004; Olson et al., 2011).

34.4.2.13 Defensins and Cathelicidins

These are antimicrobial peptides found in the lysosomes of macrophages, polymorphonuclear leukocytes, and keratinocytes. They are a part of the innate mammalian immunity system that helps fight bacterial infections. Cathelicidins obliterate the lipoprotein membranes of microbes enveloped in phagosomes after fusion with lysosomes in macrophages.

34.5 REASONS FOR THE USE OF ENZYMES AS THERAPEUTIC AGENTS

An enzyme's utility as a therapeutic agent leaves some good reasons behind. The low risk of resistance with lysins due to the selective interaction with components of the cell wall-like enzymes act on the peptidoglycan of the bacterial cell wall, and therefore are not likely to

adversely affect mammalian cells, at least not directly. Humoral immune response can decrease the efficacy of protein therapeutics but still can be reduced by coupling enzyme molecules to polyethylene glycol (PEG). Lysins control bacterial pathogens, particularly those found on the human mucosal surface. It specifically kills pathogens on mucous membranes without affecting the surrounding normal flora, thus reducing a significant pathogen reservoir in the population. Many antimicrobials are found to be effective in vitro, but lose their activity in vivo. Renata-Cegielska et al. (2009) found that products obtained after lysozyme modification and containing polymeric forms of the enzyme keep up their effect on fresh meat, which enhances their use to extend the shelf life of meat.

34.6 CONCLUSION

Antibiotic resistance is a primary issue creating an enormous clinical and financial encumbrance on the healthcare system that is wide-reaching. It requires an intricate resolution incorporating the public sector, the private sector, and industry. To overcome the problem of resistant organisms, measures must be taken and strictly followed in regard to prevention, infection control, and the judicious use of antibiotics that provide antibiotic-resistant pathogens a free environment. Enzymes used as therapeutics such as lysozymes, lysins, bacteriocins, defensins, and cathelicidins have a different mode of antimicrobial action than those of traditional antibiotics. In order to control and decontaminate areas polluted by biological weapons or pathogenic or toxic bacteria that have been accidentally released, these therapeutic enzymes should be used in combination with mild chemicals agents or physical treatments. The use of these microbial enzymes has increased greatly during the past in both the industrial and pharmaceutical fields. Enzymes are also used in clinical lab diagnosis with a hope of auxiliary development in the near future. Development in the field of clinical application of enzymes is also seen. Apart from the benefits of enzyme therapy, there are some side effects such as the release of preformed bacterial toxins from the cytoplasm of bacteria during the lysis phenomena. In this regard, the autolysins of some bacterial species are also found to be responsible for the pathogenesis of infections based on the release of different toxins. After acquiring resistance to antibodies, diseases can also be treated by microbial enzymes. If these enzymes are used in combination with routine drugs, the synergistic effect for the treatment of various diseases along with fewer side effects is shown. At present, therapeutic enzymes are available in the market as pills, capsules, powders, and food supplements. Considering the entire pros and cons of enzyme therapy, it can be concluded that more research is still required on these biomolecules so that they could prove a new path of success in overcoming the resistance problem.

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CHAPTER

35

Transforming the Healthcare System Through Therapeutic Enzymes

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35.1 INTRODUCTION

Enzymes are biocatalysts having great affinity and specificity that enhance the rate of reaction. Enzymes play a remarkable role in industry due to their participation in efficient and economical biocatalytic conversions that lead to sustainable industrial development (Beilen and Li, 2002). Their find their applications in food and beverages, textiles, leathers, detergents, dairy, brewing, and, most significantly, pharmaceuticals. According to a report, the global market for industrial enzymes in the 2016 was valued at $4.61 billion and is estimated to grow at a CAGR of 5.8% from 2017 to 2022 to reach $6.30 billion by 2022 (Markets and Markets, 2016). The reason for the rapid growth and development of the enzyme industry is the furtherance of enzyme-based processes, cheaper production methods, new application fields, and new enzyme discoveries. The technological advancement of recombinant DNA technology and genetic engineering make it more feasible to tailor enzymes specifically according to their application and process conditions (Beilen and Li, 2002).

Enzymes have a significant contribution to the pharmaceutical industry. There are approximately 150 executed biocatalytic processes in industry and most of them are going on in the pharmaceutical sector. The employment of enzymes in the pharmaceutical industry makes the process greener, sustainable, and economical. The contribution of enzymes is not only limited to curing diseases (Woodley, 2008). The various roles enzymes play are summarized in Fig. 35.1. The detailed usefulness is discussed in the different sections of this chapter.

35.2 NUTRACEUTICALS AND DISEASE TREATMENT

The term "nutraceutical" was coined by Dr. Stephen DeFelice in 1989. It is derived from the amalgamation of two words: "nutrition" and "pharmaceutical" (Kalra, 2003). The term nutraceutical can be described as food or related products that provide health benefits to

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00035-9

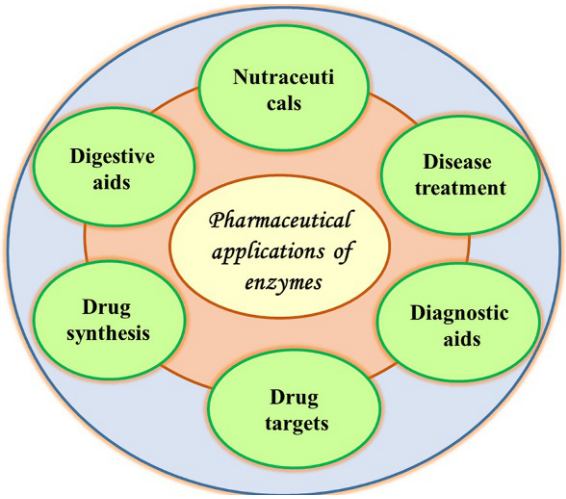

FIG. 35.1 Various application of enzymes in the pharmaceutical industry.

the consumer either by curing or preventing disease. They fulfill the nutritional and energy requirements of the body that should be ideally present in a healthy diet. The health benefits nutraceuticals offers are: (1) no side effects as used as a natural dietary supplement, (2) easy availability and economical, (3) detoxifying the body, (4) avoiding vitamin and mineral deficiencies, (5) restoring healthy digestion and dietary habits, and (6) improving the human medical condition (Chauhan et al., 2013).

35.2.1 Inorganic Mineral Supplements

There are several physiological and metabolic activities regulated by a variety of elements. Most of these demands are fulfilled through diet; however, their deficiency causes abnormal body functions and diseases. Calcium, magnesium, zinc, phosphorous, silicon, iron, copper, and boron are some of the elements that are required for the normal growth and maintenance of human health.

35.2.2 Probiotics

There have been various definitions of probiotics given by different scientists over the years. In 1989, Fuller defined probiotics as "A live microbial feed supplement (that) beneficially affects the host animal by improving its intestinal balance." Havenaar (1992) redefine it as "a viable mono or mixed culture of bacteria, which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora" (Havenaar, 1992). Guarner and Schaafsma (1998) defined it as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" (Gilliland et al., 2001).

Probiotics are primarily composed of the following groups of bacteria:

1. Lactobacilli, whose examples are *L. acidophilus*, *L. casei, L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, and *L. cellobiosus.*

2. Gram-positive cocci, which include *Lactococcuslactis*, *Streptococcus salivarius* subsp. *thermophilus*, and *Enterococcus faecium.*
3. Bifidobacteria, which are *B. bifidun*, *B. adolescentis, B. infantis, B. longum,* and *B. thermophilum.*

The health benefits that probiotics offer include treatment of gastrointestinal (GI)-related diseases such as lactose intolerance, acute diarrhea, and antibiotic-associated GI side effects. The use of probiotics also reduces the risk of systemic conditions such as allergies, asthma, cancer, and several other infections of the ear and urinary tract (Das et al., 2012).

35.2.3 Prebiotics

Prebiotics can be defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon a host's well-being and health" (Roberfroid, 2007). A food ingredient is said to be prebiotic if it resists gastric acidity, hydrolysis by mammalian enzymes, and absorption in the upper gastrointestinal tract. It should also be fermented by the intestinal microflora. Lastly, it should selectively stimulate the growth and/or activity of intestinal bacteria that are potentially associated with health and well-being (Slavin, 2013).

35.2.4 Dietary Fibers

Dietary fibers have a significant role as nutraceuticals. They are of two types, soluble and insoluble dietary fibers. Soluble fibers dissolve in water and help make stools soft and easy to pass, therefore helping treat constipation. They help in the prevention of many diseases such as diabetes, cardiovascular disease, colon cancer, and obesity (Slavin, 2013).

35.2.5 Phytochemicals

Phytochemicals are plant-derived nonnutritional chemicals that have disease preventive properties. Different phytochemicals have different pharmaceutical properties such as antioxidant properties, hormonal action, stimulation of enzymes, interference with DNA replication, antimicrobial properties, etc.

Antioxidant

Phytochemicals such as flavonoids, carotenoids, and polyphenols possess antioxidant properties to act as anticancer, antiaging, and protective actions for cardiovascular diseases, diabetes mellitus, obesity, and neurodegenerative diseases (Zhang et al., 2015).

Hormone-like properties

Some phytoconstituents have human hormone-like properties, including phytoestrogens that are diphenolic compounds similar to the human hormone estrogen. They helps in the avoidance of cardiovascular diseases, menopausal symptoms, postmenopausal osteoporosis, neuroprotective effects, and hormone-dependent cancers (breast and endometrium cancer) through dietary intake (Sunita and Pattanayak, 2011).

Enzyme modulation

The phytochemicals present in diet modulate the activity of metabolic enzymes. They cause induction of drug-metabolizing enzymes. The effect can be both positive and negative.

The example of the positive effect is cancer prevention while the negative effect is pharmacokinetic interactions with coadministered drugs (Mandlekar et al., 2006).

Interference with DNA replication

One of the examples of phytochemicals that interfere with DNA replication is saponins. They are used as an anticancer agent because they cease tumor proliferation by interfering with DNA replication in the tumorous cell (McKeown, 2012). They are also used as an antiviral drug to check the replication of viral DNA in the host. Some fatty acids, polyketides, and sterols also interfere with DNA replication (Eggers and Carcache de Blanco, 2015).

Antimicrobial properties

Phytochemicals such as allicin (from garlic) and isothiocynates (wasabi) possess antibacterial activity (McKeown, 2012). Spice phytoconstituents such as eugenol and cinnamaldehyde (cinnamon) also have antimicrobial properties (Pei et al., 2009).

35.2.6 Herbs as Functional Foods

Herbs such as ginseng and ginkgo have great therapeutic values as they deliver health benefits and prevent many chronic diseases such as cancer and cardiovascular and neurodegenerative disorders. The phytoconstituents act through different mechanisms such as antioxidant activities, mitochondrial stabilizing functions, metal chelating activities, inhibition of apoptosis of vital cells, and induction of cancer cell apoptosis to combat chronic and lifestyle-related diseases (Ferrari, 2004).

35.3 ENZYMES OF MEDICINAL VALUE

The concept of utilization of enzymes in the pharmaceutical industry began about five decades ago. The enzymes possess a wide range of medicinal importance and thus play a significant role in the medical field. There are two inimitable qualities of enzymes that make them excellent drugs and increase their utility over conventional chemical drugs. The unique characteristics include their catalytic properties, converting multiple target molecules to the desired product, and their high affinity and specificity toward the target (Vellard, 2003). There are a wide variety of diseases where the successful use of enzyme therapy is commencing. A few of them are summarized in Fig. 35.2 and discussed below in detail.

35.3.1 Cancer Treatment

As per the World Health Organization report, cancer is one of the major causes of human mortality throughout the world. In 2014, approximately 8.2 million people lost their lives due to cancer and about 14 million new cases were reported. In the next two decades, 70% increase in new cases of cancer will be expected (Fernandes et al., 2017). However, there are various form of cancers that can be successfully treated using enzyme therapy.

L-Asparaginase

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1, ASNase) is an enzyme renowned for its chemotherapeutic applications. For nearly five decades, it has been used in the treatment of acute lymphoblastic leukemia in children. It also finds utility in treating

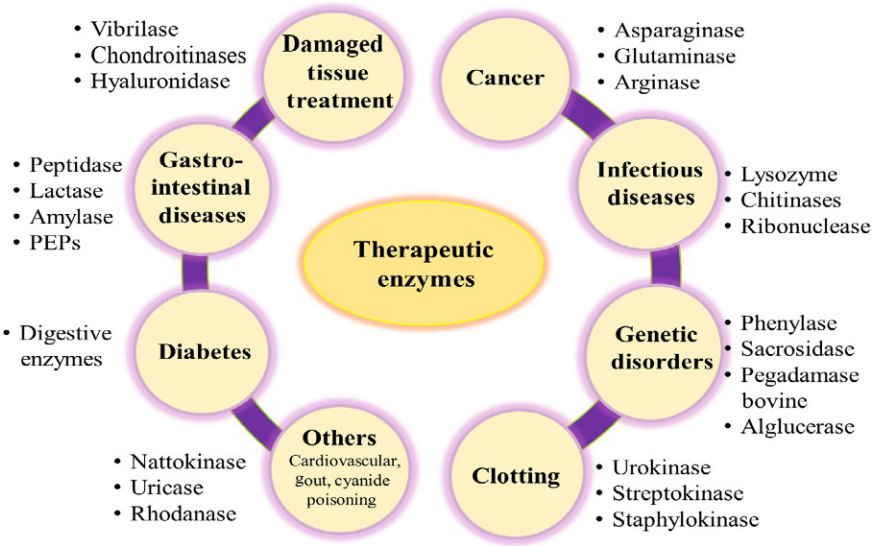

FIG. 35.2 Therapeutic enzymes and their applications in the treatment of various diseases.

other forms of cancer such as Hodgkin's disease, acute myelomonocyticleukemia, lymphosarcoma, and melanosarcoma (Vimal and Kumar, 2017a). There are presently three forms of this enzyme in clinical practice that are native enzymes derived from *Escherichia coli* (*E. coli*-ASP), it's PEGylated form (PEG-ASP), and the enzyme isolated from *Erwinia chrysanthemi*, Erwinia asparaginase (*Erwinia*-ASP) (Lopez-Santillan et al., 2017). In fact, PEG-L-asparaginase (Oncospar; Enzon) is the first polymeric drug to receive approval from the US Food and Drug Administration (USFDA), which happened in 1994 (Petros and Desimone, 2010). It is used in combination therapy with vincristine and a glucocorticoid (e.g., dexamethasone) (Batool et al., 2016). The enzyme kills the cancerous cells by starving them from the essential amino acid L-asparagine present in the plasma pool. It hydrolyzes the L-asparagine present in blood into L-aspartate and ammonia. The growth of tumor cells is inhibited in the absence of this essential amino acid. Similarly, exploiting the amino acid metabolism pathway, the L-arginase enzyme has been positively used in the treatment of breast, rectal, and colon cancers in in vitro studies. However, commercial success has yet to be achieved (Fernandes et al., 2017).

35.3.2 Diabetes Treatment

Diabetes is a serious health threat associated with impairment of sugar metabolism while also affecting the fat and protein metabolisms. Therefore, the digestive enzyme could be used to enhance the proper digestion and nutrient absorption in diabetic patients. The use of three main digestive enzymes—amylase, lipase, and protease—as a dietary supplement will help in improving sugar, fat, and protein digestion. Apart from digestive support, these enzymes impart better circulation, boost the immune system, decrease inflammation, and decrease kidney complications and gastroparesis. In addition, the intake of other supplements such as probiotics, aged garlic extract, L-carnitine, L-glutamine, and Coenzyme CoQ10 would be beneficial for the diabetic patient (Vanta, 2015).

35.3.3 Genetic Disorders Cure

There are many genetic disorders that occur due to the deficiency or absence of a particular enzyme involved in a specific metabolic pathway. The replacement of such an enzyme with an external supply is a way of curing such a disorder, and is called "enzyme replacement therapy" (ERT).

Gaucher disease (GD)

GD is a rare autosomal recessive genetic disorder in which a deficiency of glucocerebrosidase (GCase) leads to an accumulation of glucosylceramide in macrophages. It is caused by mutations in the *GBA1* gene. The disease is characterized by cytopenia, splenomegaly, hepatomegaly, and bone lesions. There are three forms of GD: type-1, type-2, and type-3. Type-1 is generally nonneuronopathic GD while the other two are neuronopathic GD. In the 1990s, alglucerase extracted from the human placenta was used to fulfill the need for GCase in the cell, particularly in Gaucher cells. Presently, it is treated using intravenous ERT using either of three recombinant preparation that are imiglucerase (Cerezyme, Sanofi-Genzyme), velaglucerase (Vpriv, Shire, authorized in 2010), or taliglucerase (Elelyso, Pfizer) (Stirnemann et al., 2017).

Fabry disease (FD)

Fabry disease is another example of a genetic disorder where successful implementation of ERT is achieved. It is a metabolic storage disorder related to the GLA gene at Xq22. In FD, massive accumulation of intralysosomal glycosphingolipids occurs due to a deficiency of a-galactosidase A (a-gal A). The main symptoms of this disease are cardiac complications leading to morbidity and mortality. The primary treatment uses one of two commercially available, genetically engineered, highly purified recombinant a-galA preparations. The first preparation is agalsidase alfa (Replagal) and the second is agalsidase beta (Fabrazyme). The first is produced using a human cell line whereas the second is produced using CHO cell lines (Chinese hamster ovary cell lines). Both forms are capable of reducing globotriaosylceramide concentrations and preventing cardiovascular malfunctioning (Tadevosyan, 2016).

Phenylketonuria (PKU)

PKU is an autosomal recessive inborn error of the metabolism caused by mutations in the phenylalanine hydroxylase (*PAH*) gene, which, in turn, leads to a deficiency of hepatic enzyme phenylalanine hydroxylase (PAH). The enzyme deficiency causes the accumulation of phenylalanine that hampers the intellectual ability of a person. Other symptoms include autistic behavior, motor deficits, eczematous rash, and seizures. Its primary treatment is dietary restrictions. However, it has some limitations that are (a) dietary compliance due to unpalatability of the diet, (b) persisting neurological or psychosocial issues, (c) potential nutritional deficiencies, and (d) financial burdens (Stirnemann et al., 2017). It can be treated with recombinant phenylalanine ammonia lyase (PEGylated) that converts phenylalanine to ammonia and *trans*-cinnamic acid, later excreted from urine in the form of hippurate (Ho and Christodoulou, 2014).

35.3.4 Infectious Disease Treatment

Lysozyme

A lysozyme is a hydrolytic enzyme that is present in body fluids such as serum, saliva, gastric juice, milk, and airway mucus secretions; it also has antimicrobial activity. Studies show

that lysozyme complexed with other components exhibits great antimicrobial activity against both gram-positive and gram-negative bacteria. An example is where scientists made a synthetic complex of equine lysozyme and oleic acid that showed a bactericidal effect against *Streptococcus pneumoniae.* The complex accumulates in the cell membrane of the pathogen and causes irreversible depolarization of membranes; ultimately, bursting of the cell occurs (Żelechowska et al., 2016). Another example of lysozymes in the treatment of infectious disease is where a dimerized lysozyme (KLP-602) was used to treat rainbow trout (*Oncorhynchus mykiss*). It is reported to suppress the pathogenic action of infectious pancreatic necrosis virus (Siwicki et al., 1998). The example of in vivo action of lysozyme is in the following diseases, such as Barrett's oesophagitis, chronic gastritis, gluten-induced atrophic duodenitis (coeliac disease), collagenous colitis, lymphocytic colitis, and Crohn's colitis. In these GI infections, lysozymes are upregulated in their expression to prevent chronic infections (Rubio, 2014).

35.3.5 Anticlotting Agents

Thrombosis is the major cause of myocardial infarction and other cardiovascular diseases that occur due to fibrin in blood vessels. The enzyme-based thrombolytic therapy includes the use of fibrinolytic enzymes such as urokinase (UK), tissue type plasminogen activator (t-PA), and streptokinase (SK).

Urokinase (UK)

The use of UK as a drug in the treatment of thrombotic disorders started in 1958. It was first isolated and purified from human urine and was found to have fibrinolytic activity. However, a few limitations are associated with its use such as a relatively large dosage, a short half-life, frequent dosages, and side effects such as retroperitoneal hemorrhage and intracranial hemorrhage. This shortcoming can be overcome by fabricating the enzymes on nanoparticles. One such attempt was made by Hao and Sun in 2013, where they attached the UK on a chitosan nanoparticle (Hao and Sun, 2013).

Streptokinase

Streptokinase (a nonenzymatic protein) is a protein that has applicability in the treatment of thromboembolic diseases in humans. Its fibrinolytic activity is due to the conversion of inactive plasminogen to active plasmin (Ali et al., 2014). The side effects such as immunogenic reactions, clearance by the antibody, and a short half-life limit its use. Scientists are overcoming this limitation by using PEGylated streptokinase, streptokinase, liposome-encapsulated streptokinase (LESK), and PEG-microencapsulated streptokinase (MESK) (Mohamad et al., 2014).

Nattokinase (NK)

Nattokinase is one of the enzymes that possesses anticlotting properties. It dissolves the blood clot and can be used as a medicating aid in the treatment of cardiovascular diseases. Presently, it is under phase-II clinical trials for atherothrombotic prevention. It is produced during the fermentation process while preparing Natto (a Japanese traditional food). Its most common producer is recombinant strains of *Bacillus subtilis, E. coli,* and *Lactococcus lactis*. It functions by hydrolyzing fibrin and plasmin, converting prourokinase to urokinase, degrading PAI-1 (plasminogen activator inhibitor-1), and elevating the tissue plasminogen activator (t-PA), which supports fibrinolytic activity. It has little or no side effects as compared to other fibrinolytic proteases (Weng et al., 2017).

35.3.6 GI Disorders Treatment

There are a wide range of GI disorders such as cystic fibrosis, lactose intolerance, etc., where digestion is impaired due to a disturbance in the body's metabolism. The external supplementation of digestive enzymes is a possible solution to deal with these diseases by improving fats, carbohydrate, and proteins digestion. A few of them are discussed below:

Exocrine pancreatic insufficiency (EPI)

The external supplementation of the pancreatic enzyme is a way to manage various disorders that occur due to inefficient production or a lack of production of digestive enzymes naturally. Exocrine pancreatic insufficiency (EPI) in chronic pancreatitis, pancreatic cancer, cystic fibrosis (CF), or diabetes is an example of this type of digestive defects.

Lactose intolerance

About 75% of the worldwide population suffers from hypolactasia or some decrease in lactase activity, which causes lactose intolerance (Ianiro et al., 2016). To overcome this problem, patients are supplemented with a lactase enzyme tablet orally that is quite helpful in the management of this condition (Francesconi et al., 2016).

Celiac sprue

Celiac sprue is an inflammatory disease of the small intestine in which a patient is intolerant to the gluten present in dietary intake. Generally, patients are recommended to avoid gluten in order to prevent any immunogenic reaction. However, an alternative to this is an oral enzyme therapy in which the patient is supplemented with glutenase enzymes that participate in the proteolysis of the gluten present in food and thus, helps in the effective management of this GI defect (Bethune and Khosla, 2012). Prolyl endopeptidases (PEPs) are a group of serine proteases that are also found effective in digesting gluten and treating celiac disease (Ianiro et al., 2016).

35.3.7 Repairing Damaged Tissue

Chondroitinase ABC (ChABC)

Damage to body tissues can occur due to various reasons such as injuries, burning, etc. Here, enzymes can also be utilized for repairing tissues and restoring their normal functions. Chondroitinase ABC (ChABC) is used in the treatment of spinal cord injuries by degrading chondroitin sulfate proteoglycans (restrict with tissue regeneration). ChABC doesn't interfere with other therapies. Therefore, it can be used as a part of combination therapy along with cell implantation, growth factors, myelin-inhibitory molecule blockers, and ion-channel expression (James et al., 2015; Zhao and Fawcett, 2013).

Hyaluronidase

The enzyme *hyaluronidase* is used in the treatment of upper limb spasticity, a movement disorder caused due to neurologic injury such as a brain tumor, cerebral palsy, or spinal cord injury. It hydrolyzes hyaluronan, eases muscle stiffness, and helps in regaining joint movement (Raghavan et al., 2016).

35.4 DIAGNOSTIC ENZYMES

35.4.1 Enzymes as Markers of Disease and Pathology Diagnosis

Enzymes play a vital role in human metabolic activities. Any abnormalities/disorders in normal physiology cause disturbances in the enzyme concentration in the human body.

The change in enzyme concentration (either an increase or a decrease) is exploited for diseases diagnosis. The enzymes whose concentration is remarkably changed during such malfunctioning are called "marker enzymes" for that particular disease. There are a wide range of diseases where enzymes are used as markers, including cancer, diabetes, autoimmune diseases, liver and heart malfunctions, etc. Examples of a few such enzymes are discussed below:

Lactate dehydrogenase (LDH)

LDH is an important biological biomarker for diagnosing various diseases such as heart, liver, and muscle-related disorders along with some forms of cancer. Its normal concentration is 60–250 IU/L. Any diversion from this indicates health-related problems. It is helpful in detecting various forms of cancers, including lymphoma, leukemia, breast cancer, and colon cancer (Hemalatha et al., 2013). It has also been reported that its concentration elevated during advanced renal cell carcinoma and was used as a prognostic biomarker for this disease (Maroto and Rini, 2014). It is also a marker of ischemic myocardial injury.

Along with aspartate aminotransferase (AST), creatine kinase (CK), and isoenzyme MB, it is used in the early diagnosis of suspected cases (Fontes et al., 1999).

Creatinine phosphokinase (CPK)

CPK is another significant marker enzyme whose normal concentration ranges from −60 IU/L in the human body. The three major forms of these enzymes are CPK-1, CPK-2, and CPK-3; each form is used in the diagnostic of different disorders. CPK-1 is the marker for brain and lung injuries while the increase in CPK-2 indicates heart-related problems. In the third form, CPK-3 is elevated from its normal concentration during bone and skeletal muscle-related defects (Uppangala, 2010).

Alkaline phosphatase

Alkaline phosphatase is another biological marker of prime importance. Its increased concentration indicates liver damage, hyperthyroidism, and bone defects (rickets, osteomalacia, osteoblastic activity). The decrease in concentration is observed in anemia, scurvy, and defective calcification (Uppangala, 2010).

Acetylcholinesterase and butyrylcholinesterase (AChE and BChE)

AChE and BChE are homologous enzymes that are expressed in the serum. Both enzymes are biomarkers for parasympathetic malfunctioning (Shenhar-Tsarfaty et al., 2014). The level of BChE is also altered in both hypercholesteremia and hypocholinesterasemia and thus, is a biological marker for the same. Fluctuation in its concentration in plasma is also observed in hepatocellular carcinoma, chronic liver diseases, and poisoning with carbamates or organophosphates that can be easily diagnosed by activity assay (Pohanka, 2013).

Cyclooxygenase-2 (COX-2)

It helps in the early detection and prevention of many cancers. Studies suggest that COC-2 expression is significantly high during the process of tumorigenesis and plays a vital role in tumor progression (Hemalatha et al., 2013).

35.4.2 Enzymes in Immunoassays

Apart from disease markers, enzymes play a vital role in the detection of diseases through their applicability in different immunoassays. They are better substitutes for radioisotopes as they are readily available and less hazardous. The most common type of immunoassays where enzymes are used are the enzyme-linked immunosorbent assay (ELISA), the

enzyme immunoassay (EIA), and the enzyme-multiplied immunoassay test (EMIT). ELISA is a highly sensitive assay used for the detection of both antigens and antibodies. The most common enzymes used in this assay are Horseradish peroxidase, alkaline phosphatase, and β-galactosidase. They are very useful for the detection of infectious diseases caused by bacterial, viral, mycotic, or parasitic organisms (Raja et al., 2011). They also have utility in detecting noninfectious diseases where hormones, drugs, serum components, oncofetal proteins, or autoimmune diseases are involved. ELISA is also a very important tool in detecting drug pharmacokinetics, pharmacodynamics response, clinical efficacy, and patient safety. It detects the antidrug antibodies induced by biologic therapeutics through bridging assays (Partridge et al., 2016). EMIT is another enzyme-labeled immunoassay that detects thyroxine by the use of malate dehydrogenase (Raja et al., 2011).

35.4.3 Biosensor-Based Diagnosis

A biosensor can be defined as "a self-contained analytical device that combines a biological component with a physicochemical device for the detection of an analyte of biological importance" (Hasan et al., 2014). It consists of three major components: (1) a sensing element (an enzyme, a microorganism, or biomolecule) that recognizes the analyte and generates a signal, (2) a signal transducer (electrical, optical, electronic, etc.), and (3) a reader device. The use of enzyme-based biosensors has a vivacious role in biomedical diagnostics due to their high selectivity, rapid analysis of biological and chemical species, high sensitivity, and direct application (Cao et al., 2011).

Diabetes

Diabetes is a typical example where enzyme-based biosensors found applicability. The amount of blood sugar can be easily monitored with this help and thus, is very helpful in diabetes management. A glucose biosensor is comprised of glucose oxidase (GOx, a standard enzyme) coupled with an electrochemical system that measures the blood sugar level by sensing oxygen consumption or H_2O_2 production. The basic reaction involves the oxidation of β-D-glucose by molecular oxygen-generating gluconic acid and hydrogen peroxide by Gox, along with cofactor flavin adenine dinucleotide (FAD). The series of reactions that take place are:

$$\text{Glucose} + \text{GOx} - \text{FAD}^{+} \rightarrow \text{Glucolactone} + \text{GOx} - \text{FADH}_2$$

$$\text{GOx} - \text{FADH}_2 + \text{O}_2 \rightarrow \text{GOx} - \text{FAD} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 \rightarrow 2\text{H}^{+} + \text{O}_2 + 2\text{e}^{-}$$

GOx is highly selective for glucose, readily available, tolerates extremes of pH, has ionic strength, and temperature-relaxed storage norms increase its utility (Yoo and Lee, 2010).

Renal and liver disorders

The end product of protein metabolism in the human body is urea that can be detected using an enzyme-based biosensor having the urease enzyme (EC 3.1.3.5) immobilized in it. The model biocatalyst urease catalyzes the hydrolysis of urea inti ammonia and bicarbonate (Monošík et al., 2012).

$$\text{Urea} + \text{O}_2 \xrightarrow{\text{Urease}} 2\text{NH}_3 + \text{CO}_2$$

The urea cycle starts in the liver and the urea formed is carried out to the kidneys so that it can be filtered and eliminated from the body in the form of urine. A low level of urea in the body is an indication of liver dysfunction and low protein intake. The increase in blood urea occurs due to a severe renal disease that is known as uremic syndrome (Koncki, 2007).

Renal, muscular, and thyroid dysfunctions

The elevated level of creatinine in the body indicates renal, muscular, and thyroid dysfunctions. It is also useful in the diagnosis of acute myocardial infarction and the quantitative description of hemodialysis therapy (Hemalatha et al., 2013). The monoenzymatic creatinine biosensors quantify the ammonia generated during creatinine hydrolysis that is catalyzed by creatinine deiminase (creatinine iminohydrolase, EC 3.5.4.21). On the other hand, three enzyme-based systems are based on the hydrolysis of creatinine to creatine, then creatine to sarcosine, followed by oxidation of sarcosine to glycine (Monošík et al., 2012).

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatinine amidohydrolase}} \text{creatine}$$

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatine amidinohydrolase}} \text{sarcosine} + \text{urea}$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{Sarcosine oxidase}} \text{glycine} + \text{formaldehyde} + H_2O_2$$

Lactate sensor

Lactate levels upturn in several pathological conditions such as cardiogenic or endotoxic shocks, respiratory failure, liver disease and systemic disorders, renal failure, and tissue hypoxia. L-Lactate oxidase and L-lactate dehydrogenase are two common enzymes used in lactate biosensors. L-Lactate oxidase catalyzes the oxidation of L-lactate to pyruvate and to generate hydrogen peroxide that could be quantitatively detected by the sensor (Rassaei et al., 2014).

$$\text{L} - \text{Lactate} + O_2 \xrightarrow{\text{L-lactate oxidase}} \text{pyruvate} + H_2O_2$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

Lactate dehydrogenase-based catalysis occurs in the presence of coenzyme (NADH or NADPH), as shown below:

$$\text{L} - \text{Lactate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{pyruvate} + \text{NADH}$$

35.5 TARGETING ENZYMES FOR DRUG DISCOVERY

The discovery of new drugs is a multistep process that begins with target identification. The occurrence of new epidemics, multiple drug resistance, and drug side effects are some reasons that encourage researchers to search for novel drug targets.

35.5.1 Reason of Selection of Enzymes as Drug Targets

Enzymes are a general drug target class and are highly validated. There are two main reasons for targeting enzymes for drug discovery. First, the enzymes play a vital role in human

physiological activities that are essential for all life processes and are generally modified during a diseased state. Second, they possess a unique binding site where drug molecules can bind and modulate the activity (Copeland et al., 2007).

35.5.2 Role of Enzymes in Human Diseases

Although enzymes are essential for all life processes, fluctuations from their normal activity lead to disease. For instance, in the case of infectious diseases, the expression of certain enzymes increases that provide a survival benefit to the pathogen inside the host and also helps in their proliferation. This can be illustrated with the example of the L-asparaginase enzyme. In pathogens such as *Salmonella typhimurium, Shigella flexneri, Yersinia* sp., and *Helicobacter pylori,* the enzymes suppress the T-cell-mediated immune response in the host and help the pathogen in its survival within the host. The enzyme is also found to play an essential role in the colonization of *Campylobacter jejuni* inside the liver of the host (Vimal and Kumar, 2017b). *Streptococci, staphylococci*, and certain *Clostridium* species produce hemolysins to destroy red blood cells and invade the host. Streptokinase, hyaluronidase, and collagenase are also produced by pathogens to combat the host immune system. Streptokinase is a streptococcal enzyme that dissolves blood clots. Hyaluronidase (produced by *Streptococci, Staphylococci,* and *Clostridia*) destroys the hyaluronic acid of connective tissue. Neuraminidase secreted by *Vibrio cholerae* and *Shigella dysenteriae* causes degradation of the neuraminic acid of the intestinal mucosa. Collagenase produced by *Clostridium* sp. disrupts the collagen of connective tissue in a way that causes infection (Ranjan et al., 2015). In contrast, *Staphylococci* produces coagulases that clot the blood. It helps the pathogen to escape from the body's phagocytes and contributes to its pathogenicity (Cheng et al., 2010). Enzyme activities are also altered in other diseases caused due to genetic and/or environmental alterations, as they disturb the natural expression or posttranslational modification (Copeland et al., 2007).

35.5.3 Enzyme Inhibitors as Drugs

Almost 50% of drug molecules that are in clinical use are enzyme inhibitors Their pathophysiologic importance and drugability make them the choice of for potential targets. A few examples where enzymes are inhibited by small molecules in order to cure the particular disease where the enzyme modulation is involved in a virulence mechanism are presented in Table 35.1.

35.6 ENZYMES IN DRUG SYNTHESIS

35.6.1 Enzyme-Catalyzed Organic Synthesis

The use of the traditional (nonbiological) organic synthesis approach has now been replaced with enzyme-catalyzed chemical transformations. The reason behind this is that enzymes are highly specific and function at room temperature under neutral aqueous conditions without the need of substrate functional group protection. The enzyme-mediated organic synthesis may use a single enzyme, multiple enzymes, or a combination of enzymes and nonbiological catalysts. Both natural and genetically engineered enzymes are used in the pharmaceutical industry (Koeller and Wong, 2001).

TABLE 35.1 Enzymes Involved in Disease-Causing Mechanisms and Their Potential Inhibitors as Drugs

S. No.	Enzyme	Disease	Inhibitor
1.	Sphingosine kinase	Cancer	SKI-II ([2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole]
2.	Guanosine 5′-monophosphate reductase	Trypanosomosis	Mycophenolic acid
3.	Dihydrofolate reductase	Infectious diseases	Methotrexate
4.	Histone deacetylase	Cancer	Trichostatin A
5.	Glycogen Synthase Kinase-3	Alzheimer's disease and Type 2 diabetes	Tideglusib and LY-2090314
6.	Cyclooxygenase	Inflammatory diseases	Aspirin and the other NSAIDs
7.	5α-reductase inhibitors	Benign prostate hyperplasia	Finasteride and Dutasteride
8.	HMG-CoA reductase	Preeclampsia	Statins
9.	Renin (angiotensinogenase)	Hypertension, heart failure, diabetic nephropathy, and chronic kidney disease	Aliskiren
10	Tyrosinase	Hyperpigmentation	Arbutin

35.6.2 Synthesis of Chiral Drugs

It is noticeable that drug molecules are generally chiral and both the enantiomers show different biological activity. Therefore, it is necessary to produce/synthesize the anenantiopure form of the drug (either of the one form that possesses drug property). It can be achieved either through chemical or chemoenzymatic means. However, the enzymatic processes are preferable due to high enantioselectivity, regioselectivity, economical efficiency, and reusability (through immobilization). Apart from these, the process can be feasible at ambient temperatures and atmospheric pressures that help in overcoming problems such as isomerization, racemization, epimerization, and rearrangement (Patel, 2004). The various enzymes used in the synthesis of chiral drugs are summarized in Table 35.2.

35.6.3 Enzyme Synthesis of Rare Sugars—Precursors for the Synthesis of Drugs with Few Side Effects

Rare sugar has numerous commercial as well as pharmaceutical applications such as acting as precursor molecules used as drugs against many diseases. The drug that is synthesized from rare sugar has very few side effects in comparison with drugs synthesized from natural sugars (Langan, 2014). There are three classes of enzymes that are involved in the synthesis of rare sugars: (1) isomerase, (2) epimerase, and (3) oxidoreductase (Langan et al., 2014). One of these enzymes is xylose isomerase (EC 5.3.1.5), which catalyzes the interconversion of D-xylose and D-xylulose. Researchers found that this enzyme is also involved in the epimerization of the D- and L-forms of xylose and arabinose, forming lyxose and ribose, respectively. L-Ribose thus synthesized has grabbed attention, as it is used as the precursor of different antiviral and anticancer drugs (Langan et al., 2014).

TABLE 35.2 Enzymes Used in Chiral Drug Synthesis for the Treatment of Diseases

S. No.	Compound	Enzyme Involved in Synthesis	Therapeutic Specialization	Treatment of Disease
1	Atazanavir	Leucine dehydrogenase and Formate dehydrogenase	Antiviral	HIV
2	Indinavir	Naphthalene dioxygenases and Naphthalene monooxygenase	Antiviral	HIV
3	Lobucavir	Lipase	Antiviral	Herpes virus and hepatitis B
4	Ribavirin	Lipase	Antiviral	Hepatitis C
5	Pleuromutilin or mutilin	–	Antiinfective	Infectious disease
6	Paclitaxel	Taxolase, deacetylase and xylosidase	Anticancer	Cancer
7	Oral taxane	Lipase	Anticancer	Mammary gland cancer, colon cancer, ovarian cancer
8	Buspirone	L-Amino acid acylase	Antidepressant	Anxiety and depression
9	Saxagliptin	Phenylalanine dehydrogenase	Antidiabetic	Diabetes
10	Abacavir	γ-Lactamase	Antiviral	HIV and Hepatitis-B
11	Crixivan	Toluene and naphthalene dioxygenases as well as a naphthalene monooxygenase	Antiviral	HIV
12	Carbovir	Adenosine deaminase	Antiviral	HIV
13	Zanamavir	Aldolase	Antiviral	Type A and B influenza
14	Epivir	5′-Nucleotidase and alkaline phosphatase	Antiviral	HIV and Hepatitis-B

35.7 RECOMBINANT PHARMACEUTICAL ENZYMES

35.7.1 Microbial Strains Used for Production

The use of genetic engineering recombinant DNA technology enables the safer, economical, and desired amount of protein/enzymes production at controlled conditions. The production therapeutics on a large scale in the industry require a host. There are about 151 recombinant pharmaceuticals that are approved by the USFDA and/or by the European Medicines Agency (EMEA). Their production is mediated through the use of microbial cells, either bacteria or yeast, as hosts and they are often termed microbial factories. The most common choice of bacteria is *E. coli*. Other than this, *Bacillus subtilis* is also used. However, its use is restricted when a posttranslation modification (PTM) is desirable in the protein produced. To meet the

requirement of PTM, yeast cells (*Saccharomyces cerevisiae*) are used. Apart from these, insect cell lines, hybridoma cell lines, hamster cell lines, and human cell lines are also preferred (Ferrer-Miralles et al., 2009).

35.7.2 Industrial Production of Recombinant Pharmaceutical Enzymes

Enzymes are a major part of the biopharmaceutical industry and have a wide range of applications, as discussed earlier in this chapter. The enzyme with specific characteristics and large quantities can be produced by employing recombinant DNA technology. The technique enables tailoring the enzyme according to its desired application. At the beginning of commercial-scale enzyme production, they were derived from either animal or plant sources. This causes low availability and high prices. The use of microbial sources results in higher production. Also, it is relatively simpler and easier to use, more economical, and can be easily manipulated at the genetic level to get the desired qualities in the enzymes. Presently, about 50% of enzymes are made by yeasts and molds and 30% by bacteria whereas animal and plants provide 8% and 4%, respectively (Demain and Vaishnav, 2009). The few examples of commercially marketed recombinant enzymes are recombinant dornase alpha (Pulmozyme for the treatment of cystic fibrosis), alteplase (Activase, dissolves blood clots), galsulfase (Naglazyme, for the treatment of mucopolysaccharidosis VI), idursulfase (Elaprase, for the treatment of Hunter's disease), velaglucerase (VPRIV, for the treatment of type 1 Gaucher disease), taliglucerase alpha (ProtalixBiotherapeutic, also Gaucher disease), Imiglucerase (Cerezyme, Gaucher disease), miglustat (Zavesca, Gaucher disease), and alfagalsidase (Replagal, Fabry's disease) (Demain and Vaishnav, 2009).

35.8 IMMOBILIZED ENZYME APPLICATIONS IN PHARMACEUTICALS

Enzymes holds tremendous potential for numerous applications with great affinity and efficacy. The further enhancement of their efficiency could be possible with technology such as enzyme immobilization. The advantages immobilization offers are recycling, economical efficiency, and control over the process. The immobilized enzymes are associated with two functions that are catalytic and noncatalytic. The catalytic activity is associated with the activity and stability of the enzyme and the noncatalytic with shape as well as mechanical and chemical stability of the carrier. The few examples of immobilized enzymes are NZ 435, produced by Novozymes, which is Candida Antarctica lipase B absorbed on macroporous beads; covalent immobilized penicillin G acylase for the production of antibiotics; and immobilized glucose isomerase for the continuous production of fructose syrup (Schoevaart, 2008). One of the major support systems for enzyme immobilization is nanoparticles. The applications of these immobilized systems are in diagnosis (nanoparticle-based biosensors), delivery agents for drugs (enzyme-nanoparticle conjugates), and the synthesis of biomolecules (e.g., enzyme-nanoparticle conjugates for chiral drug synthesis).

35.9 ENZYME DELIVERY SYSTEMS

35.9.1 Oral or Inhalable Enzymes

Apart from digestive enzymes, enzymes generally used as drugs are delivered through intravenous injection. Oral administration of enzymes is very challenging. However, with the advancement of technology and delivery systems, there are various enzyme formulations that are delivered orally. A chemical modification such as enteric coating and the use of a mucoadhesive polymeric system shield them against stomach acid and intact enzymes could absorb through the intestinal wall (Shaji and Patole, 2008). An example is glutenases, which is used in the treatment of celiac sprue, an inflammatory disease of the small intestine. In this disorder, the person shows an inflammatory response against the dietary component gluten. Glutenases are used as a part of oral enzyme therapy to help in proteolysing gluten and reducing inflammation (Bethune and Khosla, 2012). Another example is the delivery of enzyme 2 (ACE2) and its enzymatic product, angiotensin-(1–7) [Ang-(1–7)] to cure pulmonary hypertension (pH). PH is caused due to deficiency of ACE2 and Ang-(1–7) and repetitive dosage is a requirement of the therapy. In order to provide stability and protection against gastric enzyme degradation, scientists developed a novel approach by encapsulating the enzyme within the plant cell. They employed transplastomic technology, through which chloroplasts produce the therapeutic enzyme/protein within the plant leaves. This process is relatively economical and safer as there is less chance of endotoxin contamination. Another, similar example where plant-based therapeutics are used is taliglucerasealfa (Elelyso). It is approved by the USFDA and used in the treatment of Gaucher disease (Shenoy et al., 2014).

Therapeutic enzymes can also be delivered through the nasal passage and such enzymes are called inhalable enzymes. The most common example of an inhalable enzyme is Dornase alfa (Pulmozyme), which is used in the treatment of cystic fibrosis, a pulmonary disorder. It is a purified solution of recombinant human deoxyribonuclease (rhDNase) that enhances the clearance of lung secretions by promoting digestion of airway extracellular DNA released from neutrophils (Yang et al., 2017). In another attempt, researchers developed DNase I-loaded poly-lactic-*co*-glycolic acid-microparticles (PLGA-MP) for mucus clearance in cystic fibrosis. This formulation is highly biocompatible, enhances enzyme stability, and preserves its activity (Osman et al., 2013).

35.9.2 Nanoparticle Immobilized Enzyme

There are many nanoparticle delivery systems used for various drug deliveries: polymeric, liposomal, magnetic, gold, silver, quantum dots, carbon nanotubes, dendrimers, etc., as summarized in Fig. 35.3.

Nanostructured biomaterials and nanoparticles, in particular, have particularly remarkable physicochemical properties such as a very small size (size range 1–1000 nm), large surface area, high reactivity, and a functionalizable structure. They offer several advantages such as improved serum stability of the drugs, prolonged systemic circulation, target delivery of drugs, sustained and controlled release of the drug, concurrent delivery of multiple drugs to the same cell in combination therapy, etc., making them a promising drug delivery system (Zhang et al., 2010). There are many enzymes that are being immobilized on nanoparticles

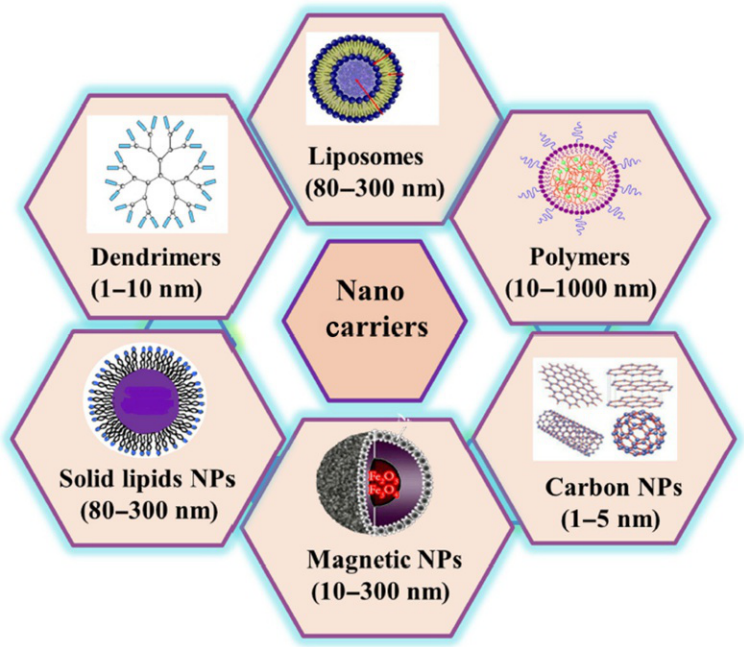

FIG. 35.3 Different types of nanoparticles as enzymatic drug delivery agents.

and used as drugs. In 1994, PEG-L-asparaginase (Oncospar; Enzon) became the first polymeric nanoparticle therapeutic to receive USFDA approval for the treatment of acute lymphocytic leukemia (Petros and Desimone, 2010). Some enzymes of medicinal significance that are immobilized on nanoparticles are presented in Table 35.3.

35.10 CONCLUSION AND FUTURE PROSPECTS

The advancement of scientific knowledge and technology leads to significant developments in the pharmaceutical industry. The latest enzyme technology enables us to produce safer, cheaper enzymes with enhanced potency and specificity that contribute to a healthy life. About 2500 years ago, Hippocrates said, "Let food be thy medicine and medicine be thy food," a statement that is quite relevant in today's scenario. The self-awareness and consumption of nutraceuticals under expert supervision will be useful in maintaining a healthy life and combating lifestyle-related diseases such as obesity, diabetes, and cardiovascular diseases. Nutraceuticals have tremendous potential for nutritional and medicinal effect. However, there is a need to be careful about their negative aspects, such as microorganism count, adulterants, fatty acids, and inorganic pollutant concentrations (Das et al., 2012).

In this chapter, we have discussed many enzymes of medicinal value and their role in curing diseases. However, sometimes their clinical implementations are restricted because of issues related to their practical use, such as poor stability and immunogenicity as well as potential systemic toxicity. The solution to this problem is targeted delivery of therapeutic

TABLE 35.3 Enzymes Immobilized on Varieties of Nanoparticles and Their Therapeutic Applications

S. No.	Enzyme	Nanoparticle Composition	Application	Special Feature
Polymeric nanoparticle				
1	L-Asparaginase	Polyethylene glycol	Chemotherapy	Increased solubility and stability, decreased immunogenicity
2	L-Glutaminase	Polyethyleneglycol (PEG)-polyhydroxybutyrate (PHB)	Chemotherapy	Increased stability, decreased immunogenicity
3	Hyaluronidase	Chitosan-hyaluronidase-5-fluorouracil polyethylene glycol-gelatin	Chemotherapy	Increased bioavailability, controlled release
4	Streptokinase	Chitosan	Thrombolytic therapy	Increased half-life, high loading capacity
5	Urokinase	Chitosan	Thrombolytic therapy	Biocompatibility, controlled release
6	Uricase	Poly(lactic-*co*-glycolic acid)	Gout treatment	High skin permeation
7	Collagenase	Mesoporous silica	Skin cancer treatment	High penetration and slow degradation
Liposomes				
8	Lysozyme	L-α-Phosphatidylcholine, egg yolk and cholesterol	Antimicrobial Agent	Control drug release and bioavailability
9	α-Chymotrypsin	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	Anti-inflammatory	Thermal stability
10	Glucose oxidase (GO)	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	Diagnosis of glucose in blood	Stability
Dendrimers				
11	Trypsin	Polyamidoamine	Anticoagulant Anti-inflammatory Digestive aid	Thermal stability
12	Lipase	Poly(phenylenesulfide)	Biocatalysts for the synthesis of pharmaceutical products and organic compounds	Improved the optimum pH and caused the temperature, Thermal Stability
Solid lipid nanoparticles				
13	Superoxide dismutase	Compritol and oleic acid	Skin ulcer lesion especially due to burns	High skin penetration and high stability
14	Catalase	Lecithin, triglyceride and Poloxmer 188	Antioxidant	Stability and resistant to proteolysis

TABLE 35.3 Enzymes Immobilized on Varieties of Nanoparticles and Their Therapeutic Applications—cont'd

S. No.	Enzyme	Nanoparticle Composition	Application	Special Feature
15	Coenzyme Q10	Compritol 888 ATO, Poloxamer 188 and Tween 80	Dermal disease	Increased skin penetration and stability
Magnetic nanoparticle				
16	L-Asparaginase	Hydrogel-magnetic nanoparticles	Chemotherapy	Increased biocompatibility and stability
17	Lysozyme	Superparamagnetic iron oxide/silica nanocomposites	Antimicrobial agent	Stability and controlled release
18	Trypsin	Chitosan magnetic nanoparticles (Fe_3O_4-CTS)	Digestive aid	Thermal and pH stability
19	β-Glucosidase	Starch-coated magnetic iron oxide nanoparticles	Chemotherapy	Site directed delivery and thermal stability
Carbon nanotubes				
20	Superoxide dismutase	Multi-walled carbon nanotubes	Antioxidant	Imparts stability, accessibility, selectivity, and reduces leaching
21	α-Glucosidase	Amine-functionalized multi-walled carbon nanotubes	Biosensor-measuring antidiabetic potential of medicinal plants	Good sensitivity, rapid response, reusability

enzymes. There are many approaches to targeted delivery that depend on the nature of the enzyme and its intended use. It may be the use of other proteins (e.g., conjugation of urokinase to fibrinogen), magnetic carriers (e.g., magnetite-based ferromagnetic colloid and aspirin), liposomes (e.g., tPA-loaded liposomes), RBCs (e.g., RBC-tPA conjugates), nanoparticles, etc. (Maximov et al., 2009). Nanoparticles as the delivery agent hold tremendous potential and a wide horizon regarding the same has yet to be explored. They provide stability, increase half-life, and decrease immunogenicity to the drug.

The diagnostic enzyme is another considerable aspect of enzyme utility in the pharmaceutical industry. They are markers of many diseases and help in the detection of many life-threatening diseases. Commercial success is achieved in many cases but there are still areas for improvement. The improvement needed is in terms of reusability, stability, accuracy, sensitivity, process automation, and cost effectiveness. The use of nanotechnology-based sensors and lab-on-a-chip technologies (microfluidics) offer a solution toward this goal (Song et al., 2006). The medical relevance of enzymes can further be extended as potential drug targets. The advancement of structural biology, molecular modeling, and high-throughput screening are the powerful tools that make drug discovery much faster (Copeland et al., 2007).

The enzymes are also extensively used in the process of drug synthesis. Enzymes such as lipase are very useful in chiral drug synthesis to provide the enantiopure drug. The modern techniques such as Dynamic kinetic resolution use a combination of enzymatic resolution

and transition metal-catalyzed racemisation (Huerta et al., 2001). The enzymes isolated from extremophiles need more exploration so they can tolerate harsh industrial conditions (Ghanem, 2007). The demand of the present scenario is the development of biocatalytic processes with a high acceleration process rate and stereoselectivity and that offer a low cost of production.

These are the few areas where enzymes are being successfully used in the pharmaceutical industry, having a positive impact on transforming healthcare. In spite of much accomplishment, there is a further scope for development with the growing demand and advancement of the science of technology.

Acknowledgment

We are incredibly grateful to the support provided by NIT Raipur.

Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER

36

Enzymes in the Pharmaceutical Industry for β-Lactam Antibiotic Production

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36.1 INTRODUCTION

In the pharmaceutical industry, the discovery and development of β-lactam antibiotics (BLAs) are considered to be a primary finding (Demain and Elander, 1999). Most β-lactams inhibit bacterial cell wall biosynthesis, others act as antifungal agents (e.g., some clavams), and even others act as beta-lactamase inhibitors (e.g., clavulanic acid). These compounds were discovered in filamentous fungi; however, they have also been identified in actinomycetes and gram-negative bacteria (Liras and Martin, 2006). Naturally occurring β-lactam are classified in four basic structural groups: penicillins/cephalosporins, carbapenems, clavams, and monocyclic β-lactams (Tahlan and Jensen, 2013). β-Lactams contain a four-member beta-lactam ring, and their second ring structure allows the classification (Liras and Martin, 2006). Beta-lactam antibiotics, particularly penicillins and cephalosporins, represent one of the largest biotech markets in the world, with annual sales up to $15 billion, which accounts for approximately 65% of the total antibiotic market. Semisynthetic beta-lactam antibiotics make up the bulk of antibiotic sales worldwide: 3×10^7 kg/year out of a total of 5×10^7 kg/year (Chandel et al., 2008). The annual consumption of immobilized PGA, for the production of

https://doi.org/10.1016/B978-0-12-813280-7.00036-0

beta-lactam antibiotics, is estimated in the range of 10–30 million tons (Souza et al., 2005). Penicillin G is known as the first molecule with antibiotic characteristics to be used for the treatment of various infectious diseases. In 2000, a global production of 40,000 tons was estimated and is still considered to be one of the fastest growing areas (6%–15% per year). The 6-aminopenicillanic acid (6-APA) is a very important intermediate, mainly from the medical point of view because it is widely used for the production of semisynthetic antibiotics such as amoxicillin and ampicillin, among others. The 6-APA is mainly produced in China, India, and Europe. For its part, China has generated 6-APA for more than 10 years and in 2001, manufactured about 4000 tons (Demain and Elander, 1999).

The β-lactam ring in penicillin/cephalosporin is formed by isopenicillin N synthase. In clavam compounds this ring is manufactured by β-lactam synthetase while in carbapenem this ring is set by carbapenem synthetase (Tahlan and Jensen, 2013). In cephamycin synthesis, the other two enzymes (lysine 6-aminotransferase and piperideine-6-carboxylic acid dehydrogenase) play an important role. The gene lat for lysine 6-aminotransferase is located in the cephamycin gene cluster (Martin, 1998). Most carbapenem BLAs have been isolated from *Streptomyces* species (Bodner et al., 2011) while the simple carbapenem biosynthesis requires three enzymes (CarA, CarB, and CarC). Thienamycin, also known as Thienpenem, was the first among the naturally ocurring class of carbapenem antibiotics to be discovered and isolated. However, its manufacture is more complex, although it is initiated in the same manner as other di-substituted carbapenems, and to the simplest carbapenem sharing only two common steps before diverging (Bodner et al., 2011). A nonribosomal peptide synthetase is responsible for penicillin, cephalosporin, and cephamycin synthesis. In this case, these antibiotics are obtained by the condensation of L-alpha-aminoadipic acid, L-cysteine, and L-valine (Martin, 1998).

36.2 β-LACTAM ANTIBIOTICS

Beta-lactam antibiotics are the most widely utilized antibacterial agents in the world. These compounds efficiently inhibit the bacterial transpeptidases known as penicillin-binding proteins (PBPs), which are involved in the biosynthesis and remodeling of the peptidoglycan structure of the bacterial cell wall (Llarrull et al., 2010; Singh et al., 2017; Wilke et al., 2005). Structurally, the BLAs contain a 4-membered β-lactam ring fused to a 5-membered thiazolidine ring and a unique side chain. The penicillin derivatives (penams), cephalosporins (cephems), and carbapenems represent the most successfully used BLAs in the treatment of bacterial infections. Exceptions to this chemical feature are the monobactams, which are compounds with a monocyclic β-lactam ring (Aminov, 2017; Singh et al., 2017). The group of penicillins comprises penicillins G and V, which are active against gram-positive cocci; penicillins resistant to penicillinase, such as nafcillin, which is active against *Staphylococcus aureus*; ampicillin and other drugs with a broad spectrum against gram-negative bacteria, in particular when combined with an inhibitor of β-lactamase; and penicillins of the extended spectrum such as piperacillin, which is active against *Pseudomonas aeruginosa* (Miller, 2002). It is possible to naturally produce some penicillins, according to the chemical composition of the fermentation medium used to grow *Penicillium*. Penicillin G (penicillin benzathine or benzylpenicillin) is the one with the highest antimicrobial activity of this class and the only natural penicillin used in humans (Demain, 1991).

Beta-lactam antibiotics also include the group of cephalosporins that is classified by generations: the first generation includes drugs with activity against gram-positive bacteria

and scarce activity against gram-negative bacteria; the second generation includes cephalosporins with little activity against gram-negative bacteria, and include some agents with activity against anaerobic microorganisms; the third generation consists of drugs with antimicrobial activity against gram-positive bacteria and a greater activity against Enterobacteriaceae, and a subgroup with activity against *P. aeruginosa*; and the fourth generation comprises the antimicrobial spectrum of all drugs of the third generation but with a greater stability against hydrolysis by β-lactamases. Some compounds are considered as the fifth generation of cephalosporins and currently are under investigation (Percival, 2017). Carbapenem antibiotics, which include imipenem, doripenem, ertapenem, and meropenem, possess the maximal antimicrobial spectrum of BLAs (Papp-Wallace et al., 2011). The broad spectrum of activity of carbapenems is associated with their intrinsic resistance to nearly all β-lactamases. The stability to β-lactamases is due to the trans-α-1-hydroxyethyl substituent at the 6 position of carbapenems; this is unique when compared with the side chains of penicillins and cephalosporins, which have cis configurations (Zhanel et al., 2007). Monobactams (aztreonam) exhibit a wide spectrum against gram-negative bacteria, and are unique among the BLAs because of their poor binding affinities to PBPs in gram-positive bacteria (Bush and Macielag, 2010).

36.3 CHEMICAL SYNTHESIS ROUTES

The basic structure of penicillins consists of a thiazolidine ring fused to the β-lactam ring, which is attached to a side chain. The penicillin nucleus is the structural basis for biological activity. Metabolic transformations or chemical alterations of this part of the molecule lead to loss of antibacterial activity. The lateral chain determines the pharmacological characteristics of each type of penicillin (Miller, 2002). Cephalosporin C contains a side chain derived from a d-α-aminoadipic acid, which is condensed with a β-lactam ring and a dihydrothiazine ring, forming 7-aminocephalosporanic acid. This compound was modified by the addition of different side chains producing a complete family of cephalosporins. The compounds containing this acid are relatively stable in diluted acid and very resistant to penicillinase, whatever the nature of its side chains and its affinity for the enzyme. Apparently, the modifications at position 7 of the β-lactam ring produce alterations of antibacterial activity, and substitutions in the position 3 of the dihydrothiazine ring generate changes in the metabolism and pharmacokinetic properties of drugs. Cephamycins are similar to cephalosporins, but they have a methoxy group at position 7 of the β-lactam ring of the nucleus of 7-aminocephalosporanic acid (Garau, 1998). Carbapenems are β-lactams containing a fused β-lactam and a pentameric ring system that differs from penicillins (penams) because they contain a carbon atom instead of a sulfur atom at position 1 and an unsaturated bond between C2 and C3 in the five-membered ring structure (Papp-Wallace et al., 2011). Monobactams are β-lactam compounds that not fused with another ring. The antimicrobial activity of monocyclic β-lactams lacking the ionizable group at N1 indicates that new antibiotics for treatment of infections caused by resistant bacteria without the need for coadministration of β-lactamase inhibitors are possible (Konaklieva, 2014). Several strategies for the preparation of β-lactam compounds have been developed and reported in the literature. The ketene-imine cycloaddition (the Staudinger reaction) and the chiral ester enolate-imine cyclocondensation (the Gilman-Speeter reaction) are the most used methods for the synthesis of β-lactams with excellent

enantiopurity (Kamath and Ojima, 2012; Singh, 2004). However, other notable methods are sometimes employed such as the ring expansion of aziridines by metal-catalyzed CO insertion (Alper reaction), the intramolecular cyclization of suitable amides (the Mitsonobu reaction), the coupling of nitrones with propargyl moieties catalyzed by copper salts (the Kinugasa reaction), the metal-catalyzed cyclocarbonylation of allyl derivatives with imines (the Torii reaction), the intramolecular cyclization, heterocyclic rearrangement (Troisi et al., 2010), photo-induced rearrangements, and radical cyclizations (France et al., 2004). Recently, a protocol for the synthesis of β-lactam compounds has been reported based in the halocyclization followed by conversion to N-sulfonyloxy derivatives. The BLAs obtained by this way display activity against gram-positive bacteria (Carosso and Miller, 2015). On the other hand, Vandekerckhove and D'Hooghe (2013) reported the obtention of purine-β-lactam chimeras by N-alkylation of 6-benzylamino- or 6-benzyloxypurine with β-lactam compounds. Interestingly, these hybrid molecules exhibit antiviral actions.

36.4 BIOCATALYSIS IN THE SYNTHESIS OF β-LACTAM ANTIBIOTICS

Penicillins and cephalosporins are the principal groups of BLAs. While the early works demonstrate that penicillin F and G both contain a β-lactam nucleus, many efforts have been made with the aim to improve production of this class of antibiotics. The first approaches to obtain substituted forms of penicillin varying the fermentation conditions did not show very promising results (Behrens et al., 1948). Since the successful production of penicillin V by the acetylation of chemical synthesized 6-APA (Sheehan, 1959), semisynthetic β-lactam has been developed in a continuous and systematic fashion (Kong et al., 2010). The complex and labile structure of these compounds make necessary a bunch of complicated conditions to carry out the chemical synthesis processes (protection/deprotection reactions, environmentally unfriendly solvents, very low temperatures, etc.) (Mateo et al., 2005b). On the other hand, green chemistry has become not only an environmental necessity, but an adequate and beneficial option for the development of industrial processes in the modern economy (Ferreira-Leitão et al., 2017). An example of this is that nearly 60% of the industrial scale-produced compounds that need a specific chirality in the pharmacy industry are obtained using a biocatalyzed process based on enzymes (Downey, 2013).

The Organization for Economic Cooperation and Development has reported that more than 300 industrial processes used in several pharmacy industries are based on enzymes (Ferreira-Leitão et al., 2017). However, two of the principal areas of enzymatic biocatalysis in industry are in the process of β-lactam production, and there are no new discoveries: hydrolysis of penicillin G to produce semisynthetic antibiotics using penicillin-G-acylase and hydrolysis of cephalosporin C using D-amino acid oxidase and glutaryl acylase; both processes were reported more than 30 years ago (Savidge, 1984; Whitesides and Wong, 1985). The first effort to reach an enzymatic synthesis of a β-lactam was reported in the 1960s (Cole, 1969a, b). Nevertheless, BLAs currently command more than 60% of the global total sales of antibiotics (Sklyarenko et al., 2015a, b). Since those first trials, the pharmacy industry has taken two principal approaches to integrate enzymatically controlled processes: the *thermodynamically controlled synthesis* (TCS) and the *kinetically controlled synthesis* (KCS). The

first uses unmodified substrates for enzymes with acylase activity (Savidge, 1984) and the second uses activated acyl-donor (Kasche et al., 1987). The strategy of TCS has fewer complications due to being controlled by the thermodynamic constant of the process. Its aim is to transform the maximum possible quantity of antibiotic nuclei into active compounds using an excess of substrate (Ulijn et al., 2002). Factors such as pH, acyl donor concentration, and the use of organic cosolvents must be attended in order to improve the obtained antibiotic yield (Mateo et al., 2005b). The KCS approach is relatively more complicated because of its necessity to be carried out with no stabilized enzyme. Also, undesired natural reactions exist that can be catalyzed by acylase in the system. The most important parameters to attend are the capacity of the enzyme to absorb the antibiotic nucleus, the use of an enzyme from an adequate source (it can modify the system kinetic conditions), and reaching enzyme saturation at a low concentration of the antibiotic nucleus (Mateo et al., 2005b). The biggest problem of both options is that their industrial implementation needs to be as similar to an ideal process as possible. Although the use of these strategies looks for a better yield of antibiotic production and, additionally, minimizes process costs, is important to note that design and implementation for industrial purposes must pay special attention to the principles of waste prevention, innocuous solvent use, and pollution prevention. This is necessary (in the process design/implementations context) because of requirements regarding the excess of acetylated donors (such as phenylacetamide, phenylglycine amide, and pehylacetic acid) (Alkema et al., 2003) and the use of organic solvents as a potential danger to the environment (e.g. methanol, ethanol, ethyl acetate, carbon tetrachloride, triglyme, and diglyme) (Sklyarenko et al., 2015a, b). Although many of these components have been reported to be "practically nontoxic," some of these substances have security data sheets where important toxicologically information appears as "no data available" (Sigma-Aldrich Quimica, 2008, 2015). This is an important issue to keep in mind when looking for a β-lactam enzyme-catalyzed synthetic process.

36.5 PRINCIPAL ENZYMES FOR β-LACTAM ANTIBIOTIC PRODUCTION

Continuous research and improvement of the biocatalyzed synthase of BLAs have developed diverse strategies to improve the yield of antibiotics produced and the reutilization rate of the enzyme, but one enzyme has been used throughout: penicillin acylase (PA) (EC 3.5.1.11, aka penicillin amidase). PA belongs to the hydrolases class, the amidohydrolases subclass, and the N-terminal nucleophilic hydrolases family. It presents a heterodimeric functional form built by two chains: α (of 23.9 kDa) and β (of 61.5 kDa), linked by noncovalent bonds (Alkema et al., 2000; Hewitt et al., 2000). The reaction catalyzed by PA is based on a nucleophilic mechanism performed by the active-site serine (βS1; chain β, Serine, Position 1) and targeted over the carbonyl carbon of amide or the ester bond in the substrate (Duggleby et al., 1995). Then, an acyl-enzyme is formed and can be deacylated by water, finally yielding the hydrolysis product (a semisynthetic antibiotic) and the regenerated enzyme (Alkema et al., 2003).

In recent years, research efforts have been focused on creating a system to overexpress the enzyme, modifying its stability and catalytic properties by targeted mutagenesis and immobilization systems that allow the enzyme an increased capacity. Nevertheless, PA is the

star enzyme in β-lactam synthesis. Its structure and mechanism of assembly are not fully understood, making difficult the development of an efficient process to improve its function (Sklyarenko et al., 2015a, b). PA has been tested with numerous acyl donors and key amino acids (the two principal reactants in the synthesis of BLAs) for the preparation of different semisynthetic penicillins and cephalosporins. Each experimental procedure has different operative conditions: pH can vary from 4.5 to 7.5 (even in gradient); a temperature range of 2–40°C; crude PA extract or immobilized enzyme; a concentration of acyl donor from 1.2–400 M and the β-lactam nucleus of 0.2–100 mM; reaction time can be 50 min, 2, 3, 7, 8, 11, 24, or 36 h and at the process end, the product recuperation rate can be between 2.5 and 90% (Sklyarenko et al., 2015a, b). A combination of the different values of all these factors will throw different results on each process. It is evident that chemical, kinetic, and thermodynamic approaches for the development of better BLA synthesis processes made improvements since the first works of Sheehan (1959). It is also evident that only a multidisciplinary strategy involving all these disciplines will allow adding this improvement to the existing techniques and successfully scaling the process to obtain all these active substances in the necessary quantity and in an environmentally friendly manner.

36.6 MICROORGANISM FOR β-LACTAM ANTIBIOTIC SYNTHESIS

Observations on the antagonism by fungi belonging to the *Penicillium* genus against bacteria were recorded by many scientists during the second half of the 19th century, including Tyndall, Lister, Huxley, and Duchesne. However, it was Fleming's discovery that led to penicillin, the first chemotherapeutic agent produced by a microbe, thus initiating the golden age of drugs (Demain and Elander, 1999). Following on the heels of penicillin production by *Penicillium chrysogenum* (Fig. 36.1) came the discoveries of cephalosporin formation by *Cephalosporium acremonium*, cephamycin, clavam, and carbapenem production by actinomycetes, and monocyclic β-lactam production by actinomycetes and unicellular bacteria.

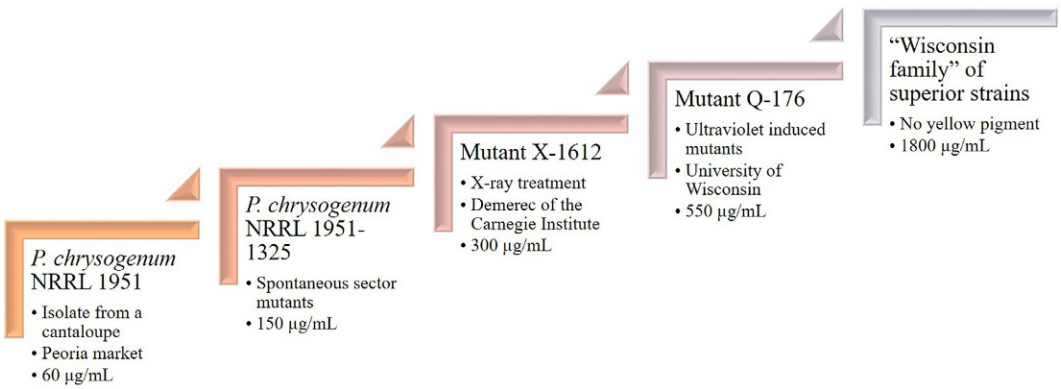

FIG. 36.1 *Penicillium chrysogenum* strain improvement for penicillin production from 1946 to 1955.

Pioneering studies on the biosynthesis of penicillin using cell-free systems led to the crucial observation that the *penam* nucleus of penicillins and the *cephem* nucleus of both cephamycins and cephalosporins are formed by the condensation of three precursor amino acids: L-α-aminoadipic acid (Aad), L-cysteine (Cys), and L-valine (Val) (Aharonowitz et al., 1993) by a mechanism designated as nonribosomal peptide synthesis. After the formation of tripeptide Aad-Cys-Val, it is cyclized to form isopenicillin N (IPN) by the isopenicillin N synthase (IPN synthase or cyclase). This enzyme was described and purified to near homogeneity from *P. chrysogenum* and in some actinomycetes (*Streptomyces clavuligerus, Streptomyces lipmanii*, and *Nocardia lactamdurans*) (Pang et al., 1984). IPN is an intermediate that contains an L-α-aminoadipyl side-chain attached to the *penam* nucleus, which is later exchanged for phenylacetic acid in penicillin-producing fungi but not in cephalosporin producers. The responsible enzyme is isopenicilin-N acyltranferase, which has been reported in *P. chrysogenum* and *Aspergillus nidulans* (Martin et al., 1987). Penicillin G acylase (PGA) is a widely used catalyst for the synthesis of several BLAs through kinetically controlled *N*-acylation (*kcNA*) of cephalosporinic nuclei. In the case of three functionalized cephalosporins (Cefamandole, Cefonicid, and Cefazolin), two enzymatic approaches can be pursued (Terreni et al., 2005; Ulrich, 2004). The outcomes of these reactions are strictly influenced by the catalytic properties of the used acylase. Broad-specificity penicillin acylases from *Escherichia coli coli, Cluyvera citrophila,* and other microorganisms are the most often used in BLA production. However, the acylase isolated from *E. coli* is the most used one, although not always with good results (Ulrich, 2004). Acylase from *Arthrobacter viscosus* is an alternative enzyme. The sequence of the gene encoding enzyme is known and has been successfully cloned and expressed in *E. coli* and *Bacillus subtilis* (Ohashi et al., 1989). Unlike other gram-negative microorganisms that accumulate the enzyme in the periplasmic space, *A. viscosus* is a penicillin acylase producer that secretes the enzyme into the culture medium (Ohashi et al., 1989). With the aim of comparing performances of PGA from *E. coli* and *A. viscosus*, acylase from the latter was immobilized both on Eupergit C and glyoxyl-agarose and tested in the *kcNa* synthesis of cephalosporins. Eupergit C is a hydrophobic acrylic epoxy support and glyoxyl-agarose immobilizes the enzyme through the richest Lys area of the protein surface; both have been used for the immobilization of *E. coli* PGA (Mateo et al., 2005a). Results from this work showed that *A. viscosus* displayed higher synthetic performances than PGA from *E. coli* (Terreni et al., 2007), noting the importance of the microbial source for selection of enzymes as biocatalysts in the kinetically controlled syntheses of BLAs. In the case of aminoglycosides, biosynthetic gene clusters have been isolated and characterized for streptomycin, fortimicin A, butirosin, tobramycin, and gentamicin. However, molecular biological studies concerning kanamycin have been restricted to its resistance genes until the description of the kanamycin biosynthetic gene cluster from *Streptomyces kanamyceticus* (Yanai and Murakami, 2004).

36.7 PROCESS OPTIMIZATION (TIME, TEMPERATURE, ENZYME CONCENTRATION)

Beta-lactam antibiotics have sales reaching $20 billion a year, which is approximately 65% of the total world market of antibiotics. Chemical synthesis requires protection and deprotection steps and harsh conditions involving chemicals (Shaw et al., 2000). On the other hand,

enzyme biocatalysis has slowly displaced chemical synthesis for economic considerations and for better compliance with the principles of green chemistry (Bahamondes et al., 2012). Penicillin acylase is massively used in the production of β-lactam; however, the same enzyme can catalyze the reverse reaction of synthesis, leading to the production of semisynthetic BLAs (Shewale and Sudhakaran, 1997) and enantiopure amino acids (Gong et al., 2011). This PA is always used in an immobilized form and new strategies to produce robust and efficient biocatalysts are still emerging (Zuza et al., 2011). For this reason, the process optimization of production is extremely important. Solvent pH, the ratio of enzymatic synthesis, and hydrolysis rates are among the factors that can be controlled.

36.7.1 The Role of pH

For a direct synthesis of BLA, a low pH range is suboptimal for the functioning of β-lactam acylases. In this sense, synthesis of benzylpenicillin (BP) from phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA), and that of cephalotin from thienylacetic acid (TAA) in the presence of polar solvents, such as acetone, dimethyl sulfoxide, dioxane, N,N,-dimethylformamide (DMF) and 2-ethoxyethanol, have been evaluated. When using an aqueous organic medium containing 50% DMF, the pK of the carboxyl group of PAA increased by more than 0.5 units, while 1.8 and 1.7 units were observed for PAA and TAA in the cellusolve-water mixture (50% v/v) (Fernandez-Lafuente et al., 1996a, b). By this approach, they achieved a cephalothin yield of 55%–60% in penicillin acylase-catalyzed synthesis. In the case of DMF (aqueous organic medium, 40% v/v), a 90% yield of cephalotin was achieved by a decrease of pH from 7.2 to 6.0 and increasing TAA pK (Fernandez-Lafuente et al., 1996a, b).

36.7.2 The Role of Solvents

Organic solvents affect the activity and stability of enzymes, and for the selection of solvent, two criteria must be considered: (1) the inhibitory effect of the solvent on enzyme activity, and (2) the desired increase in abundance of the nonionized form of the side chain (Rosell et al., 1998). Also, the ratio of synthesis and hydrolase activity of an enzyme is used to evaluate the inhibitory effect of solvents (Sklyarenko et al., 2015a, b). An organic solvent may also increase substrate solubility, alter p*K*a values, and favor synthesis by reducing water activity (Shaw et al., 2000). Among the solvents evaluated, the effect of methanol on apparent PAA dissociation constants has been investigated and also its water mixtures in the process of BP synthesis and pivaloyloxymethyl ester of this compound from 6-APA. In the case of pivampicillin synthesis, no improvement was observed. Similar effects were observed for dihydric alcohol and diethylene glycol during synthesis of chimeric compounds by direct PAA condensation with amines (Abian et al., 2008).

36.7.3 Ratio Synthesis/Hydrolysis

This is often used for the quantitative assessment of processes occurring during acyl transfer synthesis and mainly depends on enzyme catalytic properties, the Bergman cyclization (BC) form used, and the reaction conditions. For this purpose, different nucleophiles are

evaluated. In the case of penicillin acylase from *E. coli*, 7-aminocephalosporinic acid (7-ACA) is better than 3-[(5-methyl-1,3,4-thiadiazol-2-yl) thiomethyl]-7-aminocephalosporanic acid (MMTD-7-ACA) (Terreni et al., 2005).

36.7.4 Substrate Concentration

It has been reported that the synthesis of ampicillin, amoxicillin, and cephalexin occurs at very high substrate concentrations. However, solubility at this condition is limited and depends on pH and temperature, having a strong impact on conversion of the limiting substrate. Bahamondes et al. (2012) evaluated the synthesis of cephalexin with PA at high substrate concentration at an acyl donor (nucleophile molar ratio = 3) in aqueous and ethylene glycol media, varying pH (6.5–7.5), temperature (10–20°C), and enzyme/substrate ratio (31.25–125 IU/mmol), finding that at very high substrate concentration, the use of organic cosolvents was not required, complying with principles of green chemistry.

36.8 STRUCTURE OF BIOSYNTHESIZED β-LACTAM ANTIBIOTICS

During many years, the structure of beta-lactam antibiotics resulted in a successful tool for treatment against bacterial infections before the appearance of multidrug resistance microorganisms. The basic structure of this class of antibiotics consists of a highly reactive chemical nucleus capable of reacting with components of the bacterial cellular wall biosynthesis process. The final step during cell wall formation requires a transamidation reaction that is catalyzed by a cell wall transamidase enzyme known as penicillin binding protein 1 (PBP-1). Although other PBPs were reported (Schweizer et al., 2017), their functions are not well demonstrated. The highly reactive beta-lactam nucleus reacts with the serine hydroxyl group of PBP-1 during the bacterial peptidoglycan synthesis, leading to an irreversible inhibition of this enzyme and resulting in bacterial cell death. Basically, this highly reactive nucleus of beta-lactam antibiotics consists of two moieties: one, the 6-aminopenicillinic acid, formed by the union of one tiazolidinic ring with another beta-lactam ring, and two, a lateral chain joined to the C6 of the beta-lactam nucleus. Every penicillin and related antibiotic shares this basic chemical structure, although different ring systems exist with high antibacterial activity observed in the structure of beta-lactam antibiotics currently used in clinical therapy. Penicillins differ because different substitutions of the lateral chain in the beta-lactam ring where, depending on the modification of this chain, the antibacterial activity of the molecule can be altered as well as the pharmacokinetic parameters shown in each one. Modification of these groups has allowed the synthetic and semisynthetic creation of a wide variety of related molecules with antibacterial activity. Cephalosporins are another group of beta-lactam-related antibiotics with natural origin from *C. acremonium*. The structure of cephalosporins consists of a basic nucleus formed by a beta-lactam ring and a dihydrotiazinic ring, giving rise to the 7-aminocephalosporinic acid. Further, substitutions on position 7 of cephalosporinic have been related to their antibacterial activity. Meanwhile, substitution on position 3 has been shown to modify pharmacokinetic parameters of the molecules. There are other related antibiotics such as monobactams and carbapenems that share the beta-lactam nucleus.

Carbapenems are a unique class of beta-lactam antibiotics that shows the widest spectrum known. Lee et al. (2016) mentioned that these antibiotics can be divided according to the dependency on divalent cations for enzyme activation into metallo-carbapenemases, zinc-dependent class B, and zinc-independent classes A, C, and D. The chemical advances on the structure of these new molecules attempt to improve the diagnosis of infectious diseases that are resistant to first-line antibiotics. Due to the high efficiency shown by the beta-lactam antibiotics to inhibit the bacterial cell wall biosynthesis process, this class of molecules represents the first line of defense against a variety of bacterial infections. By the way, the appearance of multidrug-resistant bacteria has made difficult the treatment of patients with infectious diseases. It has been reported that resistance to this class of drugs can be the result of antibiotic target site alteration, prevention of antibiotic access by altered permeability or forced efflux, or antibiotic degradation (Wilke et al., 2005). The structure of beta-lactam antibiotics makes them susceptible to different degradative processes, which contributes to the resistance showed by different bacteria. The beta-lactam ring can be opened, producing an inactive metabolite, the penicilloic acid.

36.9 GENES FOR β-LACTAM ANTIBIOTIC BIOSYNTHESIS

Hydrophobic penicillins that have aromatic side chains are produced only by fungal strains, among them *P. chrysogenum* and *A. nidulans*, while hydrophilic cephalosporins are found in fungi (e.g. *Acremonium chrysogenum*) and bacteria (e.g. *S. clavuligerus*) (Garcia-Estrada et al., 2010). Ogawara (2016) reported that the *Streptomyces* genome *has* diverse β-lactamases genes of A, B, and C β-lactamase classes. On the other hand, Martin (1998) reported novel enzymes involved in methoxylation at C-7 and carbamoylation at C-3′ of the cephem nucleus, isolated from *N. lactamdurans* and *S. clavuligerus*. Recently, genes for β-lactam were identified in the arthropod springtail *Folsomia candida* (Collembola), where one phosphopantetheinyl transferase was found (Suring et al., 2016). Understanding the genes involved during antibiotic production and their expression is important for its artificial overexpression. Different genes for penicillin and cephalosporin production have been identified, isolated, and cloned. Examples include pcbAB, which was encoded for Aad-Cys-Val synthetase, and pcbC, involved in isopenicillin N (IPN) synthase expression. These two genes are common to penicillin and cephalosporin producers (Martin, 1998). These two genes were cloned from *P. chrysogenum* and *A. nidulans* (Martin and Gutierrez, 1995). The cloning of genes involved in beta-lactam biosynthetic pathways will allow understanding the channeling precursors and bottlenecks in these pathways (Martin, 1998). Nucleotide sequences of β-lactam genes are extremely conserved (Martin and Gutierrez, 1995) and these genes are clustered (Garcia-Estrada et al., 2010). For these reasons, their origin and evolution are very interesting. It has been proposed that β-lactam genes have resulted from mutation and natural selection (Fischbach, 2009). Although different β -lactam genes have been reported from different organisms, there is evidence such as pathway intermediates, characteristics of the involved enzymes, and lack of introns that suggests that all these genes have evolved from an ancestral gene cluster of bacterial origin (Liras and Martin, 2006). Nonbacterial organisms acquired these genes through horizontal gene transfer. However, successful integration of β-lactam genes into an animal metabolism requires the precursor L-α- aminoadipic acid and a phosphopantetheinyl transferase

(Suring et al., 2016). Once receptor strains acquire fragments of the original bacterial cluster, some may have differential loss, especially in more recently evolving species (Suring et al., 2017). There are reports of paralogues of the penicillin *penDE* gene in several filamentous fungi that do not produce penicillin (Garcia-Estrada et al., 2010). On the other hand, other receptor strains may insert new genes into the clusters, acquiring new functions (Liras and Martin, 2006). IPNS and ACVS genes were placed in a phylogenetic clade between bacteria and fungi (Suring et al., 2017). Genes involved in β-lactam synthesis may be located in one or more clusters. Martin and Gutierrez (1995) mentioned that β-lactam genes in *P. chrysogenum* are clustered in chromosome I (10.4 Mb), in *Penicillium notatum* are located in chromosome II (9.6 Mb), and in *A. nidulans* are clustered in chromosome VI (3.0 Mb), but each gene is expressed as a single transcript with separate promoters. In the cephamycin clusters of *S. clavuligerus* and *N. lactamdurans,* \genes such as the β-lactamase (bla), the penicillin-binding protein (pbp), and the transmembrane protein have been found (cmcT) (Martin, 1998). This cluster extends for about 30 kb in these microorganisms, and also includes the *lat* gene which encodes lysine-6-aminotransferase (Martin and Gutierrez, 1995).

In *Nocardia uniformis,* 14 open reading frames involved in antibiotic production, resistance, and export have been reported (Gunsior et al., 2004). In *Pseudomonas syringae*, ORFs with similarity to β-lactam synthase and clavaminic acid synthase, as well as amino acid synthesis enzymes, were found. Also the tabtoxin biosynthetic genes that are adjacent to the lysC tRNA gene were identified (Kinscherf and Willis, 2005). The thienamycin cluster was isolated from *Streptomyces cattleya* by Núñez et al. (2003). These authors reported genes homologous to carbapenem and clavulanic acid biosynthetic genes along with genes for methyltransferases, cysteinyl transferases, oxidoreductases, hydroxylase, regulatory genes, and genes involved in exportation and/or resistance as well as a quorum sensing system. Mellado et al. (2002) consigned the presence of 10 ORFs in the upstream region of the *car* gene of the clavulanic acid cluster from *S. clavuligerus* while the *β*-lactam gene cluster in *F. candida* genome is formed by an isopenicillin N synthase, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine synthetase, and two cephamycin C genes (Suring et al., 2017). The distribution of β-lactam biosynthesis genes in two clusters has been mentioned. Martin and Gutierrez (1995) indicated that genes involved in cephalosporin biosynthesis in *C. acremonium* are distributed in two clusters. In the first cluster are located the pcbAB and pcbC genes, which are very similar to those encoding enzymes in penicillin biosynthesis and encode the first two enzymes in this pathway. In the second cluster are found the cefE, cefF, and cefG genes, which express the last three enzymes of the pathway. The antibiotic gene clusters in the *Streptomyces* genome are reported as gene islands (Liras and Martin, 2006). In addition, the convergent evolution of distinct gene clusters for antibiotic production and the merger of distinct gene clusters into a single functional unit have been mentioned (Fischbach, 2009).

36.10 IMPROVEMENT OF ENZYME ACTIVITY THROUGH PROTEIN BIOENGINEERING

The BLAs and related β-lactamase inhibitors remain as the most clinically relevant antibiotic drug class (Lee et al., 2011). Most, but not all, B-lactam antibiotics can be divided into hydrophobic and hydrophilic fermentation products or via modification of fermented

intermediates (Demain, 1991). The desire for more efficient routes to existing antibiotics and for access to new and synthetically challenging ones stimulates continued interest in B-lactam biosynthesis (Hamed et al., 2013). Classical strain improvement technologies (via iterative random strain mutagenesis and screening for improved productivity) have resulted in significant improvements in industrial fermentation titers of BLAs (Hamed et al., 2013; Weber et al., 2012). However, these technologies do not result in the directed production of new products. One goal of "post-genomic" metabolic studies on BLA biosynthesis is to achieve rational optimization of production through improvement of enzyme activity by protein bioengineering (Hamed et al., 2013). There are currently multiple examples of successful protein bioengineering for the production of new and more powerful BLAs. One of the best-studied examples is the case of penicillin G acylase (PGA) from *E. coli* (Akkaya et al., 2012). PGA can catalyze the coupling of an acyl group to penicillin- and cephalosporin-derived lactam nuclei, a conversion used for the industrial synthesis of BLAs (Jager et al., 2008). Interestingly, the effects of mutations on positions +2 and +4 relative to the start codon in the translation region of the *E. coli pac* gene, encoding periplasmic PGA, led to increased amounts of PGA activity. This increase is also observed in mutants with extended spacer regions between the ribosome binding site and the ATG start codon. This evidence indicated that a wild-type sequence of the *pac* gene does not provide maximum expression levels and it is possible to improve the production of PGA in *E. coli* (Akkaya et al., 2012). Specifically, the analysis of the crystal structure of *E. coli* PGA has shown that residues R145 and F146 undergo extensive repositioning upon binding of large ligands to the active site, suggesting that these residues may be good targets for mutagenesis aimed at improving the catalytic performance of PGA. Therefore, site-saturation mutagenesis was performed on both positions and a complete set of 33 variants showed improved synthesis of ampicillin, indicating the importance of the mutated residues in PGA-catalyzed acyl transfer kinetics (Jager et al., 2008).

Likewise, site-directed mutagenesis based on a predicted modeled structure of PGA from *Bacillus megaterium* (BmPGA) was followed to increase its performance in the kinetically controlled synthesis of cephalexin with high reactant concentrations. Mutants were directed to amino acid residues close to the active site that were expected to affect the catalytic performance of penicillin acylase. In particular, Y144, F145, and V24 were mutated, showing that synthesis/hydrolysis ratios increase up to 1.3–3.0 times over the wild-type. This can be interpreted as an accumulation of up to twofold more cephalexin at significantly higher conversion rates (Wang et al., 2007). On the other hand, within improvements in the synthesis of BLAs are those that are related to modifications at the genetic level of the strains that produce them. An example is cephalosporin-acid synthetase produced by the *E. coli* strain VKPM B-10182 that has specificity for the synthesis of BLAs of the cephalosporin acids class (cefazolin, cefalotin, cefezole etc.). Genomic comparisons with other *E. coli* show multiple mutations, indicating the long selection history, including mutations in the genes of RNase and B-lactamases that could enhance the level of enzyme synthesis and reduce the degree of degradation of the synthesized cephalosporin acids (Eldarov et al., 2015). For the purpose of increasing cephalosporin C (CPC) yield by fermentation, *cefEF* and *cefG* genes, which encode the ultimate and penultimate steps in CPC biosynthesis, were transformed in various combinations into an industrial strain of *A. chrysogenum*. Both PCR and Southern blotting indicated that introduced genes were integrated into the chromosome of *A. chrysogenum*. Multiple transformants containing an additional copy of *cefG* showed a significant increase in CPC production.

This was the first report where it was demonstrated that an increase in the number of copies from particular genes had a synergistic effect in a CPC high production (Liu et al., 2010). On the other hand, although the availability of purified and active proteins is the starting point for the search for new BLA biosynthetic enzymes, the use of affinity tags in the purification process can negatively affect structure and/or activity measurements (Majorek et al., 2014; Waugh, 2011). However, it has been demonstrated that glutaryl-7-aminocephalosporic acid acylase (Gl7ACA acylase) from *Brevundimonas diminuta* (BrdGl7ACA), a commercial enzyme widely used for the manufacture of B-lactam antibiotics, is functionally active both in wild-type as well as in the modified version by attaching affinity groups (Khatuntseva et al., 2007). Considering that yields are determined by the properties of the employed enzyme, an intense search for enzymes with properties more suitable for producing different antibiotics has been developed. Thus, many different enzymes have been identified, each adequate for a specific kind of β-lactamic antibiotic (Gabor et al., 2005; Volpato et al., 2010).

36.11 FUTURE PERSPECTIVES

Bacterial resistance to BLAs is a worldwide health problem. Exposure to an inadequate dose of β-lactam agents or the coinfection of multiple pathogen bacteria cause some strains to develop resistance mechanisms that include the expression of genes or β-lactamases enzymes that reduce the efficacy of BLAs. Nevertheless, the BLAs still are an important therapeutic option for the treatment of bacterial infections. Therefore, in the future, efforts should be undertaken to improve the synergic action between different classes of BLA and/or β-lactamase inhibitors; to design and develop agents with improved stability to β-lactamases using combinatorial chemistry, polymeric materials, and enzymatic engineering; and to identify the resistance mechanisms of pathogenic bacteria through genetic, microbiological, and molecular tools.

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CHAPTER

37

Enzyme Immobilization Methods and Applications in the Food Industry

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37.1 INTRODUCTION

Enzymes are biological catalysts that play an immense role in the food industry. In 1877, Wilhelm Friedrich Kuhne, a physiology professor at the University of Heidelberg, first coined the term enzyme, which is a Greek word meaning "in leaven" (Gurung et al., 2013). The desirable characteristics of enzymes are hindered by their long-term operational stability, shelf storage life, recovery, and reuse. These drawbacks can be overcome by enzyme immobilization, which enhances the functional efficiency and reproducibility while requiring less labor input and reducing contamination. Therefore, this boosts the stability of the products. There are various methods to immobilize enzymes that are useful for the industry that are explained in this chapter.

37.1.1 History of Enzyme Immobilization

The first scientific observation that led to the discovery of immobilized enzymes was in 1916 by Nelson and Griffin, and the development resulted in modernized immobilization techniques by 1950–1960. The phases of immobilization are divided into the following periods.

1. Early phase (1916–40): Development in matrices belonging to the inorganic group, such as glass-alumina, was seen, and a reversible immobilization method was in progress.
2. Underdeveloped phase (1945–65): Chemical or irreversible methods were initiated to increase specificity and durability. Matrices such as phosphocellulose, DEAE-cellulose, cellulose, and synthetic polymer were used.
3. Developing phase (1965–70): This phase improved enzyme immobilization by the covalent method with the aid of chemical cross-linkers. Glutaraldehyde was used for

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https://doi.org/10.1016/B978-0-12-813280-7.00037-2

enzyme immobilization onto the insoluble carrier. Synthetic carriers were developed with active functional groups such as poly anhydride, polyisothiocyanate, etc.

4. Developed and post-developed phase (1970–80): Many new submethods such as affinity binding, coordination binding, and variations were developed. Encagement, covalent multilayer immobilized enzymes, organosoluble lipid-coated enzymes, and the introduction of genetically engineered tags were initiated. (Nisha et al., 2012).
5. Rational design phase (1990–present): Advanced immobilization techniques that have a similar catalytic ability to that of a soluble enzyme with higher operational stability and green/sustainable development are emphasized.

37.2 IMMOBILIZATION TECHNIQUE

The selection of the proper immobilization method is a pivotal part of the immobilization process. It plays a crucial role in determining the enzyme activity and characteristics in a particular reaction. Process specifications for the catalyst, enzymatic activity, efficiency of protein utilization, enzyme deactivation, regeneration characteristics, immobilization cost, toxicity of immobilization reagents, and the desired final properties of the immobilized enzymes are factors that should be considered (Chiou and Wu, 2003). The enzyme can couple to the carrier/matrix by reversible physical adsorption or irreversible chemical bonds such as a covalent bond. The matrix is of vital importance to determine the function and use of the immobilized enzyme. The properties required include hydrophilicity, physical resistance, biocompatibility, low cost, and resistance to microbial attack. Among various materials (Table 37.1) used for enzyme binding, polymers represent an excellent class of support materials for the immobilization of enzymes with bioactivity in comparison to that of enzymes in dissolved buffer solutions (Azarikia et al., 2015). Polymers bear attractive physical (high molecular weight, viscosity, film-forming ability, solvent, temperature, and pH stability) and chemical properties (hydrophilic, hydrophobic, ionic, and other desired functional groups). These make them suitable for bioconjugation with enzymes offering dramatically improved stability, catalytic activity, and sensing properties and can adsorb a high surface density of enzyme biocatalysts via covalent and noncovalent interactions controlled by the surface chemistry of the polymer (Rao et al., 2006).

37.3 CLASSIFICATION OF METHODS

There are various approaches in classifying immobilization techniques in a broad section; they are widely classified as reversible and irreversible. The binding strength of the enzyme to the adsorbent is reciprocally related to the ease with which it can be reversed. Further, it can be classified depending on the physical or chemical method used for immobilization. The classification is depicted below (Fig. 37.1):

Physical methods or reversible: (1) adsorption, and (2) disulfide bond formation.

Chemical modification or irreversible: (1) covalent bonding, (2) cross-linkage, and (3) entrapment.

TABLE 37.1 Classification of Supports Materials/Carriers For Immobilization of the Enzyme

Classification	Example	References
Organic polymer		
Natural polymers	Agarose, calcium alginate	Mahajan et al., 2010
	Chitosan, gelatin succinylated gelatin Starch polymeric blend	Costa et al., 2005
	Protein: collagen	Hanachi et al., 2015
	Carbon	Costa et al., 2005
Synthetic polymers	Polystyrene Polyisobutylene Polynorbornenes Poly(styrene-*co*-maleic anhydride) Poly(acrylic acid) Poly(ethylene glycol) Polyglycerol	Lu and Toy, 2009
Inorganic polymer	Silica	Homaei, 2016
	Processed materials Lassy carbon PANCMA nanofibers PANCMPC nanofibers	Costa et al., 2005; Fang et al., 2011

37.4 ADSORPTION

Adsorption is a simple technique where the enzyme is immobilized by being mixed with an adsorbent under suitable conditions and washing loosely bound proteins after an appropriate incubation time. In adsorption, the enzyme molecule is bound to the carrier matrix by hydrogen bonding, van der Waals forces, ionic bonding, and covalent bonding. The attractive force is weak, but it does not affect the topography of the enzyme and the active sites without affecting the activity. Every carrier matrix can be used on any enzyme for adsorption, but not every enzyme can be immobilized on all carriers. A carrier molecule can be successfully used on an enzyme molecule when the affinity between the carrier and the enzyme molecule is high. It depends on the presence of active groups on the carrier surface area, particle size, and pore structure. The carriers used for enzyme immobilization can be classified into organic and inorganic origin. Inorganic molecules can be silica, silica gel, and hydroxyapatite while organic molecules can be chitin, starch, alginate, collagen, polyacrylamide, and polyurethane. The carriers for the enzyme adsorption can be prepared in different forms of membranes, for example, porous hollow fiber membranes, and cotton flannel cloth and nylon membranes (Jesionowski et al., 2014). Noncovalent immobilization or adsorption could be reversed if the pH, ionic strength, temperature, or polarities of the solvent are reformed. The method involves seaming the enzyme internally or externally to the substratum of carrier molecules by preventing the enzyme's physical and chemical modification. Adsorption can be simultaneously used for immobilization and purification of proteins (asparagines on CM-cellulose). In simple adsorption, the leaching of the

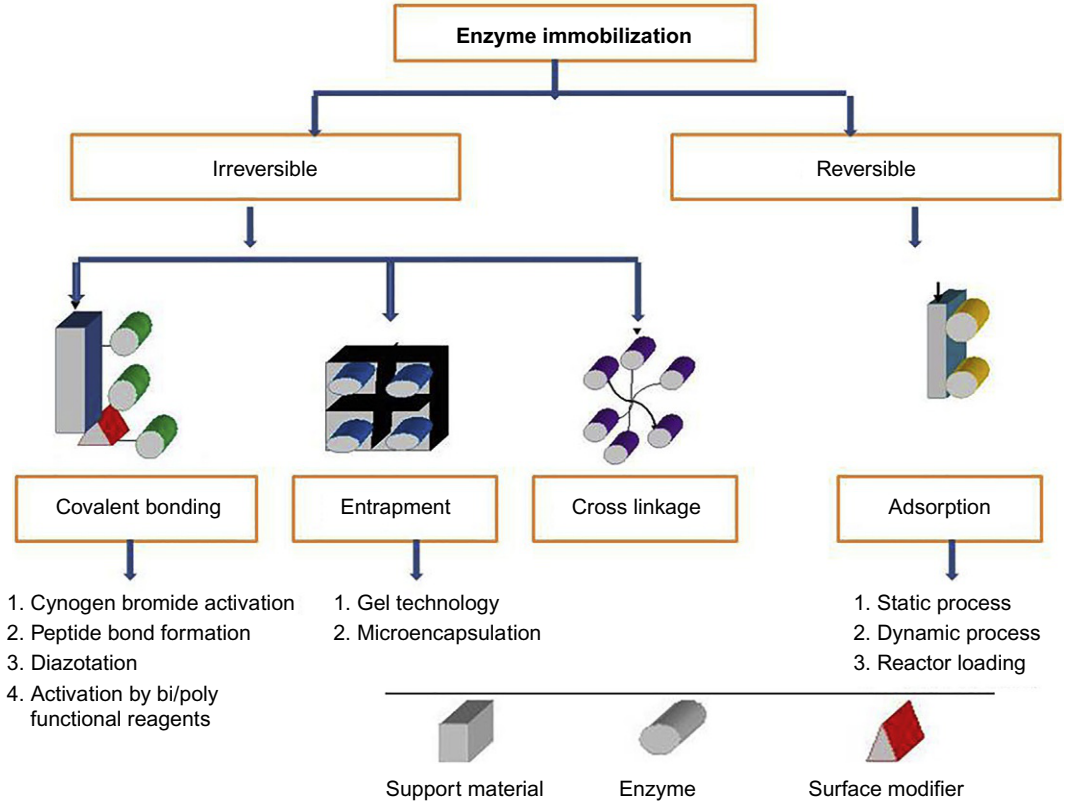

FIG. 37.1 Classification and representation of different enzyme immobilization techniques.

enzyme adsorbed to the custom-made adsorbent can be prevented by compounds such as silicone. For example, novozyme 435 (*Candida antarctica* lipase B) is coated with a silicon polymer to prevent leaching of the enzyme (Katchalski-Katzi and Kraemer, 2000).

37.5 CARRIER MOLECULES

37.5.1 Inorganic Carriers

Among a large number of available carriers, silica is most profoundly used due to its disseminative characteristics such as pore size and morphology. In recent trends, nanoparticles of silica are used in the immobilizations of mesoporous silica (SBA-15, MUS-H, and MCM 41) with pore sizes (2–30 nm), and research is being done on the effect of size and surface area on specific enzymes. The β-galactosidase enzyme is preferentially adsorbed either in mesopores or macropores, depending on its initial concentration. The selective adsorption, arising from the oligomeric complexation of the protein (monomer/dimer/tetramer), has an effect on the catalytic activity of the material. Indeed, the enzyme encapsulated in macropores is more active than the enzyme immobilized in mesopores (Pavel et al., 2017). Silica is highly used followed by gold, titanium, zirconium alumina gel, and bentonite.

37.5.2 Organic Carriers

Chitosan is a nontoxic, biocompatible, and gel-forming cationic compound that can be prepared in different geometrical configurations such as membranes, beads, nanoparticles, fibers, hollow fibers, or sponges (Krajewska, 2005). Chitosan coated with a magnetic nanoparticle is used in lipase immobilization (Ziegler-Borowska et al., 2017).

Cellulose is a polysaccharide that is a thermoplastic but hardly combustible polymer; it is also insoluble in water. Magnetically modified bacterial cellulose has been used for enzyme immobilization (Baldikova et al., 2016). The combination of organic and inorganic carriers has been developed resembling metal organic frameworks (MOF). Synthetic polymers are in extensive use, for example, poly(2-ethyloxazoline) was used as a polymer component for electrospinning of different enzymes in an aqueous medium. (Plothe et al., 2017)

37.5.3 Surface Modifiers

Adsorption requires a reliable carrier with specific functional groups that facilitate the process. The modifying agent functions along with the reactive groups in its molecule. One group enables it to anchor on the carrier chemically while the other enables it to physically interact with the enzyme. Based on this concept, silica nanoparticles were modified with poly(*n*-isopropylacrylamide) and poly(ethylene glycol) (PNIPAM-PEG) to improve the controlled release of protein by Erick et al. (2017). Also, Ozturk et al. (2016) reported an immobilized *C. antarctica* lipase B (CALB) by physical adsorption using organomodified forms of sepiolite and montmorillonite.

Modification by adsorption solves critical problems such as enzyme reaction activity that gets conducted only in an aqueous solution and not in organic solvents. Enzymes can be used for the transformations of hydrophobic substrates that can only be performed in organic solvents (Carrea and Riva, 2000; Iyer and Ananthanarayan, 2008). Similarly, enzymes preserve higher activity in aqueous than solvents by immobilization, for example, immobilized lipases on functionalized silica particles had higher potential for the synthesis of fructose oleate in an organic solvent/water system (Vescovi et al., 2017). Additionally, along with the use of the specific organic solvents, the properties such as chemo-, regio-, and enantioselectivity of the enzymes after immobilization may be customized for a particular purpose (Carrea and Riva, 2000; Klibanov, 2001). Adsorption increases the stability and can be well exemplified in the case of adsorption of papain on gold nanoparticles, which preserves its activity and also enhances the stability. This allows the reuse of the linked enzyme several times without any significant loss of its catalytic performance. In particular, k_{cat} and K_m values remain substantially unchanged while the immobilized enzyme exhibited higher activity in a wider pH range, retaining 80% residual activity at 90°C, as has been reported by Homaei et al., 2014.

The enzyme can be immobilized by adsorption

(a) *Static process*: The enzyme molecule is brought into physical contact with the carrier without agitation.
(b) *Dynamic process*: The enzyme adheres to the carrier by the process of mechanical shaking.

(c) *Reactor loading*: The enzyme binds to a carrier by the dynamic process but in large reactors that are used for the commercial production of enzymes.
(d) *Electrodeposition*: The carrier is placed in proximity with electrodes in an enzyme bath and electric current is passed through it, which initiates the process of enzyme adherence to the carrier.

The adsorption method is simple, low cost, and chemical-free. The adsorption method requires minimum activation energy and intact enzyme topology. There are many carrier molecules that give the opportunity to immobilize every class of enzymes but are liable to change with factors such as pH, temperature, and ionic strength.

37.6 COVALENT BONDING

Covalent immobilization includes the formation of a strong covalent bond between the enzyme and the carrier matrix. This method emphasizes unmodified proteins but relies on functional groups such as thiol amine ester and cysteine residues. The common method employed is the addition of an enzyme solution to competitive inhibitors to block the active site of the enzyme before immobilization to prevent a loss of activity. The coupling reaction can be divided into two types: activation of the matrix by the addition of a reactive function to a polymer, and modification of the polymer backbone to produce an activated group (Brena et al., 2013). The reaction involves forming electrophilic groups on a carrier, which bind to strong nucleophiles on the proteins. This method requires a matrix (agarose, sephadex) with reactive groups such as hydroxyl, amine, carboxyl, or spacer arm that are artificially attached to a model by chemical reaction. The matrix selection plays a major role in immobilization by a covalent bond, which depends on factors such as its cost, availability, binding capacity, hydrophilicity, structural rigidity, and durability during various applications. This method demands nonessential amino acids (other than active site groups) on the enzyme, leading to minimal conformational changes. It promotes the higher resistance of immobilized enzymes toward extreme physical and chemical conditions.

The enzyme can be immobilized on prefabricated adsorbents. In the case of *C. antarctica* lipase B (CaLB), the enzyme is adsorbed on a macroporous acrylic resin that can be leached easily to prevent surface-functionalized acrylic resins; for example Eupergit C can be used. Eupergit C, a macroporous copolymer of *N*, *N*′-methylene-bi-(meth acrylamide), glycidyl methacrylate, allyl glycidyl ether, and meth acrylamide, is widely used for the immobilization of enzymes (Katchalski-Katzi and Kraemer, 2000; Jesionowski et al., 2014). Eupergit C is highly hydrophilic and stable, both chemically and mechanically, over a pH-range 0–14; it does not swell or shrink even upon drastic pH changes in this range.

Carriers are activated by cynogen bromide, carbodiimide triazine, etc. Cyanogen bromide (CNBr)-agarose and CNBr-activated sepharose containing carbohydrate moiety and glutaraldehyde as a spacer arm have imparted thermal stability to covalently bound enzymes. The thiol group is also often employed for protein immobilization and readily undergoes conjugate addition with unsaturated carbonyls to form stable thio ether bonds (Cang-Rong and Pastorin, 2009). Currently, the photo-triggered reactions represent a unique class that illustrates the advanced management of the reaction initiation and interval along with better

resolution with space and time amid a suitable light source with a defined wavelength, light intensity, and contact time without the need for metal catalysts and ligands. Thus, the photo click chemistry to trigger the pyrazoline formation from tetrazoles is gaining importance due to the ease of detection of the progression of the reaction by the formation of a fluorescence compound; however, it is still not commercially employed.

The different groups used for covalent bonding various methods are

- Cynogen bromide activation:
  The inert support materials cellulose, sepharose, and sephadex contain glycol, which gets activated by CNBr, which then binds to enzymes and immobilizes them.
- Diazotization:
  Support materials such as amino-benzyl cellulose, amino derivatives of polystyrene, and amino-silanized porous glass are subjected to diazotization on treatment with $NaNO_2$ and HCI, enabling them to bind covalently to tyrosyl or histidyl groups of enzymes.
- Peptide bond formation:
  Enzyme immobilization is achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and vice versa of enzymes. The support material is principally chemically treated to form active functional groups.
- Activation by bi- or polyfunctional reagents:
  Reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support, for example, aminoethylcellulose, albumin, and amino alkylated porous glass.

The activity of the covalently bonded enzyme depends on the size and shape of the carrier material, the nature of the coupling method, the composition of the carrier material, and specific conditions during coupling. This method is stable and prevents the leakage of protein into the production stream; it is not susceptible to environmental changes. However, it is expensive and complicated, leading to pressure on the enzyme and drastic changes in the conformational and catalytic properties of the enzyme with the matrix (Dwevedi, 2016). The three-dimensional structure of the protein is modified after the attachment to the support and leads to a significant loss of the initial activity of the biocatalyst.

37.7 ENTRAPMENT

In recent years, the entrapment technique has become the focus of interest and has been widely used for the entrapment of many biomolecules including enzymes, catalytic antibodies, antigens, fungi, and plant and animal cells. The enzymes are physically entrapped within the lattice of a polymer matrix through the covalent or noncovalent bonds. This creates a negligible impact on the catalytic properties without significantly modifying their structures and functions; it also involves no high temperatures or harsh chemical reactions (Nawaz et al., 2015). It essentially binds a polymer solution where the enzymes with the solution are polymerized and entrapped in its lattice. It is subdivided into matrix entrapment and membrane entrapment (microencapsulation). Usually, the matrixes used are a water-soluble polymer (Guzik et al., 2014). There are various matrices that are used to entrap them and they are divided into organic and inorganic materials. Organics are polysaccharides, proteins, carbon,

vinyl and allyl polymers, and polyamides, for example, Ca-alginate, agar, K-carrageenan, collagen. Inorganics are activated carbon and porous ceramic.

The commonly used matrix is alginate. Alginate is an anionic polysaccharide distributed widely in the cell walls of brown algae and often utilized for the formation of beads capable of entrapping different macromolecules in the form of calcium-alginate beads. The porosity can be controlled to an appropriate degree by the judicious selection of precursors, modifiers, and polymerization conditions (Pizarro et al., 1997). Efficient entrapment has been achieved with alginate-gelatin-calcium hybrid carriers, preventing enzyme leakage and providing increased mechanical stability. Calcium alginate is a cost-efficient and biocompatible matrix for the entrapment of different enzymes (α-amylase, protease, and pectinase). Lipases entrapped in j-carrageenan have been reported to be highly thermostable and organic-solvent tolerant (Nawaz et al., 2015).

The sol-gel technique, or solution-gel technique, is frequently used to entrap enzymes. This method uses orthosilicate such as tetramethyl orthosilicate, tetraethyl orthosilicate, or methyltrimethoxysilane as the precursor. The hydrolysis of these precursors results in the formation of the oligomer. Then the oligomer is further hydrolyzed to form an aqueous solution that is gelatinous in nature. This gelation process leads to the development of a three-dimensional network, and the biomolecules are finally trapped into the cages of the network (Pizarro et al., 1997). Excellent activities on the entrapment of lipase by utilizing alkoxysilane derivatives as a gel precursor are observed. Sol-gel matrices with supramolecular calixarene polymers have been used for the entrapment of *Candida rugosa* lipase (Psomaa et al., 2010).

37.8 COPOLYMERIZATION

Copolymerization or cross-linking of enzymes was developed in the 1960s. Initially, the protein was cross-linked with NH_2 groups with the bifunctional chemical cross-linker glutaraldehyde. This had several drawbacks such as low activity retention, low mechanical stability, poor reproducibility, and difficulties in handling the gelatinous cross-linked enzymes. The cross-linking of enzymes was improved by linking the enzymes in a gel matrix or on a carrier, but this also led to many drawbacks. The enzyme linkage was focused on carrier-bound enzymes and was used for industrial methodology and commercialized which had significant stability without getting denatured and were thermostable. The initial studies were done using cross-linked crystals of thermolysin (EC 3.4.24.4), of interest in the manufacture of aspartame, and the method was shown to apply to various ranges of enzymes. They were highly active, and their particle size ranged from 1 to 100 nm. Their operational stability and ease of recycling ideally suited them for industrial biotransformations.

Recent reports from Sheldon (2007) on a cross-linked enzyme crystal of chloroperoxidase (CPO) from Caldariomyces fumago showed a high thermal stability and tolerance to organic solvents, moreso than the free CPO. However, the few disadvantages of cross-linked enzyme crystals demanded a high purity and crystallization. These were improvised by the addition of salts, or nonionic polymers, to aqueous solutions of proteins, which led to the precipitation of molecules binding noncovalently without disturbing their tertiary structure and

denaturation. It was found that cross-linking these physical aggregates would render them permanently insoluble while maintaining their preorganized superstructure and catalytic activity. This indeed proved to be economical and led to the development of immobilized enzymes, cross-linked enzyme aggregates (CLEA) (Talekar et al., 2012). The first examples of CLEAs were derived from penicillin G amidase, an industrially important enzyme that is used in the synthesis of semisynthetic penicillin and cephalosporin antibiotics.

37.9 ENCAPSULATION

The encapsulation strategies are simple, inexpensive, and of interest because they entrap a huge number of biomolecules. The encapsulation method is based on the molecule in a polymer matrix; it limits the movements but permits substrate recognition and catalysis (Jadhav and Singhal, 2014). There are two methods of encapsulation that include the gel technology method and encapsulation in the matrix.

Gels are widely used as a support material and are of natural or synthetic origins. Natural sources are derived from agar, k-carrageenan, or alginate; synthetic gels are derived from acrylamide. Acrylamide is traditionally used as an inert matrix to lodge enzymes. Acrylamide-derived gel is a cross-linked network of polymers with high molecular weight; they adsorb polar solvent by the capillary forces, osmosis, and polymer/solvent molecular interactions. The gel's internal structure depends on the relative quantities of its components, that is, the monomer and the cross-linker (Nawong et al., 2016). Accurate selection of the monomer and cross-linking agent proportions forms the network by modification of the porous structure and pore size. An optimized gel would allow entrapment without the risk of the enzymes parting the polymer matrix with any severe diffusional resistances (Chena et al., 2004).

Agar is a polysaccharide in the cell walls of some red algae; it consists of agarose and agaropectins. Agar or agarose can be enzymatically degraded using two types of agarases by their hydrolysis patterns. The two agarases, namely, α-agarase and β-agarase, hydrolyze α-1, 3 linkages and β-1,4 linkages in agarose, respectively. α-Agarase cleaves the α-L-(1,3) linkages of agarose to produce oligosaccharides of the agarose series with 3,6-anhydro-L-galactopyranose at the reducing end (Datta et al., 2013). By contrast, β-agarase cleaves the β-D-(1, 4) linkages of agarose to produce neoagaro-oligosaccharides with D-galacto pyranoside residues at the reducing end. Neoagaro-oligosaccharides from β-agarase have recently received much attention because of their numerous biological functions, such as inhibition of bacterial growth, retardation of starch degradation, improvement of food qualities as low-calorie additives, and skin moisturizing and whitening. Therefore, agar-derived oligosaccharides have broad applications in the food, cosmetic, and medical industries. Agarase can be directly used to produce agar-derived oligosaccharides but is often easily devitalized and difficult to segregate from the reaction system during recovery and recycling. Therefore, the enzymatic activity, stability, and recovery of agarase should be improved before its application as an industrial biocatalyst (Tischer and Wedekind, 1999). Based on the various methods discussed above, the properties or characteristics of different enzyme immobilization techniques are summarized in Table 37.2.

TABLE 37.2 Properties of Different Enzyme Immobilization Techniques

Properties	Adsorption	Covalent Binding	Entrapment	Copolymerization	Encapsulation
Production	Simple	Difficult	Difficult	Simple	Simple
Cost	Low	High	Moderate	Cheap	Low
Binding force	Variable	Strong	Weak	Strong	Variable
Enzyme leakage	Yes	No	Yes	No	Yes if molecules are small
Applicability	Wide	Selective	Wide	Wide	Wide
Operational issues	High	Low	High	Low	Low
Matrix effects	Yes	Yes	Yes	No	Yes
Considerable diffusional barriers	No	No	Yes	No	Yes
Microbial safety	No	No	Yes	No	No

http://www1.lsbu.ac.uk/water/enztech/immethod.html
http://www.easybiologyclass.com/enzyme-cell-immobilization-techniques/

TABLE 37.3 Various Applications of Immobilized Enzymes.

Enzyme	Application
Trypsin	β-Lactoglobulin production
β-Galactosidase	Removal of lactose from milk
Pectinase	Pectin solution production
Cardosin A (Protease)	α-Lactoglobulin production
Tyrosinase	Detect phenolics in red wine
Lipase from *Candida rugosa*	Production of oil and grease
β-Galactosidase and amyloglucosidase	Hydrolyze lactose in whey/whey permeates, skimmed milk
Pectin lyase	Degrade esterified pectin
Pectinase from *Aspergillus aculeatus*	Degrade pectin
β-Galactosidase	Enhance flavor of muscat wine
Candida molischiana Glucomylase	Degrade starch and hydrolyzed mannose starch
Laccase from *Pyricularia oryzae*	Wine, fruit juice, and beer processing

37.10 APPLICATIONS OF IMMOBILIZED ENZYMES

Various applications of the immobilized enzymes are presented in Table 37.3. Among the different methods of immobilizations, calcium alginate is a cost-efficient and biocompatible matrix for enzymes, namely α-amylase, protease, and pectinase. Maltase from *Bacillus licheniform* KIBGE-IB4 was immobilized using an entrapment technique within calcium alginate beads, as reported by Nawaz et al. (2015). The sol-gel methods using sodium silicate silica precursor are being used to immobilize whole cells of *Methylomonas* sp. strain GYJ3 (Chena et al., 2004). The effect of entrapment in different sol-gel matrices was evaluated by the enzyme activity and stability by using methyltrimethoxysilane as a precursor. Some challenges faced in this method are that the enzyme occasionally leaks into the solution and leads to reduced stability and activity. Hence, proper modification of the matrix or membrane is essential.

Magnetic nanoparticles for immobilizing enzymes have recently been considered for the commercial application of biocatalyst-processed products. This technique depends on the solid-phase magnetic feature, which can be rapidly separated and recovered from the reaction medium using an external magnetic field because it reduces the capital and operation costs (Guzik et al., 2014; Chena et al., 2004). Entrapment by nanostructured supports such as electrospun nanofibers and natural materials has revolutionalized the world of enzyme immobilization with its wide-ranging applications in the field of fine chemistry, biomedicine, biosensors, and biofuels (Table 37.3). Prevention of friability, leaching, augmentation of entrapment efficiency, and enzyme activity by *C. rugosa* lipase entrapped in chitosan has been reported by Wen-qiong et al., 2017. It is nontoxic, biocompatible, and amenable to chemical modification as well as being highly affinitive to protein due to its hydrophilic nature. Entrapment by mesoporous silica is attributed to its high surface area, uniform pore distribution, tunable pore size, and high adsorption capacity (Premaratne et al., 2017).

37.11 PROSPECTS OF ENZYME IMMOBILIZATION

The methods used for immobilizing enzymes aim at improving the retention activity of enzymes as well as their cost effectiveness and safety. The methods available now have their own characteristic advantages and disadvantages (Table 37.4). In recent years, genetic engineering technology has been clubbed with enzyme immobilization, which can help to increase the enzyme's yield and kinetics. Enzymes obtained from cloning and non-GRAS status microorganisms may be cloned into safe, high-potential microorganisms by increasing the number of gene copies of the enzyme to increase the production. Designing a new catalyst structure by utilizing bioinformatics to generate a more efficient enzyme than the existing ones needs to be explored.

TABLE 37.4 Advantages and Disadvantages of Enzyme Immobilization Methods

Methods[a]	Enzyme Support Material	Enzyme	Advantages[a]	Disadvantages[a]	References
Physical adsorption (Weak bondshydrophobic, Van der Waals or ionic interactions)	Olive pomace powder	Thermomyces lanuginosus xylanase	Simple and cheap	Desorption Nonspecific adsorption	Jesionowski et al., 2014
Encapsulation (Noncovalent bonding)	Carageenan	Lipases	Simple and oriented immobilization	High cost	Wen-qiong et al., 2017
Covalent binding (Chemical binding between functional groups of the enzyme and support)	Eupergit C	*Candida antarctica* Lipase B	No enzyme leakage, potential for enzyme stabilization	Matrix and enzyme are not regenearble, Major loss of activity	Vescovi et al., 2017
Entrapment (Occlusion of an enzyme within a polymeric network)	Calcium alginate	Protease	Wide applicability	Mass transfer limitations, enzyme leakage	Chena et al., 2004; Nawaz et al., 2015
Cross-linking (Enzyme molecules are cross-linked by a functional reactant)	Starch and glutaraldehyde (25%) Ammonium sulfate and DNSA (3,5-dinitrosalycyclic acid)	Alpha-amylase	Biocatalyst stabilization	Cross-linked biocatalysts are less useful for packed beds	Talekar et al., 2012

[a] *Brena et al., 2013; http://www.easybiologyclass.com/enzyme-cell-immobilization-techniques/:*

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CHAPTER

38

Enzymes in Biosensors for Food Quality Assessment

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38.1 INTRODUCTION

The quality of food that reaches consumers has a direct impact on their health and nutrition, and is therefore an essential prerequisite of food manufacturing. Incidents of microbial contamination, spoilage, the presence of pesticides, and adulteration have demonstrated the necessity of having strict quality checks to ensure that the quality of food is not compromised at any step of the manufacturing and supply chain, right from the raw produce until the final product. Food safety and security agencies make sure that manufacturers adhere to strict quality standards, and various techniques have been standardized to test the physicochemical, biological, and organoleptic properties of the food samples along with the overall nutritional value. However, some food samples have to be tested onsite, which may not be possible if the testing equipment is not portable. Additionally, employing skilled labor to perform the tests and to accurately interpret the results may not be possible. Conventional techniques of food analysis may be extremely tedious and time consuming. It is therefore essential to replace such conventional methods with rapid and easy-to-use techniques.

Biosensors are showing great promise in this area, mainly due to their simplicity and quick results. They allow the testing of multiple samples for multiple characteristics with minimum error. Unlike medical applications, where blood, serum, or urine are the major sample matrices, the food industry presents a wide variety of samples with varied compositions that cause difficulty in designing and optimizing biosensors (Monosik et al., 2012).

38.2 BIOSENSORS

A biosensor is an analytical device that converts a biological response into an electrical signal (Velusamy et al., 2010). Typically, a biosensor consists of two main components: a bioreceptor and a transducer. The bioreceptor or biorecognition element interacts with a target

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00038-4

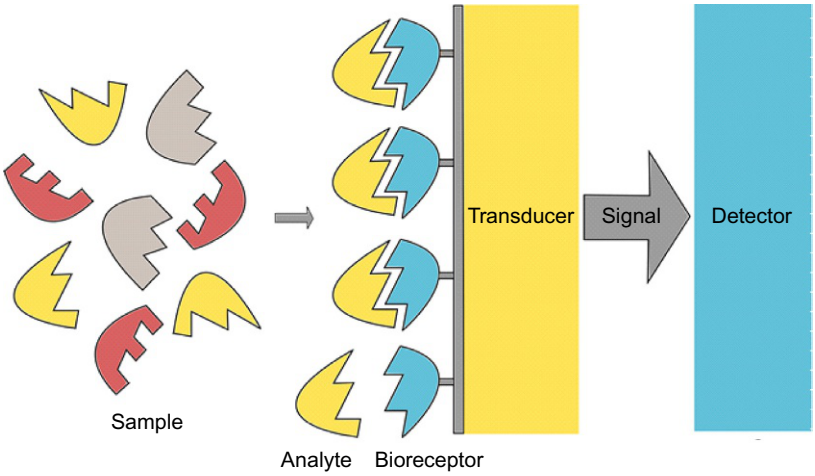

FIG. 38.1 The working of a typical biosensor.

molecule with high specificity. This interaction is then converted into a measurable signal by the transducer (Fig. 38.1).

Biosensors can be classified on the basis of the biorecognition element as well as the transducer used. Biorecognition elements can be whole living cells, bacteriophages, proteins, enzymes, antibodies, nucleic acids, etc. Similarly, a transducer can be optical, electrochemical, piezo-electric, etc. (Fig. 38.2). Based on the type of applications, biosensors are constructed using multiple combinations of biorecognition elements and transducers, each having its own advantages and limitations. This chapter will focus on the use of enzyme-based biosensors for food quality assessment.

Enzymes are biocatalysts that interact with their substrates with high specificity. Their specificity and selectivity make them ideal candidates for use as bioreceptors in biosensors. Indeed, the first biosensor developed in 1962 that employed a bioreceptor was a glucose oxidase (GOD) enzyme-based sensor for analyzing oxygen concentration (Clark and Lyons, 2006). Enzyme-based biosensors have since found a wide range of applications in the food industry and medicine. Recent developments in biosensors demonstrate the use of nanotechnology and redox mediators. Going through this chapter, readers will see many examples of biosensors utilizing electrodes modified with gold nanoparticles (AuNPs), gold nanorods (AuNRs), carbon nanotubes (CNTs), grapheme, etc. Nanomaterials such as CNTs have a high specific surface area and electrical conductivity (Tîlmaciu and Morris, 2015), which are desirable for biosensors. Also, it will be evident that most enzymatic biosensors discussed here are electrochemical sensors. Electrochemical biosensors consist of a working electrode, a reference electrode, and a counter electrode. Enzymatic electrochemical biosensors are based on the generation of charged species via enzyme-catalyzed reactions, which in turn give rise to a current or a change in voltage (Tîlmaciu and Morris, 2015). Further, it should be noted that all electrochemical biosensors discussed here have used some type of electrochemical stations for amperometric, potentiometric, and other types of electrical measurements, unless otherwise stated.

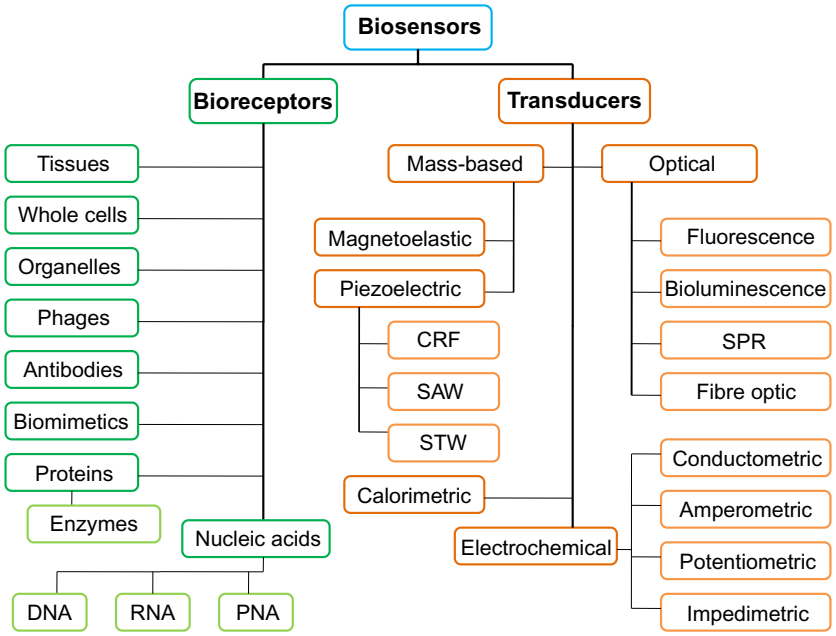

FIG. 38.2 Various biorecognition elements and transducers.

Enzyme-based biosensors have been developed for a variety of aspects significant to food quality and acceptability, such as nutrient content, adulteration, toxins and toxic substances, pathogen detection, spoilage, etc. Examples of biosensors relevant in this regard are described further.

38.3 NUTRIENT CONTENT

The health conscious 21st century consumer understands the significance of the nutrient content of food and intends to make informed decisions when buying food products. It is a requirement for most packaged foods to display a nutrition facts label, and food manufacturers are expected to ensure that their products adhere to the nutrient content listed on their labels. Examples of various enzyme biosensors designed to determine the concentrations of important nutrients in food samples are explained further (Table 38.1).

38.3.1 Sugars

Sugars are an integral component of the food and beverage industry, and as such, are important indicators of quality. Of the most important sugars, glucose, is of importance for both the food industry as well as medicine. Glucose sensors form the basis of almost 85% of the biosensor industry (Nenkova et al., 2010). Examples of enzyme-based sugar biosensors employing various enzymes are described.

TABLE 38.1 A Quick Glance at Some Examples of Biosensors

Enzyme	Analyte	Food Sample	Reference
Glucose dehydrogenase	Glucose	Fruit drinks and wines	Monošík et al., 2012
Fructose dehydrogenase	Fructose	Fruit juice, honey, soft drinks, energy drinks	Antiochia et al., 2013
Fructose dehydrogenase, Invertase	Glucose, fructose, sucrose	Condensed milk, infant food reference	Vargas et al., 2013
Glucose oxidase, Invertase, β-galactosidase, α-glucosidase, Mutarotase	Glucose, maltose, lactose, sucrose	NA	Soldatkin et al., 2013
Malate quinone oxidoreductase	Malic acid	White, rose, and red wines	Molinero-Abad et al., 2014
Malate dehydrogenase, Diaphorase	Malic acid	Red wine samples	Giménez-Gómez et al., 2017
Lactate oxidase	Lactic acid	Wines and ciders	Loaiza et al., 2015
Lactate oxidase, Horseradish peroxidase	Lactic acid	Wine and beer	Pérez and Fàbregas, 2012
Citrate lyase, Oxaloacetate decarboxylase	Citric acid	Apple, strawberry, tomato, and carrot juices	Kim, 2006
Ascorbate oxidase	Ascorbic acid	Commercial juices, vegetable crops	Wen et al., 2012
Alcohol dehydrogenase	Ethanol	Beer, red wine, and white spirits	Li et al., 2013
Alcohol oxidase	Ethanol	Beer, legen, and tapai samples	Kuswandi et al., 2014
Tyrosinase	Hydroquinone and other polyphenolics	Red wine samples	Sýs et al., 2013
Peroxidase	Tert-butyl hydroquinone	Salad dressings	Regina de Oliveira et al., 2014
Laccase	Formetanate hydrochloride	Spiked mangoes and grapes	Ribeiro et al., 2014
Laccase, tyrosinase	Carbamate	Spiked orange, lemon, and tangerine	Oliveira et al., 2014
Organophosphate hydrolase, Acetylcholine esterase	Organophosphates and nonorganophosphates	Spiked apples	Zhang et al., 2015
Horseradish peroxidase	Palytoxin	Spiked shellfish and seawater samples	Gao et al., 2017
Urease	Patulin	Fruit juices	Soldatkin et al., 2017
Acetylcholine esterase	Aflatoxin B1	Spiked walnut, pea, and sesame	Stepurska et al., 2015

TABLE 38.1 A Quick Glance at Some Examples of Biosensors—cont'd

Enzyme	Analyte	Food Sample	Reference
Alkaline phosphatase	Aflatoxin M1, Ochratoxin A	Red wine, milk	Karczmarczyk et al., 2017
Urease	Urea	Spiked milk	Ramesh et al., 2015
Acetylcholine esterase	Melamine, urea	Spiked milk	Ezhilan et al., 2017
Xanthine oxidase	Xanthine	Fish, chicken, beef, and pork	Devi et al., 2013
Xanthine oxidase	Xanthine	Fish	Dervisevic et al., 2015
Peroxidase	Inosine monophosphate	Stored muscles	Sun et al., 2016
Diamine oxidase, horseradish peroxidase	Histamine	Sardines, mackerel, greater weever	Pérez et al., 2013
Histamine dehydrogenase, putrescine oxidase	Histamine and putrescine	Octopus	Henao-Escobar et al., 2016
Tyrosinase	Tyramine	Spiked Sauerkraut	Apetrei and Apetrei, 2013

Their detailed explanation and method of working are described further. NA: not applicable; no real sample analysis performed.

Traditionally, glucose biosensors have used GOD and sometimes peroxidase (POD) enzymes. The reactions catalyzed by the two enzymes are as follows:

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-Gluconic acid} + H_2O_2$$

$$\text{Electron donor} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Oxidized electron donor} + H_2O$$

Glucose biosensors constructed using commercially available FAD-dependent glucose dehydrogenases from *Aspergillus* sp. and *Aspergillus oryzae* immobilized on a nanocomposite electrode have been described (Monošík et al., 2012). The electrode consisted of multiwalled carbon nanotubes (MW-CNTs) entrapped between layers of chitosan. The dehydrogenases catalyzed the conversion of β-D-glucose to D-glucono-1,5-lactone, and reduced the mediator. The oxidation of the reduced mediator at the electrode generated a current. The biosensors employing the enzymes from *Aspergillus* sp. and *Aspergillus oryzae* showed linearity in the range from 50 to 960 μM and from 70 to 620 μM, respectively. The response time was 60 s, with a limit of detection of 4.45 and 4.15 μM, respectively. No change in sensitivity was observed after 100 consecutive measurements and storage for 4 weeks at 4°C. Testing with commercial fruit drinks and wines showed good correlation between the results obtained with the biosensors and HPLC.

An amperometric fructose biosensor utilizing fructose dehydrogenase (FDH) was described (Antiochia et al., 2013). The enzyme was immobilized in a carbon nanotube paste (CNTP) electrode using osmium polymer hydrogel and albumin hydrogel for entrapment in two different sets. FDH catalyzed the conversion of D-Fructose to 5-keto-D-Fructose, simultaneously reducing the osmium polymer mediator. Detection was achieved by the generation of a current when electrons were released at the electrode by the reduced mediator

at 200 mV. With a fast response time of 4 s and a limit of detection of 1 μM, the biosensor showed linearity in the range of 0.1–5.0 mM. The biosensor retained 80% of sensitivity after 4 months, and utilized only 5 U of the enzyme. When tested on commercially available fruit juice, honey, soft drinks, and energy drinks, the results obtained were consistent with commercial reference kits.

In addition to the detection of a target sugar, researchers have reported biosensors that allow the detection of multiple sugars simultaneously. An integrated amperometric biosensor based on a self-assembled monolayer modified gold disk electrode that involved coimmobilization of invertase, FDH, and tetrathiafulvaline (TTF) was reported (Vargas et al., 2013). The sensor could simultaneously detect glucose, fructose, and sucrose, and was successfully tested to detect sugar concentrations in condensed milk and infant food reference material. The reaction cascades involved are as follows:

$$\text{Sucrose} \xrightarrow{\text{invertase}} \alpha\text{-D-glucose} + \beta\text{-D-fructose}$$

$$\alpha\text{-D-glucose} + \beta\text{-D-fructose} \xrightarrow{\text{fructose dehydrogenase}} \text{5-keto-D-fructose}$$

Further redox reactions involving the pyrroloquinolinequinone group and TTF led to the release of electrons in the solution. With a detection limit of 0.36 μM, the biosensor showed linearity in the range of 0.012 μM to 3 mM sucrose.

A conductometric biosensor involving reaction cascades catalyzed by multiple enzymes for simultaneous detection of maltose, lactose, sucrose, and glucose was developed (Soldatkin et al., 2013). Invertase, β-galactosidase, and α-glucosidase were used to decompose their corresponding substrates, that is, sucrose, lactose, and maltose to α-D-glucose while mutarotase was used to transform the α-D-glucose to β-D-glucose. Finally, GOD was used to convert β-D-glucose to D-Glucolactone. D-Glucolactone hydrolyzes to give D-gluconic acid, which in turn dissociates to give a proton. The important reaction cascades involved in this process are as follows:

$$H_2O + \text{Maltose} \xrightarrow{\alpha\text{-glucosidase}} \mathbf{2}\,\alpha\textbf{-D-glucose}$$

$$H_2O + \text{Lactose} \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \alpha\textbf{-D-glucose}$$

$$H_2O + \text{Sucrose} \xrightarrow{\text{invertase}} \beta\text{-D-fructose} + \alpha\textbf{-D-glucose}$$

$$\alpha\textbf{-D-glucose} \xrightarrow{\text{mutarotase}} \beta\text{-D-glucose}$$

The β-D-glucose so formed undergoes traditional conversion to gluconic acid by GOD, which leads to the formation of H_2O_2, and, ultimately, protons. The detection time for the biosensor is 2 min, and the detection limits for all substrates are 1 μM. The biosensor retained 90% of its activity after a month in dry conditions at 4 °C.

38.3.2 Organic acids

Organic acids are common constituents of wines and fruit juices. They help in stabilizing pH-sensitive colors, impart taste, and have antimicrobial and antioxidant properties

(Nelson et al., 2016). As such, they make for excellent markers of quality of beverages. Some examples of biosensors for detection of organic acids are hereby described.

38.3.2.1 Malic acid

An amperometric biosensor employing malate quinone oxidoreductase enzyme for the detection of malic acid was developed (Molinero-Abad et al., 2014). The enzyme was immobilized on a screen-printed carbon electrode modified with AuNPs using bovine serum albumin. TTF was used as the redox mediator. The FAD-dependent enzyme catalyzed the conversion of malate to oxaloacetate where FAD gets reduced to $FADH_2$. The $FADH_2$ then reduces the oxidized TTF to its reduced form, generating a current. The limit of detection is 2.0 μM. The biosensor is able to successfully detect malic acid concentrations in white, rose, and red wine samples.

A bienzymatic amperometric biosensor utilizing malate dehydrogenase and diaphorase enzymes entrapped in an electrosynthesized polypyrrole film for real-time monitoring of L-malate during malo-lactic fermentation was described (Giménez-Gómez et al., 2017). A thin-film gold electrode was used as transducer. The biosensor performed best in the range of 10^{-7} M to 10^{-6} M, and had a detection limit of 6.3×10^{-8} M. The biosensor retained more than 90% of its initial sensitivity after 37 days of operation. Malic acid concentrations in three red wine samples were tested to demonstrate practical use of the biosensor. It was found that the chemical decomposition of NAD^+ decreased the working stability of the biosensor.

38.3.2.2 Lactic acid

An amperometric lactic acid biosensor employing lactate oxidase immobilized onto screen-printed carbon electrodes modified by platinum nanoparticles was described (Loaiza et al., 2015). The electrodes were prepared by chemical reduction of a platinum precursor on graphitized carbon nanofibers. The enzyme was immobilized using polyethyleneimine (PEI) and glutaraldehyde (GA). The biosensor had a detection limit of 6.9 μM and retained 90% of its initial sensitivity after 3 months of storage at room temperature, and 95% of initial sensitivity after 18 months of storage at −20°C. The L-lactate concentrations in commercially available wines and ciders were tested, and the results obtained with the biosensor were consistent with those obtained with commercial kits. No significant interference was observed in the presence of other compounds commonly found in cider and wine.

An amperometric bienzyme biosensor employing lactate oxidase and horseradish peroxidase (HRP) for determination of lactic acid was reported (Pérez and Fàbregas, 2012). The enzymes were incorporated into a carbon nanotube/polysulfone membrane using phase inversion onto screen-printed electrodes. Ferrocene was used as the redox mediator, and was incorporated into the membrane along with the enzymes. With a limit of detection of 0.05 mg L^{-1}, the biosensor showed linearity over the range of 0.1–5 mg L^{-1}. Results obtained on testing with commercial wine and beer samples were consistent with the results from a commercial reference kit.

38.3.2.3 Citric acid

A potentiometric biosensor employing citrate lyase (CTL) and oxaloacetate decarboxylase (OAD) for the detection of citric acid in fruit juices was described (Kim, 2006). The enzymes were immobilized on glass beads using GA as the cross-linking agent. CTL catalyzed the

conversion of citrate to acetate and oxaloacetate, while OAD catalyzed the decomposition of oxaloacetate to pyruvate and CO_2. Carbonate ions generated in the solution by the release of CO_2 were potentiometrically detected using a carbonate ion selective electrode. The biosensor was successfully applied for detection of citrate in apple, strawberry, tomato, and carrot juices, and the results obtained were in good correlation with those obtained by gas chromatography. The biosensor showed linearity in the range of 10^{-4} to 10^{-1} M citrate.

38.3.2.4 Ascorbic Acid

An electrochemical vitamin C biosensor where the enzyme ascorbate oxidase was incorporated into a biocompatible conducting poly(3,4-ethylenedioxythiopene)-lauroylsarcosinate film using a one-step immobilization process was described (Wen et al., 2012). The biosensor had a response time of 15 s, a limit of detection of 0.464 μM, and showed high linearity in the range of 0.002–14 mM. With regards to operational stability, the biosensor retained 79% of its initial sensitivity after 10 consecutive measurements and only 66% of initial sensitivity after 20 consecutive measurements, owing to depletion of dissolved oxygen inside the film containing the enzyme. Use of the biosensor for the detection of ascorbic acid concentrations in commercial juice samples and vegetable crops was successfully demonstrated.

38.3.3 Ethanol

An amperometric biosensor employing alcohol dehydrogenase based on hybrid nanosheets made of graphene and AuNRs was reported (Li et al., 2013). The enzyme catalyzed the conversion of ethanol to acetaldehyde, where NAD+ was reduced to NADH. This NADH then underwent oxidation, generating a current. The biosensor was successfully tested on commercially available beer, red wine, and white spirit samples and showed linearity over the range of 5–377 μM. The limit of detection is 1.5 μM.

The enzyme alcohol oxidase was immobilized on polyaniline film to form a "dip stick" disposable visual biosensor for ethanol detection and successfully used for the determination of ethanol in beer, legen, and tapai samples (Kuswandi et al., 2014). The enzyme converted ethanol to acetaldehyde and H_2O_2, and the H_2O_2 oxidized the polyaniline film to give a color change from green to blue. The biosensor was paired with Java-based image processing software for quantification of color measurements. The biosensor gave a linear response to ethanol concentration in the range of 0.01%–0.8%, with a detection limit of 0.001%.

38.3.4 Antioxidants

A biosensor employing tyrosinase (TYN) enzyme immobilized in a Nafion film on a carbon paste electrode for the detection of hydroquinone and other polyphenolic antioxidant capacity was described (Sýs et al., 2013). The enzyme oxidized hydroquinone to p-quinone, which was then reduced back to hydroquinone at the carbon paste electrode, generating a current. Determination of hydroquinone in red wine samples was successfully performed. The biosensor worked well in concentrations up to 120 μM of hydroquinone, with a detection limit of 1.6 μM.

POD immobilized on Sepiolite clay mineral was employed for the detection of tert-butyl hydroquinone using square wave voltammetry (Regina de Oliveira et al., 2014). The sensor

was constructed by inserting a copper wire into a compacted paste consisting of graphite powder, MW-CNTs, POD, sepiolite, mineral oil, and nafion perfluorinated resin. The biosensor showed linearity over the range of 1.65–9.82 mg L^{-1}, and limits of detection and quantification of 0.41 and 1.25 mg L^{-1}, respectively. The biosensor was successfully used to test tert-butyl hydroquinone concentrations in commercial salad dressing samples.

Analysis of food constituents is not the only avenue for assessment of food quality. The presence of pesticide residues and toxins in food products is of great concern. Exposure to organophosphate pesticides in children has been associated with respiratory problems, asthma (Raanan et al., 2015) and attention deficit/hyperactivity disorder (Bouchard et al., 2010) in addition to the well-known neurotoxic properties of the pesticides. Aflatoxins have been shown to have carcinogenic, nephrotoxic, and immunosuppressive properties (Edite Bezerra da Rocha et al., 2014). Similarly, adulteration and food fraud greatly reduce the quality and acceptability of food products. Via substitution, modification, and dilution by physical or chemical means, adulteration adversely affects the purity of the original ingredient (Moore et al., 2012), thereby damaging food quality. Adulterants such as melamine are added to infant milk powder to falsify protein content, and have been associated with chronic kidney failure (Rovina and Siddiquee, 2016). Examples of biosensors relevant to pesticide detection in foods are described below.

An electrochemical biosensor for the detection and quantification of formetanate hydrochloride (FMT) was created and successfully tested on spiked mangoes and grapes (Ribeiro et al., 2014). The biosensor was prepared by immobilizing commercially available *Trametes versicolor* laccase (LAC) on the surface of a gold electrode using GA as the cross-linking agent. The gold electrode was previously modified with AuNPs. Detection was based on the inhibition of LAC activity by FMT. 4-Aminophenol (4-AMP) was used as the substrate for the enzyme. In the presence of FMT as the inhibitor, LAC was unable to catalyze the oxidation of 4-AMP. All electrochemical measurements were carried out using a commercially available potentiostat/galvanostat. The biosensor showed linearity in the range of 9.43×10^{-7} to 1.13×10^{-5} M, and had a detection limit of $9.5\times10^{-8}\pm9.5\times10^{-10}$ M.

A bienzymatic carbamate biosensor utilizing a hybrid film of LAC and TYN enzymes along with AuNPs entrapped in chitosan was reported (Oliveira et al., 2014). The hybrid film was electro-deposited onto a grapheme-doped carbon paste electrode. Cyclic voltammetry (CV), square-wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS) were performed using a commercial electrochemical system. In the presence of an inhibitor, LAC and TYN were unable to catalyze the oxidation of 4-AMP to quinone-imine. The optimum proportion of the enzymes LAC and TYN for best results was found to be 2:1. The biosensor showed linearity in the range of $1.68\times10^{-9}\pm1.18\times10^{-10}$–$2.15\times10^{-7}\pm3.41\times10^{-9}$ M, and remained stable up to 20 days. No significant interference was observed from glucose, citric acid, or ascorbic acid. The biosensor was successfully used to detect carbamate concentrations in spiked samples of orange, lemon, and tangerine.

A combination of organophosphate hydrolase (OPH) and inhibition of acetylcholine esterase (AChE) for a discriminative detection of organophosphorus (OP) and nonorganophosphorus (non-OP) pesticides was described (Zhang et al., 2015). Paraoxon and carbaryl were used to represent OP and non-OP pesticides respectively. The enzymes were immobilized onto carboxylated MWCNTs via NHS/EDC chemistry to form electrostatically interacted enzyme armored MWCNT-AChE and MWCNT-OPH. Using PEI and salmon sperm DNA salt

(DNA), a set of cushioning bilayers of MWCNT-PEI and MWCNT-DNA was prepared. Two layers of MWCNT-(PEI/DNA), followed by one layer each of MWCNT-OPH and MWCNT-AChE, were allowed to self-assemble in a layer-by-layer (LbL) process on a glassy carbon electrode. Oxidation of thiocholine, a hydrolysis product of ATCh catalyzed by AChE, produced a peak current at 0.61 V. Similarly, hydrolysis of OP by OPH to form p-nitrophenol led to a peak current at 0.96 V. The presence of OP was determined by the inhibition of AChE activity. A differential determination of pesticides could be carried out as OPH did not hydrolyze non-OP, and no peak current was observed at 0.96 V. The percentage of AChE inhibition was calculated as $[(I_0 - I_{Inhibition})/(I_0)] \times 100$, where I_0 and $I_{Inhibition}$ were the CV responses before and after inhibition. With a detection limit of 0.5 μM for paraoxon and 1 μM for carbaryl, the biosensor showed linearity over a range of 0.5–40 and 10–80 μM for paraoxon and carbaryl, respectively. The detection of pesticides extracted from real samples was successfully demonstrated with apples spiked with known concentrations of paraoxon and carbaryl.

38.4 MYCOTOXINS

38.4.1 Palytoxin

A biosensor employing HRP linked aptamers as bioreceptors was described for the detection and quantification of palytoxin (PTX) in artificially spiked samples (Gao et al., 2017). The method was based on a combination of biolayer interferometry (BLI) and an enzyme-linked aptamer-competitive binding assay (ELACBA). PTX was immobilized onto the surface of a commercially available amine reactive second generation (AR2G) biosensor using EDC/NHS chemistry. PTX-specific ssDNA aptamers were screened from an ssDNA library using the MB-SELEX protocol. The biosensor worked on the principle of competitive binding of HRP-aptamers to PTX. On submerging the biosensor in a solution of 3,3′-diaminobenzidine (DAB), the HRP of the HRP-aptamers bound to the sensor converted DAB to a colored polymeric product on the sensor surface, greatly changing its optical thickness and inducing wavelength interference. The BLI-ELACBA technique was successfully used to detect and quantify PTX in artificially spiked shellfish and seawater samples. The biosensor demonstrated real-time rapid detection (10 min) of PTX, with a detection limit of 0.04 pg mL^{-1}.

38.4.2 Patulin

A conductometric biosensor based on the inhibition of urease was employed for the determination of patulin (Soldatkin et al., 2017). Urease was coimmobilized with bovine serum albumin via cross-linking using GA on the surface of gold interdigitated electrodes to form a bioselective membrane. The decomposition of urea by the enzyme urease led to a change in the proton concentration of the medium, in turn changing the conductivity. However, the inhibition of enzyme activity led to a decreased change in conductivity as compared to the control, which was used as an indication of the presence of patulin. Additionally, as the inhibition of urease by patulin is irreversible, a 10 mM cysteine treatment was included for reactivation of the biosensor after inhibition. Total reactivation of the biosensor after up to 55% of inhibition by patulin could be achieved. Further, the biosensor was tested to study its suitability

to detect low concentrations of patulin as found in fruit juices, which required increasing the incubation time to more than 80 min.

38.4.3 AflatoxinB1 (AFB1)

A potentiometric biosensor utilizing pH-sensitive field-effect transistors for the detection of Aflatoxin B1 was described (Stepurska et al., 2015). The biosensor was based on the inhibition of AChE in the presence of the toxin. In the absence of Aflatoxin B1, AChE catalyzed the decomposition of acetylcholine to choline and acetic acid. The acetic acid dissociated to produce protons, leading to a change in pH. To prepare the biosensor, 1% AChE was co-immobilized with BSA on the surface of the transducer to form a biologically active membrane. Saturated GA vapor was used for cross-linking. For real sample analysis using the biosensor, uncontaminated samples of walnuts, peas, and sesame were spiked with known concentrations of AFB1 and tested successfully. The biosensor showed a dynamic range of 0.2–40 $\mu g\,mL^{-1}$. Reproducible results were obtained over a working period of 1 day, and the biosensor remained stable in storage for 1 month.

A voltammetric biosensor for the detection of Aflatoxin M1 (AFM1) and Ochratoxin A (OTA) based on a competitive immunoassay utilizing a secondary antibody conjugated with the enzyme alkaline phosphatase was developed (Karczmarczyk et al., 2017). α-Naphthyl phosphate disodium salt (α-NP) was used as a substrate. The sensor featured modified gold screen-printed electrodes where BSA-toxin conjugates were immobilized onto a self-assembled monolayer of 3-mercaptopropionic acid via NHS/EDC chemistry. For real sample analysis, certified red wine and milk standards were spiked with known concentrations of toxins. The detection of toxins was based on the competition between toxin molecules and BSA-toxin conjugates to bind to the primary rabbit antibody. In the presence of toxin molecules in the sample, only those primary antibody molecules that did not bind to the toxin molecules bound to the BSA-toxin conjugates. In the next step, a corresponding number of secondary antibody molecules were bound to the primary antibodies. The alkaline phosphatase converted α-NP to α-naphthol, which was detected by differential pulse voltammetry (DPV).

$$\alpha\text{-Naphthyl phosphate} + H_2O \xrightarrow{\text{Alkaline phosphatase}} \alpha\text{-naphthol} + HPO_4^{-2}$$

The limits of detection for OTA in red wine and AFM1 in milk were 15 $ng\,mL^{-1}$ and 37 $pg\,mL^{-1}$, respectively.

38.5 ADULTERANTS

38.5.1 Urea

A potentiometric urea biosensor employing *Arthrobacter creatinolyticus* urease immobilized on a poly(acrylonitrile-methanemethacrylate-sodium vinylsulfonate) (PAN) membrane was described (Ramesh et al., 2015). Glutaraldehyde was used as the cross-linking agent. Urease catalyzes the following reaction

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

The detection of urea in samples was based on the formation of ammonium ion produced by the decomposition of urea by urease. Potentiometric measurements were carried out using a commercially available electrochemical workstation.

For real sample analysis, milk samples were spiked with known concentrations of urea and tested using the biosensor. The potentiometric readings obtained were in good correlation with the spiking concentrations. The biosensor showed linearity over the range of 1–100 mM and a limit of detection of 0.3 mM. It retained sufficient sensitivity for 13 consecutive cycles. It could be stored at 4°C for 70 days.

38.5.2 Melamine

A voltammetric biosensor for the simultaneous detection of melamine and urea based on the inhibition of AChE was described (Ezhilan et al., 2017). The biosensor employed a platinum (Pt) electrode modified by zinc oxide nanospheres prepared by drop-casting a mixture of ZnO, AChE, and chitosan on the surface of the Pt electrode. The Pt/ZnO/AChE/chitosan electrode had a linear range of 1–20 nM for both the analytes and had a limit of detection of 3 pM for melamine and 1 pM for urea, respectively. Detection was achieved by the inhibition of AChE activity by urea and melamine. Real sample analysis was performed by spiking milk samples with known concentrations of urea and melamine in various proportions.

38.6 FRESHNESS

Chemicals formed by the decomposition of food ingredients over the period of storage are excellent targets for the analysis of freshness of food. Examples of such substances include xanthine, hypoxanthine, and biogenic amines. In fish samples, xanthine and hypoxanthine formed by the degradation of inosine monophosphate act as biomarkers for food spoilage. Similarly, biogenic amines such as histamine, putrescine, and cadaverine formed by the decarboxylation of amino acids serve as spoilage indicators in meat and meat products. Some examples of relevant enzyme biosensors follow.

38.6.1 Xanthine

An amperometric biosensor employing xanthine oxidase (XOD) for the detection of xanthine in meat samples was reported (Devi et al., 2013). The enzyme was immobilized onto citrate-capped silver nanoparticles, which were deposited on the surface of a gold electrode though cysteine self-assembled monolayers. Xanthine oxidase catalyzes the following reaction:

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{xanthine oxidase}} \text{Uric acid} + H_2O_2$$

The detection of xanthine was based on the catalytic conversion of xanthine to uric acid and hydrogen peroxide. Electrons released from the dissociation of hydrogen peroxide gave rise to a current. The biosensor showed a linear working range from 2 to 16 μM. The response time was 5 s and the limit of detection was 0.15 μM at 35°C and pH 7.0. Fish and meat samples

spiked with known concentrations of xanthine were tested using the biosensor, and the results obtained were in good agreement with those obtained by the standard enzymatic colorimetric method. The biosensor successfully measured xanthine concentrations in fish, chicken, beef, and pork samples. The electrode retained 80% of its initial sensitivity after 180 uses over 60 days when stored dry at 4°C.

Yet another XOD-based amperometric biosensor working on a similar principle was described (Dervisevic et al., 2015). Xanthine oxidase was immobilized on poly(GMA-*co*-VFc)/MWCNT nanocomposite film on the surface of a pencil graphite electrode (PGE). A commercial electrochemical analyzer was used for all electrochemical measurements. Xanthine concentrations in fish samples obtained from a local market were successfully determined using the biosensor. The biosensor showed linearity in three concentration ranges: 2–28, 28–46, and 46–86 μM. The response time was 4 s, and the limit of detection was 0.12 μM.

A bienzymatic biosensor for the determination of Inosine monophosphate (IMP) was developed and used for the analysis of freshness and quality of stored muscles (Sun et al., 2016). The biosensor employed POD and 5′ nucleotidase enzymes coimmobilized on a glassy carbon electrode modified with polypyrrole-Pluronic F-127 AuNPs. A linear range of 0.05–15 $g\,L^{-1}$ with a low detection limit of 3.85 $ng\,mL^{-1}$ could be attained.

38.6.2 Biogenic amines

In general, an amine oxidase catalyzes the conversion of an amine to a corresponding aldehyde as follows:

$$R\text{-}CH_2NH_2 + H_2O + O_2 \xrightarrow{\text{amine oxidase}} R\text{-}CHO + H_2O_2 + NH_3$$

A bienzymatic amperometric biosensor for the detection of histamine in fish samples was developed (Pérez et al., 2013). Diamine oxidase (DOx) and HRP enzymes were coimmobilized into a polysulfone/MWCNT/ferrocene membrane on screen-printed electrodes using phase inversion. Histamine dihydrochloride was used as a substrate for DOx. By the catalytic action of DOx, histamine is converted to its corresponding aldehyde, along with ammonia and peroxide. HRP reduces the peroxide so formed to release H_2O. The oxidized form of HRP then gets reduced by ferrocene, ultimately generating a current. The limit of detection was 1.7×10^{-7} M and linearity was observed over a range of 3×10^{-7} to 2×10^{-5} M. Sardines, mackerel, and greater weever samples were successfully tested with the biosensor, and the results obtained were in good correlation with those obtained with a reference ELISA procedure.

Histamine dehydrogenase (HMD) and putrescine oxidase (PUO) were employed for the simultaneous detection of histamine and putrescine and in octopus samples (Henao-Escobar et al., 2016). Two different electrode systems, one for the detection of histamine and the other for the simultaneous detection of histamine and putrescine, were described. In the first system, a three-electrode configuration was used where HMD was immobilized onto a TTF modified screen-printed carbon electrode (SPCE) to be used as the working electrode. The second electrode system used a four-electrode configuration and consisted of two working electrodes, each modified respectively with HMD and PUO enzymes. The immobilization of the enzymes along with BSA on the TTF/SPCEs was achieved via cross-linking with GA. HMD converted histamine to 4-imidazoyl aldehyde. The reduced form of HMD further

reduced TTF and the reduced TTF in turn released an electron at the electrode, generating current. Similarly, PUO catalyzed the conversion of putrescine to 4-aminobutanal, ultimately generating a current. A limit of detection of $8.1 \pm 0.7\,\mu M$ for histamine and $10 \pm 0.6\,\mu M$ for putrescine was attained.

An amperometric biosensor employing TYN for the detection of tyramine was described (Apetrei and Apetrei, 2013). The electrochemical electrode consisted of TYN immobilized onto a phosphate-modified polypyrrole film using GA as the cross-linking agent. TYN catalyzed the conversion of tyramine to dopamine, and dopamine to O-dopaquinone. The O-dopaquinone so formed was reduced at the electrode, generating current. The biosensor was successfully applied for the determination of tyramine in spiked salted sauerkraut samples. Linearity was observed in the range of 4×10^{-6} to $80 \times 10^{-6}\,M$, with a detection limit of $5.7 \times 10^{-7}\,M$.

38.7 CONCLUSION

In this chapter, enzyme-based biosensors for the analysis of various aspects of food quality such as food composition, freshness, adulteration, and the presence of mycotoxins were discussed. Research groups developing biosensors have paid a lot of attention to achieving a low response time, a low detection limit, a wide linear detection range, and long-term stability, desirable characteristics for any biosensor to be applicable for commercial usage. With increasing simplicity and portability as well as improving sensitivity and reproducibility, biosensors are rapidly emerging as an alternative to conventional methods of food quality assessment.

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CHAPTER

39

Enzyme Engineering for Enzyme Activity Improvement

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39.1 INTRODUCTION

Proteins are the macromolecules made up of one or more polypeptide chains (i.e., primary structure) formed by covalent peptide bonds and folded to form thermodynamically stable three-dimensional native functional structures (i.e., secondary, tertiary, and quaternary structures). Proteins are made up of either single or multiple polypeptide chains, and each polypeptide chain is made up of 20 common protein-forming amino acids; they rarely include some exceptional nonprotein amino acids. Other than peptide bonds, the thiol group of cysteine residue of polypeptide bonds participate by oxidation in the formation of disulfide bonds within the same polypeptide chain or cause the formation of cross-linking between different polypeptide chains. Although disulfide bond formation cross-links different polypeptide chains, it does not lead to protein branching. In some proteins, component amino acids get modified due to posttranslational modifications, namely, hydroxyproline, hydroxylysine, etc. In extreme cases, UGA and UAG, the general stop codons, also code for amino acids selenocysteine (Sec) and pyrrolysine (Pyl), respectively. These exceptional amino acids are found cotranslationally added in a few proteins (Zhang and Gladyshev, 2007) such as selenocysteine in formate dehydrogenase or glutathione peroxidase (Chambers et al., 1986; Zinoni et al., 1986) and pyrrolysine are reported in several methanogenic archaea (Hao et al., 2002; Srinivasan et al., 2002; Zhang and Gladyshev, 2007). Pyl-containing t-RNA synthetases have been engineered with multiple substrate specificity (Guo et al., 2014). Protein is the macromolecule with diverse functionality such as (1) structure/support, for example, collagen, which forms the matrix of skin, ligaments, tendons, and bones; (2) transport, for example, hemoglobin, the protein of RBC; (3) motion, for example, actin, a muscle protein responsible for muscle contraction; (4) Nutrition and dietary proteins, for example, casein, (5) regulation by proteinaceous hormones that serve as intercellular messengers, for example, insulin for blood sugar regulation; (6) defense with the help of antibodies, the globular proteins

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00039-6

that "recognize" foreign particles; and (7) biological catalysis with the specialized proteins, namely, enzymes that help biological reactions occur more easily within living systems. Enzymes are highly effective biocatalysts that have evolved over time for specific functions. These biological catalysts not only accelerate chemical reactions many times but also ensure substrate specificity in terms of enantio, stereo, and regiospecificity. These exquisite properties of the enzymatic machinery cause the continuation of all natural product synthesis found within living systems and prompt human impulses for introducing desired characteristics in enzymes for their wide range of use for commercial purposes (Lutz, 2010). Various useful and complex natural products are formed naturally. For ages these compounds have been used/exploited by humans, such as drugs and other protein-based products widely used domestically and commercially (Butler, 2008; Hertweck, 2009). These molecules are well known to display a miscellaneous range of advantageous activities; hence they have become scientifically and commercially highly valuable commodities. These natural organic molecules have a vast array of structures and functions that have generated interest in elucidating their underlying role of enzymatic catalysis, as few chemical reactions are complicated to understand and explain. By understanding such biochemcial pathways, the catalytic capability of enzymes for product heterogeneity with optimum production and synthesis of "unnatural" natural products can be exploited for human welfare.

The natural catalytic efficiency of enzymes is generally not compatible with the industrial requirements of the catalyst (Lutz and Bornscheuer, 2009). Industrial reactions for commercial molecule synthesis are not carried out at physiological conditions, instead for industrial catalysis pH, temperature, pressure, and the ionic strength of the substrate are significantly high and in nonaqueous environments. Although the industrial reaction conditions are highly specific, an increase in the process temperature or a change in the pH or organic solvents can be advantageous as these modifications reduce contamination hazards, decrease substrate viscosity with an increase in the solubility of the reactants, and increase the rate of mass transfer. These conditions may also increase the rate of reaction so the product formation can be enhanced, which also reduces the chances of formation of unwanted by-products. But all these nonphysiological conditions of the reaction environment are not suitable for structural and functional stability of the enzymes. Because enzymatic catalysis occurs at a rapid rate and at physiological conditions, these catalysts are considered more ecofriendly than traditional catalysts. It has been discovered that organisms thriving in diverse environmental conditions may have enzymes that can withstand extremes of temperature, pH, and salt concentration. Thus, discovering and/or engineering enzymes that function under unusual conditions is of growing importance as industrial processes and product demands continue to evolve (Liszka et al., 2012).

39.2 STRENGTHS OF ENZYMES FOR COMMERCIAL USE

The high degree of product as well as substrate specificity is the principal characteristic of biocatalysts. Enzymes surpass the chemical catalysts by means of chemical specificity as well as regioselectivity (He et al., 2000; Hu et al., 2010; Notomista et al., 2009; Notomista et al., 2011; Yang et al., 2014), or enantioselectivity, (Abrahamson et al., 2012, 2013; Bommarius et al., 1994; Chenault et al., 1989; Reetz et al., 2006; Wu et al., 2013). Enzymatic catalysis runs

in an aqueous cellular environment with consideration of temperature, pH, and pressure of physiological conditions, as biocatalysis occurring in aqueous surroundings of physiological conditions are more energy efficient. Therefore, enzymatically catalyzed reactions are technologically best suited for renewable raw materials.

39.3 LIMITATIONS OF ENZYMES FOR COMMERCIAL USE

Although enzymatic catalysis is dynamic and tremendously selective for substrate and products, there are structural and functional stability-related limitations that are allied with the large-scale industrial use of enzymes. Due to their proteinaceous structure, enzymes have restricted stability within a limited range of surrounding environments, namely temperature, pH, solvent polarity, and ionic strength of reactants. Thus for industrial use, in comparison to chemical catalysts, only handfuls of distinguished biocatalysts are in use as the ease and economics of commercial enzyme use get in the way of enzyme-based technology on a large scale. Further development/identification of enzymes useful for industrial purposes is a long and tedious process that generally cannot be restricted within the requirements of industrial environmental compulsions. As a consequence, redesigning natural enzymes with protein engineering is perceived for the improvement of enzymes for commercial exploitation.

39.4 SIGNIFICANCE OF ENZYME ENGINEERING FOR COMMERCIAL USE

Enzyme engineering can be applied to enhance the catalytic properties of useful enzymes by the use of mutagenesis, site-directed mutagenesis, or any other method. For the commercial exploitation of enzymes, enzyme stability and solubility, and substrate or product specificity along with catalytic activity are the major areas of concern that can be addressed by enzyme engineering for any given commercial reaction condition (Dunn and Khosla 2013; Gao et al., 2010; Johannes and Zhao, 2006; Zabala et al., 2012). In the present scenario, the commercial processes for the synthesis of chemicals of human utilization, for example, pharmaceuticals, have been a major focus of research and development. These chemicals and processes are important for the future of humans and biological catalysts in industrial use are also environment friendly. With the advancement of protein science in the area of structure and mechanism, we have paved the way for enzyme modification or novel enzyme synthesis (Bornscheuer et al., 2012; Reetz, 2013; Turner, 2009). Olano et al. (2010) used combinatorial metabolic engineering by modifying several enzymes, namely, oxidoreductases, group transferases, halogenases, cyclases, and deoxysugar biosynthetic enzymes from actinomycetes and formed modified secondary metabolites with altered solubility or receptor binding ability. It has been reported that due to a few postassembly enzymatic reactions, biologically active compounds can be derived from nonactive compounds (Jacobsen et al., 1997). If such metabolic enzymes are modified or engineered, the desired analogs/isomers of the products can be generated directly. Some tailored enzymes have been reported in the synthesis of modified natural products with medicinal properties (Kharel et al., 2012; Podust and Sherman, 2012; Walsh and Wencewicz, 2013).

39.5 EVOLUTION IN PROTEIN ENGINEERING

The application of enzymes for commercial/industrial uses has been enhanced in the recent past due to the advancements made in protein engineering. The headway for the use of biocatalysts in industry is not very rapid as every so often, enzymes do not have the desired properties for an industrial application, such as in the instability of enzymes at high temperatures of reactions as well as in various organic solvents. All these achievements can be broadly grouped into three stages of development.

39.5.1 Rational Protein Design

Revolutionary recombinant DNA technology (RDT) also pioneered protein engineering as RDT made it possible to change specifically any given amino acid in a polypeptide chain due to the precise and specific base replacement in a cloned DNA sequence instead of trial and error of mutations for desired characteristics (Hutchinson et al., 1978; Gillam and Smith, 1979a, 1979b). Thus site-directed mutagenesis was used as an elementary technique of gene manipulation due to simplified DNA manipulation that initiated protein engineering for the design and development of mutant proteins having superior working characteristics or entirely novel proteins. By the application of RDT-based, site-directed protein engineering, at first the formation of designer proteins or enzymes with predictable properties was not achieved (Lutz and Patrick, 2004; Neylon, 2004). For rational designing of proteins/enzymes, a prior knowledge of amino acid sequences and native folding of polypeptide chains, that is, a three-dimensional structure of the target protein and the correlation between structure and function is a prerequisite. These structural and functional understandings facilitate the "rational" prediction for changeable sites on the enzyme, where desired changes would provide desired properties to the targeted enzyme (Bonagura et al., 1999a, 1999b; Cahoon et al., 1997; Carter et al., 1989; Cedrone et al., 2000; Craik et al., 1985; Danielson et al., 1999; Gengenbach et al., 1999; Harris et al., 1998; Mouratou et al., 1999; Wells et al., 1987; Wilcox et al., 1998). After site-directed mutagenesis of flexible amino acids, the required properties containing mutants are selected. Hence for the protein/enzyme engineering by rational designing, the aforementioned information of the "hot spots" on the protein/enzyme is necessary. This advancement has generated considerable data on the role of the primary structure of proteins, that is, the sequence of amino acid residues and their respective role in structural and catalytic mechanisms. This data can be furthered subjugated to produce novel proteins with customized functions as industrially useful enzymes (Estell et al., 1985; Onuffer and Kirsch, 1995; Winter et al., 1982), antibodies (Jacobsen et al., 1997; Neuberger et al., 1984) and transporter proteins (Looker et al., 1992). With the use of the technique, chimeric proteins getting added specific functions of another protein are being formed, for example, the grafting of complementarity-determining regions (CDRs) or cell binding ligands from any other source to construct adapted antibodies (Abderrazek et al., 2011; Jones et al., 1986; Park, 2002; Rybak et al., 1992) and the improvement of protein functions such as hemoglobin and the transporter protein (Komiyama et al., 1995; Natarajan et al., 2011). In this method of protein engineering, gene mutation is carried out either randomly or at a distinct location and then a protein variant with the required property is screened and selected. With the directed evaluation process, the modified mutant can be further used for the next mutation and subsequent selection for more improvements (Myers et al., 1985). "Directed evolution," a widely used term, applies to various methods using a natural evolution strategy of mutagenesis with subsequent selection for varying and

improving the functions of different enzymes (Nannemann et al., 2011). Directed evolution (or molecular evolution) does not require prior sequence or three-dimensional structure knowledge, as it usually employs random mutagenesis protocols to engineer enzymes that are subsequently screened for the desired properties (Dalby, 2003; Jaeger and Eggert, 2004; Jestin and Kaminski, 2004; Tao and Cornish, 2002; Williams et al., 2004). Directed evolution-based enzyme engineering is a widely accepted approach for improving the structural enzyme-substrate complex forming as well as the catalytic efficiency of industrially useful enzymes (Lutz and Bornscheuer, 2009). The enzyme subtilisin was used as a paradigm for this approach. This serine protease has every property altered such as reaction rate, specificity for substrate, pH optima, and enzyme stability for different factors (Bryan, 2000). Directed evolution has proved to be an effective strategy for improving or altering the activity of biomolecules for industrial, research, and therapeutic applications. The evolution of proteins in the laboratory requires methods for generating genetic diversity and for identifying protein variants with desired properties (Packer and Liu, 2015). Gene shuffling is an alternative approach to directed evolution, where many protein variants already existing in nature with desirable characteristics were used for novel combinations to form variants with more desirable properties (Bommarius, 2015). There are three alternative sources for gene shuffling: (1) the natural existence of polymorphic genes within a single organism or the formation of the gene of interest by random in vitro mutagenesis, (2) isozymic enzymes and their genes in different organisms, and (3) the presence of a protein family with a protein of interest as a member as well as other members with related activities. Ness et al. (1999) used 26 subtilisin family members to form a chimeric proteases library; later, this library was used for gene shuffling. With the screening of the library for four distinct enzyme properties, it was observed that few variants had considerably superior enzymes then any of the parental enzymes. Lehmann et al., initially used phytase sequences of 13 different fungal stains to construct a consensus enzyme (consensus phytase-1) which had increased thermostability than the original parent enzymes and subsequently swapped the active site of Aspergillus niger NRRL 3135 phytase with this synthetic phytase and termed new protein as consensus phytase-7, having limited variation in catalytic reactivity (Lehmann et al., 2000a). Hence "consensus sequence" based gene shuffling can add properties diverse than the parent proteins, an innovative approach in favor of directed evolution (Lehmann et al., 2000b).

39.5.2 Combinatorial Protein Engineering

Gene libraries are created by the use of structure-based combinatorial protein engineering (SCOPE). Such libraries have genetic information regarding the structure-function relationship of encoded proteins (O'Maille et al., 2002). The comparative studies of protein structure and function create many hypotheses, which have to be probed for the connection of molecular structure and subsequent functionality. SCOPE acts as an instrument for gene library construction that encodes rationally engineered protein variants. These engineered protein variants provide the initial data to check different hypotheses using both structural and functional analyses (O'Maille et al., 2004). Traditionally, directed evolution involves a two-step procedure: random mutagenesis and in vitro recombination molecular diversity is generated followed by high-throughput screening of library members with the desired phenotype. The SCOPE-based gene library construction applies a series of PCRs with multiple reactions to generate recombination with the help of multiple primers or random fragments in a single step (Perlak, 1990; Stemmer, 1994). The SCOPE-mediated gene synthesis essentially occurs

in distinct steps, which enables controlled recombination of either a fragment of gene or a complete gene to synthesize the required crossing over. Therefore these libraries have less statistical complexity that reduces sample numbers for screening, sequencing, and functional analysis, which effectively reduces the cost of the process. With such directed recombination, sites and frequency of crossing over are not limited by homology of the gene or the linear space between various mutations (O'Maille et al., 2004). With combinatorial protein engineering, millions of proteins can be sampled. Even then, though this approach has limitations, as only limited protein sequences in comparison to the actual possible protein sequences can be analyzed. The genetic code degeneracy and preconceived experimentation techniques also pose restrictions for gene library designing (Wong et al., 2006). Combinatorial protein engineering is far more effective than rational protein designing, as with SCOPE more protein sequences can be manipulated. But for that, combinatorial protein engineering has a need of vast mutant libraries that are complicated to get because of more sequence information of the targeted protein. Random mutagenesis often causes additional inaccuracy during DNA replication, leading to frequent errors and unpredictable alterations in gene sequences. On the other hand, target DNA is digested into small pieces during recombination that are again joined randomly, causing DNA shuffling. In directed evolution, 1) equipment for high-throughput screening is required, and 2) the uncertain mutations are also the major handicap. To overcome these shortcomings by the creation of larger libraries and more intense screening/selection, researchers are now concentrating on constructing smaller but higher quality libraries.

39.5.3 Data-Driven Protein Engineering

The chemical properties and biological functions of a protein are encoded within the sequence of amino acids. The data-driven approaches for systematic exploration of the relationships between protein sequence, structure, and function have a profound impact across all areas of biology, especially medicine and biotechnology. This approach takes advantage of previous information on protein sequences and their structures and corresponding functions while also employing predictive computational algorithms, then correlating this information in selecting potential target sites and active site amino acid residues useful for protein tailoring. Due to predicted site engineering only, the library size become compact with structural and functional stability; hence these libraries are functionally superior. Therefore, with the coupling of two ideas of protein design, the library size gets smaller with more precise targeted protein engineering. Quantum mechanics (QM), molecular dynamics (MD) simulation, and free energy related studies along with sequence- and structure-based designing are critically important to evaluate the amino acid substitution effect on the structural and functional stability of the new protein. QM, DM, and free energy information are useful to predict the various adapted or additional properties of the customized protein having unchanged native biocatalytic mechanism. Semirational protein engineering practically and effectively enhances the biocatalyst engineering prospects, as it needs fewer repeating processes to recognize variants having ideal phenotypes. With the use of small high-quality libraries, more time consuming high-throughput methods in library analysis are no longer needed. With the use of fewer variants, more library members can be evaluated by the methods that aren't applicable with high-throughput libraries. Knowledge-based library designing makes available the outline for the prediction and rationalization of experimental results and makes protein engineering a more hypothesis-dependent procedure (Lutz, 2010).

39.6 STABILIZATION OF ENZYMES THROUGH PROTEIN ENGINEERING

The enhanced applications of enzymatic catalysis for industrial plus domestic areas have created the requirements for novel biocatalysts with new or improved properties. For such enzymes, novel research strategies have been greatly encouraged. The current progress of recombinant DNA technology, high-throughput technology, genomics, and proteomics have stimulated the development of novel biocatalysts and biocatalytic processes. Enzymes with the required properties such as improved activity, elevated thermostability, and specificity under industrial conditions can be acquired by optimizing process conditions and by protein engineering, which has emerged as a powerful tool to improve enzyme properties (Singh et al., 2013). The computational algorithm SCHEMA was developed to estimate the disruption caused when amino acid residues that interact in the three-dimensional structure of a protein are inherited from different parents upon recombination. Exceedingly interrelated protein modules are recombined by SCHEMA and these modules are selected from dismembered native enzymes having a basic intact structure (Meyer et al., 2003; Voigt et al., 2002). Steipe et al. (1994) broke new ground with successful protein stabilization through the consensus method for IgG-type monoclonal antibodies. The consensus method-based protein stabilization for IgG-type monoclonal antibodies was further modified and evolved by Lehmann et al. (2000a,b, 2002). Porebski and Buckle (2016) have reviewed the developments in the semirational designing of proteins. Palackal et al. (2004) thermostabilized xylanase, an enzyme used to improve a farm animal's digestion for feed hemicelluloses, with improvements in the xylanase melting temperature. The animal feed is heat-treated, hence xylanases that are stable at high temperature are needed. Palackal et al. (2004) applied dual phasic combinatorial protein engineering that used gene site saturation mutagenesis (GSSM) followed by extensive mutations at nine hot spot sites of the xylanase enzyme encoding gene. This method increased the thermal stability of the xylanase enzyme from 61 to 96°C. Also, substituting nonrenewable energy sources WITH biofuel is a good option and various efforts have been done to decrease biofuel production cost. One of the important efforts is to convert plant-based cellulosic material to biofuels with the use of enzyme cellulases. Protein engineering advancements have been made to enhance enzymatic efficiency of cellulose enzymes as per cost-benefit ratio for absolute lignocellulosic material degradation. Yet the quantities of enzyme used in the process along with total time invested in reaction are the areas of concern. Thus, thermostable cellulases with increased specific activity, microbial growth inhibition, and mass transfer ratio for a highly viscous reaction medium above all that are easily processed during downstream processing can be vastly beneficial for enzymatic biotransformation (Anbar and Bayer, 2012). This is one area where protein engineering of the enzyme has a vital use, therefore Heinzelman et al. (2009a) effectively thermostabilized diverse cellulases, which is critically useful for biotransformation of cellulosic biomass for fuel and other beneficial chemicals. Heinzelman et al. (2009a) used SCHEMA structure-guided recombination of three fungal class II cellobiohydrolases (CBH II cellulases) to get a highly thermostable CBH II chimeras compilation. In this study, the group found that during recombination of three related fungal cellobiohydrolases 2 (CBH2s), catalytic domain variants get stabilized. They also observed that distinguished mutations responsible for protein stability have an additive effect. Later Heinzelman et al. (2009b) discovered a conservative point mutation site, that is,

C313S, responsible for protein stabilization. The combination of SCHEMA amid linear regression for identification of the stabilizing amino acid sequence blocks was used for the creation of stabilized chimeras for cellobiohydrolase 1 (CBH1) thermostability and functionality at 70°C. By this technique, a combination of eight blocks from five fungal homologs were substituted to form 390,625 possible chimeras to form the active, thermostable cellobiohydrolase class I (CBH I) enzymes (Heinzelman et al., 2010). Cellulase stabilization through protein engineering was standardized by Anbar and Bayer (2012) and their study concluded that mutational changes in the amino acids that are aligned in the native protein structure have more impact on the stability of the modified protein and indispensable Boltzmann kinetics, that is, a lower energy structure is frequently a more stable structure, are followed for protein stability.

39.7 NOVEL BIOCATALYSTS THROUGH COMPUTATIONAL DESIGN

The main objective of computational design for enzyme engineering is to modify the three-dimensional structure of an active site with desired catalytic activity. The computational design for the enzyme refers to the ability to form an enzyme-substrate complex, where ground or transition state substrate is bound to the primary protein structure (Wijma and Janssen, 2013). A stable [ES] complex can be predicted by the use of a suitable molecular mechanics energy function (Boas and Harbury, 2007; Das and Baker, 2008; Vizcarra and Mayo, 2005), which helps in the recognition of minimal potential energy containing amino acid sequences and their possible conformations. A number of steps are included in computational enzyme design as initially, a well-defined target reaction with all the information of requisite functional groups and appropriate catalytic mechanisms is chosen. Then, different transition state models, both molecular and quantum mechanical, are studied. On the basis of these models, the first layer of the active site constituting the most appropriate amino acid residues is constituted. These hypothetical structures are termed "theozymes" by Tantillo et al. (1998), which were in a later step used to dock into various possible protein three-dimensional structures and the resulting complexes are in silico optimized with combinatorial investigation for alternate amino acid(s) to improve transition state binding (Dahiyat and Mayo, 1996; Hellinga and Richards, 1991; Malisi et al., 2009; Zanghellini et al., 2006). In conclusion, on the basis of catalytic orientation, substrate binding energy at the transition state and structural coherence among the designed active site and the transition state are filtered to obtain a stable structure model. At the end, details of thermodynamically viable designs obtained from in silico catalyst designing are given (Kries et al., 2013). Alteration of *Escherichia coli* protein thioredoxin by Bolon and Mayo (2001) into a primitive esterase was the first successful example of computationally designed enzyme engineering, where the binding site is engineered with the introduction of histidine, the nucleophilic center for the rate of catalysis enhancement. After that, several modified biocatalysts were created, for example, Jiang et al. (2008) formed a modified enzyme for carbon-carbon bond cleavage via retroaldolization, N–O bond cleavage, that is, the Kemp elimination catalyst by Röthlisberger et al. (2008). Designed enzymes also may have the capacity to catalyze those chemical reactions that do not occur in biological systems, namely, the multistep retroaldol transformation, the Diels-Alder cycloaddition, and proton transfer

(Hilvert, 2013 and Kries et al., 2013). Although these designed enzymes are not efficient as natural enzymes, there are always possibilities that exist for improvement through directed evolution (Althoff et al., 2012; Blomberg et al., 2013; Giger et al., 2013; Khersonsky et al., 2012). Giger et al. (2013) had applied random mutagenesis to improve the activity of the designed retro-aldolase. With enzyme engineering advances, many potentially useful activities can now be added in the enzymes as enzymatic catalysis with inert molecules, (Gustavsson et al., 2014; Liang et al., 2010; O'Reillyn and Turner, 2015; Pavlidis et al., 2016; Prier et al., 2017; Savile et al., 2010), to change the product specificity (Kampranis et al., 2007; Li et al., 2013; Murciano-Calles et al., 2017; O'Maille et al., 2008), to change the cofactor specificity (Cahn et al., 2017), biofuels formation through amino acid metabolism (Atsumi et al., 2008). Liang et al. (2010) with the directed evolution technique engineered a biocatalyst KetoREDuctase (KRED) for asymmetric reduction of (E)-methyl 2-(3-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-oxopropyl)benzoate to form the corresponding (S)-alcohol. This product of asymmetric reduction is a prime intermediary metabolite in montelukast sodium (Singulair) synthesis, which is a regulator for asthmatic and allergic symptoms. Savile et al. (2010) reported an efficient biocatalytic engineering process by a modeling and mutation approach for asymmetric hydrogenation-catalyzing, broad-spectrum enzyme development that catalyzed the antidiabetic compound sitagliptin synthesis along with the synthesis of chiral amines through asymmetric enamine hydrogenation. Chiral amines are imperative for the chemical and pharmaceutical ventures and with this, swift enthusiasm is developing for transaminases utilization for chiral amines synthesis (Abrahamson et al., 2012, 2013; Gustavsson et al., 2014; Pavlidis et al., 2016). The monoterpene synthase enzyme was protein engineered for predictive conversion of the substrate as well as product specificity by Kampranis et al. (2007). For the rational approach structure-function-phylogenetic interdependence were combined to get desired enzyme. *Salvia fruticosa* 1,8-cineole synthase (Sf-CinS1) was successfully engineered to a Sabinene synthase with few logically envisaged replacements. O'Maille et al. (2008) applied a structure-guided approach to study the comparative amino acid constituents of tobacco 5-epiaristolochene synthase (TEAS) and henbane premnaspirodiene synthase (HPS). It was found that there is approximately a 75% amino acid similarity between these enzymes. This similarity between these two enzymes was present in the form of a functionally linked nine amino acid residue subset out of the naturally occurring 135 amino acid differences between TEAS and HPS. Both these enzymes identified the same substrate but formed different products as the ionized farnesyl diphosphate (FPP) substrate was cyclized in 5-epi-aristolochene (5-EA) by TEAS enzymes and the same substrate, that is, FPP was converted to premnaspirodiene (PSD) by HPS enzymes. It was found that site-directed mutations at these nine residue subset interchange the product specificities of each enzyme. Li et al. (2013) used domain swapping and structure-based mutagenesis to identify plasticity residues between *Artemisia annua* α-bisabolol synthase (AaBOS) and *A. annua* amorpha-4,11-diene synthase (AaADS), the major enzyme for sesquiterpene lactone, Artemisinin, a malaria drug, biosynthesis. It was found that catalytic efficiency of the enzyme AaADS was improved by plasticity residue substitution, for example, T399S substitution in AaADS enhanced the synthesis of amorpha-4,11-diene. Cahn et al. (2017) applied a structure-dependent semirational strategy to alter the NADP cofactor specificity in four structurally distinct NADP-using enzymes, namely, glyoxylate reductase, xylose reductase, cinnamyl alcohol dehydrogenase, and iron-containing alcohol dehydrogenase. Atsumi et al. (2008, 2010) described a metabolic engineering dependent approach for efficient alteration in the amino

acid metabolism in favor of alcohol production by *E. coli*. The modified organism was able to produce higher alcohols, including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol from the renewable carbon source glucose.

39.8 CONCLUSION

The application of advanced protein engineering includes directed evolution, gene synthesis, sequence analysis, bioinformatics tools (Kazlauskas and Bornscheuer, 2009; Lutz and Bornscheuer, 2009; Röthlisberger et al., 2008; Turner, 2009), and computer modeling, and the conceptual advance that improves an enzyme can be more pronounced than previously expected. Although first-generation, computationally designed enzymes have relatively modest activities, they are readily optimized by directed evolution. The combination of computational methods and experimental evolution represents a particularly powerful strategy for creating practical enzymes with novel and useful activities. Computational design complements and extends other approaches to enzyme engineering, including directed evolution and catalytic antibody technology. Its unmatched versatility with respect to fold and function holds particular promise for the generation of catalysts that lack biological counterparts. Proteins, with diverse novel as well improved functions, can be designed with the computational approach either as new enzymes or as an added controller of natural reactions or as biotherapeutics. These methodologies do not just give significant data to the comprehension of sequence-structure-function relations in proteins. They additionally hold assurance for the applicability of protein designing in biomedical research (Yang and Lai, 2016). Although the de novo design of enzymes for any conceivable reaction remains a distant dream, melding computation with evolution represents a powerful strategy for investigating basic enzyme chemistry and for discovering new catalysts with tailored activities and specificities. The dynamic interplay of these approaches will likely contribute significantly to future efforts to engineer proteins with truly enzyme-like properties (Hilvert, 2013).

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CHAPTER

40

Biosensors for Food Quality and Safety Monitoring: Fundamentals and Applications

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40.1 INTRODUCTION

The food industry has grown rapidly over the last 50 years to meet the needs of the population as well as react to changing lifestyles. The consumption of ready-to-eat foods makes food as an important issue because it has socioeconomic and health impacts (Kurbanoglu et al., 2017). Food scientists and the food industry are responding by way of developing strategies and technologies for rapid, sensitive, reliable, and cost-effective analytical methods to determine the presence of foodborne pathogens and contaminants (Ibrišimović et al., 2015; Luong et al., 1997). Biosensors represent an important tool in food analysis. Their advantages over traditional methods such as chromatography, spectrophotometry, etc. are due to their economics, their ability so save time, their real-time monitoring, and, above all, their lack of need for highly trained personnel (Mello and Kubota, 2002). Basically, biosensors use biological components known as a bioreceptor and a transducer, which convert the bioreceptor response into an analytical signal. Biological binding reactions can be performed by various interactions such as enzyme/substrate, antigen/antibody, receptor/ligand, chemical interactions, and nucleic acid hybridization (Monosik et al., 2012; Thakur and Ragavan, 2013). However, recent advances in protein engineering present opportunities to improve the specificity of the target as well as the sensitivity by modifying the structure of proteins, ligand binding, etc. That will be dealt with in this chapter.

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00040-2

40.2 PRINCIPLES OF BIOSENSORS

The term "biosensor" is used to describe an analytical device based on a combination of bioreceptors such as antibodies, enzymes, tissues, organelles, and cells along with a variety of transducers, namely, electrochemical, thermal, optical, and piezoelectrical, depending on the specific purpose of the biosensor (Paddle, 1996). Biosensors can be classified either by the type of bioreceptor or the transducer (Gaudin, 2017).

40.2.1 Components of Biosensors

40.2.1.1 Bioreceptors

Bioreceptors interact with the substance of interest and the specificity of the interaction determines the efficiency of the biosensor. Bioreceptors are divided into three categories: (i) biocatalytic receptors, including enzymes, whole cells, cell organelles, or tissues,; nevertheless, enzyme sensors are widely used due to their high substrate specificity, (ii) bioaffinity receptors such as chemoreceptors, antibodies (immunobioreceptors), or nucleic acids that form a stable and specific complex with their respective ligand, and (iii) hybrid receptors that employ DNA and RNA sequences that are complementary to one specific sequence present in the target microorganism (Mello and Kubota, 2002).

40.2.1.2 Transducers

Transducers convert the biological recognition of bioreceptors into a detectable signal that is measured as well as being proportional to the target concentration. The choice of transducers depends on the type of signals emitted by the bioreceptor. Electrochemical biosensors are based on monitoring electroactive species that are either produced or consumed by the bioreceptor. Transduction is carried out by an amperometric, potentiometric, or conductometric method, depending on the specific compound. The amperometric transducer measures the current produced due to the chemical reaction of an electroactive species to an applied potential, which is directly related to the concentration of the target. However, the selectivity of the amperometric devices is governed by the redox potential of the electroactive species present in the sample. Consequently, this is disadvantageous because the signal measured could be due to the noise from another chemical species. Potentiometric transducers are based on the generation of a proportional potential of the working electrode to the concentration of the active species with respect to a reference electrode. Meanwhile, conductometric biosensors depend on the conductivity changes that occur due to biochemical reactions (Mello and Kubota, 2002). Thermal transducers are regarded as a small microcalorimeter that detects changes in temperature. Because most whole microbial cell- or enzyme-catalyzed reactions are accompanied by heat generation, thermal transducers have broader applicability. A piezoelectric transducer consists of an oscillating quartz crystal immersed partially or completely in a liquid. Based on the changes in the physicochemical properties of the sample, such as viscosity, density, and conductivity, the variations in vibration frequency of the quartz crystal coupled with enzymes, antibodies, and antigens are measured and related to the analyte of interest (Luong et al., 1997). The optical transducers are based on light phenomena such as UV-Vis absorption, bio/chemiluminescence, fluorescence/phosphorescence, reflectance or scattering,

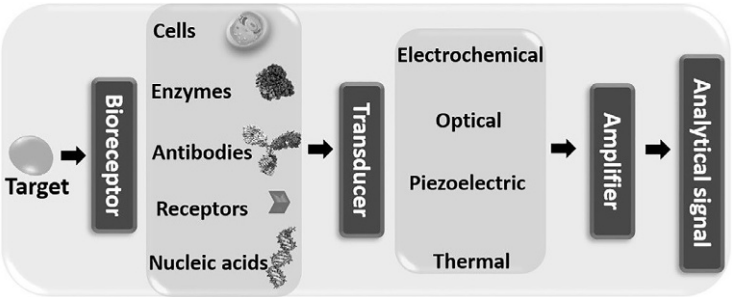

FIG. 40.1 Principles of biosensors.

and the refractive index caused due to the interaction of light with the substance to detect (Mello and Kubota, 2002). Basic principles governing biosensors are presented in Fig. 40.1.

40.3 BIOSENSORS FOR THE QUALITY OF FOOD PRODUCTS

Quality is an important character THAT determines the growth and success of the food industry. Common biosensors employed and/or developed in monitoring the quality of different food products are discussed here.

40.3.1 Beverage Industry

40.3.1.1 Biosensors for Sugar-Sweetened Beverages

40.3.1.1.1 GLUCOSE

Glucose is an important component of beverages and is present in a considerable concentration (0.7%–6.5%) in sugar-sweetened beverages (SSBs), due to the use of high fructose corn syrup (HFCS), fruit juices, or sucrose (Ventura et al., 2011). Glucose is added in beverages to confer sweetness; however, it represents the major source of calories to the human diet, resulting in metabolic disorders such as obesity and diabetes mellitus (Kahn and Flier, 2000). Consequently, biosensors for glucose determination in food products and humans are the most studied and developed, and account for nearly 85% of the biosensor market (Wang, 2007). Enzyme-based biosensors are the most utilized for the monitoring of glucose. As biological recognition elements, glucose oxidase (GOx) and glucose-1-dehydrogenase (GDH) are widely used. Among these two enzymes, GOx is the most employed due to its specificity, turnover rate, and method of obtaining.

40.3.1.1.2 FRUCTOSE

D-Fructose is a low-cost monosaccharide widely distributed in food and beverages, either as a free sugar or in association with glucose as sucrose, a disaccharide. Nevertheless, its consumption has been related to metabolic diseases (Stanhope et al., 2013). In addition, this can cause health problems in some people due to the deficit of fructokinase (Steinmann and Santer, 2016), resulting in fructosuria. Therefore, fructose monitoring in the beverage

industry is essential both for consumer protection and for beverage quality. D-Fructose-5-dehydrogenase (FDH) is the widely employed enzyme for fructose measurement. However, there are some studies in which hexokinase coupled with fructose-6-phosphate kinase (F6PK) are utilized as the bioreceptor (Bhand et al., 2010).

40.3.1.1.3 ASPARTAME

Aspartame is a low-calorie artificial sweetener widely utilized in dietetic beverages because it is 200 times sweeter than sucrose. This substance is composed of aspartic acid, phenylalanine, and methanol, which also can produce ill effects on human health (Balgobind et al., 2016). The neurotoxic effects of phenylalanine and methanol have been well documented, specifically phenylalanine toxicity for people with phenylketonuria (Medinsky and Dorman, 1995; Van Spronsen et al., 2009). Therefore, the determination of aspartame in dietary beverages to monitor permissible levels or to determine its desirable presence in nonaspartame dietetic drinks is essential. The common basis employed by aspartame biosensors is based on the hydrolysis of aspartame and the detection of its metabolites.

40.3.1.1.4 ASCORBIC ACID

L-ascorbic acid is added to SSBs for its antioxidant and stabilizing properties, which are desirable characteristics in food products (Varvara et al., 2016). Furthermore, it is a good indicator of beverage freshness because of its easy oxidation (Favell, 1998). A daily intake of ascorbic acid (70–90 mg) is reported to be beneficial for health, such as the activation of an immune response. Nevertheless, a lower or a higher consumption of ascorbic acid can produce negative effects on human health (Hu et al., 2010). Vermeir et al. (2007) constructed a biosensor for ascorbic acid detection on fruit juice samples. Ascorbic acid was measured employing ascorbate oxidase, which in the presence of oxygen produced dehydroascorbate and water. Microplate differential calorimetry (MiDiCal) technology was applied as the transducer in which the enthalpy change for ascorbate oxidase activity was correlated to the ascorbic acid concentration. The biosensor showed a linear range from 2.4–350 mM with a detection limit of 0.8 mM. Later, Barberis et al. (2015) developed an amperometric biosensor for detection of ascorbic acid in fruit juices. This biosensor was designed to simultaneously detect ascorbic acid and the antioxidant capability of either ascorbic acid or phenolic compounds. The biosensor was composed of ascorbate oxidase in combination with fullerene C_{60} (FC_{60}), fullerene C_{70} (FC_{70}), single-walled carbon nanotubes (SWCN), and multiwalled carbon nanotubes (MWNT), with a limit of detection of 0.10, 0.13, 0.20, and 0.22 μM, respectively. However, the ascorbic acid selectivity index was almost zero in sensors with ascorbate oxidase with fullerene while it was 0.13 and 0.22 for the sensors coupled with SWCN and MWCN, respectively. Bioreceptors, transducers, and the characteristics of biosensors used in the beverage industry are presented in Table 40.1.

40.3.1.2 Biosensors for Wine Component Analysis

The wine-making industry is very much interested in monitoring substrates and metabolites during the process in order to avoid economic losses. Compounds such as ethanol, glucose, and lactate play an important role in the quality, taste, and flavor of wine. Therefore, they are employed as process control indicators (Goriushkina et al., 2009).

TABLE 40.1 Analysis of Compounds in the Beverage Industry Using Biosensors

Analyte	Bioreceptor	Transducer	Characteristics	Application	References
Lactate Glucose	Lactate oxidase and Glucose oxidase	Amperometric electrodes	DL = 5×10^{-6} M	Wine	Shkotova et al. (2016)
Glucose	Glucose oxidase	Fiberoptic	LR = 0.05–5.0 mM DL = 0.01 mM	Beverages and human serum	Ho et al. (2014)
		Amperometric	LR = 1–100 μM DL = 0.32 μM	Blood samples	Rafighi et al. (2016)
			LR = 0.5–24 mM DL = 26.9 μM	Fruit juices	Ayenimo and Adeloju (2017)
			LR = 0.02–0.5 mM DL = 7.035×10^{-3} mM	Beverages	Gokoglan et al. (2017)
Fructose	Fructose dehydrogenase	Amperometric	LR = 0.1–5 mM DL = 1 μM	Honey, fruit juices, soft and energy drinks.	Antiochia et al. (2013)
			LR = 05–0.3 mM DL = 1.2 mM	Agave, cola, honey, maple syrup	Siepenkoetter and Salaj-Kosla (2017)
	Hexokinase and fructose-6-phosphate kinase	Calorimetric	LR = 0.5–6.0 mM DL = 0.12 mM	Syrup	Bhand et al. (2010)
Aspartame	Carboxyl esterase and alcohol oxidase	Amperometric	LR = 5×10^{-8} to 44×10^{-7} M DL = 3.68 μM	Soft drinks	Odacı et al. (2004)
			DL for methanol = 0.1 μM DL for aspartame = 0.2 μM	Soft drinks	Radulescu et al. (2014)
	α-Chymotrypsin and alcohol oxidase	Optical	LR = 0.056–3.07 mM	Soft drinks	Xiao and Choi (2002)
Ethanol	Alcohol dehydrogenase	Amperometric	LR = 0.1 to 2.0 M DL = 0.07 M	Wine	Samphao et al. (2015)
	Alcohol oxidase		DL = 0.52 mM	Beers	Cinti et al. (2017)

LR = linear range, DL = detection limit.

40.3.1.2.1 ETHANOL

The monitoring of ethanol in wines is crucial to controlling the desirable levels of alcohol in the final product as well as to avoid their inhibitory/toxic effect on yeast and thereby conserving the quality of wine. Enzyme-based biosensors employed to analyze alcohol utilize

alcohol oxidase (AOX) and alcohol dehydrogenase (ADH). The detection of alcohol by AOX is based on its ability to convert low molecular weight alcohols to aldehydes and hydrogen peroxide in the presence of oxygen whereas ADH is more stable and selective to ethanol without the requirements of oxygen. For this reason, ADH is the most used biosensor, although it needs the addition of NAD^+ (Azevedo et al., 2005). Pyrroloquinoline quinone-dependent alcohol dehydrogenases (PQQ-ADH) also have been used in alcohol detection (Niculescu et al., 2002). These enzymes do not require oxygen and can directly transfer electrons between their active center and the electrode. PPQ-ADH was coupled with a redox polymer Os-complex-modified poly(vinylimidazole), with poly (ethylene glycol) diglycidyl ether as a cross-linker. This device demonstrated a sensitivity of $0.336 \pm 0.025\ \mathrm{A\,M^{-1}\,cm^{-2}}$ for ethanol and a detection limit of 1 μM while functioning satisfactorily in a flow-injection system.

40.3.1.2.2 MALIC ACID AND LACTIC ACID

Malic acid is an important parameter of wine quality due to its high acidity. Its conversion to L-lactic acid decreases this acidity, contributing to wine freshness and providing stability. Hence both L-malic acid and L-lactic acid are considered for development of methods to test wine quality (Goriushkina et al., 2009).

Giménez-Gómez et al. (2016) developed an amperometric biosensor for L-lactate in wines. This biosensor is composed of lactate oxidase (LOX) and horseradish peroxidase (HRP), immobilized on a three-dimensional matrix of polypyrrole (PPy) with a thin-film gold electrode functioning as an electrochemical transducer. The role of HRP was to hydrolyze the hydrogen peroxide produced by LOX. The biosensor showed a linear range response of 1×10^{-6}–1×10^{-4} M, with a detection limit of 5.2×10^{-7} M and a sensitivity of $-13{,}500 \pm 600\ \mu\mathrm{A\,M^{-1}\,cm^{-2}}$.

Recently, Giménez-Gómez et al. (2017) constructed a biosensor for L-malic acid detection, employing malate dehydrogenase (MDH) and diaphorase (DP) enzymes as the selective receptor and NAD^+ to act as the redox mediator. Similar to the previous study, the PPy membrane was used as an immobilizing agent and a thin-film gold electrode as the transducer, which showed a sensitivity of $1365 \pm 110\ \mathrm{mA\,M^{-1}\,cm^{-2}}$, with a detection limit of 6.3×10^{-8} M.

40.3.1.2.3 POLYPHENOLS

Polyphenols are compounds of great importance to wine character and quality because they produce several sensorial characteristics and have a high antioxidant capacity. These compounds are monitored to classify wines according to their taste and color and in some cases, they are employed as indicators of quality alteration. In general, enzyme-based biosensors are utilized, and the common ones used in phenolic amperometric quantification are tyrosinase (Abhijith et al., 2007), peroxidase (Yang et al., 2006), pyrroloquinoline quinine-dependent glucose dehydrogenase (Makower et al., 1996), and cellobiose dehydrogenase (Stoica et al., 2004).

Di Fusco et al. (2010) quantified polyphenol in wines utilizing laccase-based biosensors. Laccase activity was monitored amperometrically by its oxidative action on phenols producing water and an oxidized compound. This method is similar to the method of Folin–Ciocalteau in the determination of polyphenols.

Tyrosinase, which oxidizes monophenols, was employed initially (Adamski et al., 2010), and the authors later (Adamski et al., 2016) developed another one with laccase. In both systems, a glass-carbon electrode was used for the amperometric quantification of phenolic compounds. It was reported that the limit of detection of polyphenols by tyrosine and a

laccase biosensor was 10.3 and 27.4 mg/L, respectively. Unfortunately, interferences by other wine components were observed with both biosensor systems.

40.3.1.2.4 GLYCEROL

Glycerol in wines is formed by yeasts for alternative regeneration of NAD^+ via fermentation (Wang et al., 2001). Production of glycerol is a 1:10 ratio of the ethanol formed (Mataix and Luque De Castro, 2000). It has been described that glycerol contributes considerably to the taste properties and smoothness of wine, and its sudden diminution can be related to the presence of undesirable microorganisms (Oliveira et al., 2006). Due to the established ratio of glycerol:ethanol, glycerol detection aids in the determination of a possible adulteration of wine or a change in wine quality (Mataix and Luque De Castro, 2000; Šehović et al., 2004).

Monošík et al. (2012) developed a multienzymatic biosensor for glycerol determination in wines, which consisted of five enzymes: glycerol kinase (GK), creatine kinase (CK), creatinase, sarcosine oxidase, and peroxidase. Though these enzymes were immobilized in chitosan, the authors used a gold planar electrode (GPE) for the first biosensor system and nanocomposite-containing MWNTs for the second biosensor system. Gold and nanocomposite biosensors showed a linear range of 5–640 μM and 5–566 μM with detection limits of 1.96 and 2.24 μM and sensitivities of 0.8 and 0.81 $nA\,\mu M^{-1}$, respectively; both had a response time of 70 s.

40.3.2 Dairy Industry

40.3.2.1 Lactose

Lactose is a good indicator of dairy product quality as well as for detection of mastitis in cows, which decreases the lactose content in milk (Conzuelo et al., 2010). Measurement of this carbohydrate is also crucial in functional foods such as lactose-free milk directed to people with lactose intolerance. A biosensor for lactose detection in milk using GOx and β-galactosidase (β-gal) was tested by Jasti et al. (2014). Lactose concentration was determined by the hydrolytic activity of β-gal on lactose, which produced galactose and glucose. The glucose produced was further oxidized by Gox-producing hydrogen peroxide and was detected by employing a colorimetric method. Both enzymes were immobilized on BSA-coated allyl glycidyl ether (AGE)-ethylene glycol dimetacrylate (EGDM) copolymer. The addition of glutaraldehyde after immobilization provided a high thermal stability to the enzymes, and this aided in lower limits of lactose detection (0.17 $mg\,mL^{-1}$). Tasca et al. (2013) constructed a new amperometric biosensor for lactose, which was composed of cellobiose dehydrogenase (CDH) as the bioreceptor and SWCNT facilitating the direct transfer of electrons. CDH allows this transference because of its two domain structures. At the FAD-containing dehydrogenase domain (DH_{CDH}), lactose is oxidized and electrons are subsequently transferred to the heme b-containing cytochrome domain (CYT_{CDH}). This biosensor demonstrated a detection limit for lactose of 0.5 μM with a linear range of 1–150 μM, high sensitivity (476.8 $nA\,\mu M^{-1}\,cm^{-2}$), and a fast response time of 4 s.

40.3.3 Meat Industry

40.3.3.1 Nitrates

Nitrate and nitrite are compounds of special interest in the meat industry because they are used as preservatives against pathogens and as antioxidants at the meat curing process

(Jadán et al., 2017). Nonetheless, these compounds can provoke serious health issues as nitrate in meat is reduced to nitrite, a precursor of the carcinogen nitrosamines (Tannenbaum and Correa, 1985). Hence, the determination of nitrate and nitrite in meat is essential in quality control to find whether their levels are within the permitted levels in the finished product.

Dinckaya et al. (2010) developed a voltametric biosensor for nitrate detection in meat samples using nitrate reductase that reduces nitrate to nitrite. The biosensor recorded a linear range of $5.0–90\times10^{-9}$ M and a detection limit of 2.2×10^{-9} M. Later, Jadán et al. (2017) also utilized a nitrate reductase immobilized on an oxygen electrode and an amperometric detector to evaluate the decline in oxygen concentration to hydrogen peroxide and water, which is proportional to the nitrate amount. The linear range for nitrate was 10–70 μM and the results of this sensor without oxygen interference are comparable with that of the HPLC method.

40.3.3.2 Amines

The most common biogenic amines presented in meat are tyramine, cadaverine, putrescine, and histamine (Ruiz-Capillas and Jiménez-Colmenero, 2005), causing toxicological effects in humans such as migraine, headaches, nausea, vomiting, and respiratory and blood pressure disorders (Ladero et al., 2010). Biosensors in the food industry use amine oxidase, xanthine oxidase, or hypoxanthine oxidase as bioreceptors along with either electrochemical or optical transducers. Recently, Omanovic-Miklicanin and Valzacchi (2017) developed two new chemiluminescence biosensors for putrescine in meat. This sensor is based on the enzymatic reaction of putrescine oxidase or diamine oxidase on putrescine in the presence of O_2, producing 4-aminobutyraldehyde, H_2O_2, and ammonia. Consequently, the hydrogen peroxide produced is the equivalent of putrescine levels and was quantified by measuring the chemiluminescence of the reaction between hydrogen peroxide and luminol. Putrescine oxidase and diamine oxidase biosensors showed a linear range of 1–2 mg/L with a detection limit of 0.8 and 1.3 mg/L, respectively. Previously, Pospiskova et al. (2013) also utilized a diamine oxidase bioreceptor in fiberoptic biosensors for the detection of biogenic amines. The biosensor was coupled with a ruthenium complex, and the consumption of oxygen due to amine oxidation was determined by measuring the suppression in the fluorescence lifetime of the ruthenium complex. The results obtained were favorable for putrescine and cadaverine detection with a linear range concentration of 0.075–0.1 mM L^{-1} and a detection limit of 25 and 30 μmol L^{-1}, correspondingly.

40.3.4 Bakery Industry

40.3.4.1 Acrylamide

Acrylamide is an aliphatic amide produced during baking at high temperatures due to the reaction between the reducing sugars and the asparagine present in bread. It is a neurotoxicant and a possible carcinogen; regrettably, the mechanism of acrylamide synthesis is still not fully understood. Hence, the improvement in manufacturing practices to avoid acrylamide production is still not achievable and thereby, acrylamide detection is crucial (Stobiecka et al., 2007). Silva et al. (2009) developed a biosensor for acrylamide with whole cells of *Pseudomonas aeruginosa* with amidase activity, which hydrolyzes acrylamide-producing acrylic acid and ammonia. It was detected potentiometrically with a linear response in the range of $0.1–4.0\times10^{-3}$ M of acrylamide and a detection limit of 4.48×10^{-5} M.

40.4 BIOSENSORS FOR FOOD SECURITY

The Centers for Disease Control and Prevention (CDC) have estimated that one in six Americans gets sick each year due to the consumption of contaminated foods and/or beverages (Cdc.gov., 2017). Also, more than 9.4 million episodes of foodborne illness and 1351 foodborne bacterial contaminations associated with death occur every year in the United States (Scallan et al., 2011). Food safety is a serious issue that the food industry must face due to the potential harmful agents present in processed food, such as pathogens, pesticides, antimicrobial compounds, allergens, antibiotics, etc. (Luong et al., 1997). Biosensors represent a promising tool not only to ensure food safety at the industry scale during processing and packing, but also be for consumers to verify the quality of the food they consume.

40.4.1 Pathogens

Pathogenic microorganisms are a nuisance not only to human health but also to the health of the food industry. The CDC's Food Net in 2015 reported 20,098 confirmed infections across the United States, including 4598 hospitalizations and 77 deaths related to pathogen infections per every 100,000 persons. The most common foodborne pathogens were reported to be *Salmonella*, *Campylobacter*, *Shigella*, *Cryptosporidium*, *Escherichia coli*, *Vibrio*, *Yersinia*, *Listeria*, and *Cyclospora*. Conventional methods for the detection and identification of foodborne pathogens are mostly based on microbiological and biochemical identification but are greatly restricted by along assay time that, in some cases, may take up to several days to yield results due to enrichment requirements. Biosensors for foodborne pathogens should comply with a high sensitivity (ability to detect lower population densities) as a desirable feature because some pathogens are harmful at low densities (Adley and Ryan, 2015). In recent years, biosensors have become an alternative tool over conventional methods due to their ability to perform rapid response analyses, high-throughput capacity, good selectivity, low cost, speed of operation, portability, and the ability to measure samples with minimal sample preparation (Moran et al., 2016). Different bioreceptors and transducers employed for the detection of pathogenic microorganisms present in different samples are presented in Table 40.2.

40.4.2 Pesticides

Although environmental concerns are growing, pesticide use has been estimated at 2 million tons per year (Verma and Bhardwaj, 2015). Pesticides are known for their persistence as well as their accumulation in soil, water, agricultural products, and eventually in biological systems affecting human health (Arjmand et al., 2017). This highlights the importance of detection of pesticides in foods. Organophosphate compounds (OP), inhibitors of the acetylcholinesterase (AChE), are by far the largest class of urban and rural pesticides. Amperometric AChE biosensors are generally used to detect these compounds. They are reported to have a linear range from 1 nM to 5 μM with a detection limit of 0.7 nM for paraoxon and from 5 nM to 1 μM for dimethoate (Lang et al., 2016). Further, they developed a strategy to minimize the irreversible inhibition of cholinesterase by submerging the biosensor in a cholinesterase reactivator solution and by this way, restored more than 95% of the original sensibility of the biosensor. Previously,

TABLE 40.2 Detection of the Main Foodborne Pathogens in Food Industry Using Biosensors

Pathogen	Bioreceptor	Transducer	Characteristics	Application	References
Salmonella	Immunosensor	Optical	DL = 1×10^{3} CFU mL^{-1}	Turkey breast and chicken drumsticks	Abdelhaseib et al. (2016)
Salmonella	Aptamer	Impedimetric	LR = 1×10^{1}–1×10^{8} CFU mL^{-1} DL = 6 CFU mL^{-1}	Apple juice	Bagheryan et al. (2016)
Campylobacter	Immunosensor	Optical	LR = 1.6×10^{3}–1.3×10^{4} DL = 9.7×10^{2} CFU m^{-1}	Turkey	Sapsford et al. (2004)
Campylobacter	Immunosensor	Electrochemical	LR = 1×10^{2}–1×10^{7} CFU mL^{-1} DL = 2.1×10^{4} CFU mL^{-1}	Chicken carcass	Che et al. (2001)
Shigella	Immunosensor	Optical	LR = 7.8×10^{5}– 1.3×10^{7} CFU mL^{-1} DL = 7.8×10 CFU mL^{-1}	Milk	Sapsford et al. (2004)
Cryptosporidium	Immunosensor	Optical	DL = Up to 10 oocysts mL^{-1}	Drinking water	Thiruppathiraja et al. (2011)
Cryptosporidium	Immunosensor	Piezoelectric	LR = 1×10^{2} to 1×10^{4} oocysts mL^{-1}	Drinking water.	Campbell and Mutharasan (2008)
STEC non-O 157	Immunosensor	Potenciometric	LR = $1\times10^{-1}\times10^{7}$ cells mL^{-1}	Lettuce and sliced carrots	Ercole et al. (2003)
STEC O 157	Immunosensor	Optical	LR = 4 to 4.0×10^{8} CFU mL^{-1}	Milk, orange juice	Hu et al. (2016)
STEC O 157	Immunosensor	Colorimetric	LR = 1.8×10^{3} to 1.8×10^{8} CFU mL^{-1}	Water	Park et al. (2008)
Vibrio	Immunosensor	Optical	LR = 1×10^{3} to 1×10^{7} CFU mL^{-1}	Food products	Sungkanak et al. (2010)
Listeria	Electrochemical	Amperometric	DL = 2.17×10^{2} CFU mL^{-1}	Blueberrys	Davis et al. (2013)
Listeria	Immunosensor	Impedance	LR = 3.0×10^{1} to 3.0×10^{4} CFU mL^{-1}	Lettuce	Chen et al. (2015)

LR = linear range, DL = detection limit, STEC = Shiga toxin producing *E. coli*.

Nunes et al. (1998) also developed an amperometric biosensor based on cholinesterase (ChE) for the detection of carbamate residues and its limit of detection for aldicarb, carbaryl and carbofuran, methomyl and propoxur in potato, carrot and sweet pepper samples was in the range of 5×10^{-5} to 50 mg kg^{-1}. A portable AChE screen-printed electrode biosensor was used to determine the total concentration of organophosphorus and carbamate pesticides in water and food samples with a lower limit of detection of $2\,\mu g\,L^{-1}$ (Hildebrandt et al., 2008). Iprovalicarb, a fungicide derived from carbamates, has been detected in onion or potatoes by using antibody-based biosensors with a sensitivity of 0.0128–5000 $ng\,mL^{-1}$ (Cho and Kyung, 2008). Biosensors have also been developed to determine the presence of herbicides in contaminated ecosystems, agricultural products, and foods. Sulcotrione, an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD), is a group of herbicides widely used to protect corn crops against grassweeds. This herbicide has been detected in water samples by an amperometric biosensor based on the inhibition of HPPD with a lower limit of detection of $1.4.10^{-10}$M. This detection limit is in good agreement with European Union legislation, which sets a maximum concentration of $0.1\,mg\,L^{-1}$ of drinking water for pesticides, equivalent to 3.10^{-10}M for sulcotrione (Rocaboy-Faquet et al., 2016).

40.4.3 Antibiotics

Antibiotic resistance is growing at alarming rates and is a great threat to human health (Su et al., 2014). Trace amounts of antibiotic residue present in food products could trigger adverse side effects on human health such as allergic reactions in the case of hypersensitive individuals (Conzuelo et al., 2013). As a new screening method, biosensors offer a big opportunity for a rapid and cheap detection of antibiotic residue in food products (Bargańska et al., 2011). A summary of bioreceptors, transducers, detection limits, and types of samples is presented in Table 40.3.

40.5 PROTEIN ENGINEERING FOR BIOSENSORS

The most common recognition element of a biosensor is a protein basing its action on enzyme activities, protein interactions, and posttranslational modifications (Wang et al., 2009). However, proteins in their native form are not always able to cover the limits of detection and the level of stability required for a sensor. For this reason, researcheds have looked for a mechanism to modify these characteristics by manipulating their structure and obtaining a more stable, sensitive, selective, and specific biomediator that can be employed as a biosensor. Protein engineering plays an important role in food industries because, by its application, it is possible to maintain or even enhance the quality of the final products. With recent advances in molecular biology, protein engineering offers the possibility to enhance biomolecules for catalysis, recognition, structural integrity, signaling, locomotion, and defense. Progress in technology gives the advantage of computational predictions to modify or design a protein to acquire or redefine a specific structure, specific function, or novel applications (Arnold, 2001), which later can be translated experimentally. Additionally, computational tools lend more control on biomolecular recognition (Looger et al., 2003). Despite its potential character, three main challenges are to be resolved before developing applications (Damborsky and Brezovsky, 2014; Fowler and Fields, 2014; Tiwari et al., 2012): (i) structure modeling and prediction, (ii) protein stability at the desired conditions, and (iii) protein-protein and DNA-protein interactions.

TABLE 40.3 Detection on Antibiotics Residues in the Food Industry Using Biosensors

Analyte	Bioreceptor	Transducer	Characteristics	Application	References
Penicillin G	Immunosensor	Electrochemical	LR = 3.34×10^{-3} to 3.34×10^{3} ng L^{-1} DL = 2.7×10^{-4} ng L^{-1}	Milk	Li et al. (2015)
Tetracycline	Aptamer	Optical	LR = 0.20–2.0 μg mL^{-1} DL = 0.039 μg mL^{-1}	Raw milk	Luo et al. (2015)
Chloramphenicol	Aptamer	Optical	LR = 0.01–1 ng mL^{-1} DL = 0.01 ng mL^{-1}	Milk	Wu et al. (2015)
Thiamphenicol	Immunosensor	Optical	DL = 25 μg kg^{-1}	Kidney	Thompson et al. (2017)
Kanamycin	Aptamer	Electrochemical	LR = 1.2–75 ng mL^{-1} DL = 0.11 ng mL^{-1}	Milk	Sharma et al. (2017)
Sulfonamide	Immunosensor	Electrochemical	LR = 0.12–8.41 ng mL^{-1} DL = 1 ng mL^{-1}	Milk	Conzuelo et al. (2012)
Streptomycin	Enzyme	Electrochemical	LR = 0.05–20 ng mL^{-1} DL = 10 pg mL^{-1}	Honey/Milk	Liu et al. (2013)
Nitroimidazole	Immunosensor	Optical	DL = 1 μg kg^{-1}	Kidney	Thompson et al. (2009)

LR = Linear range, DL = detection limit.

40.5.1 Protein Design

Four methods are considered for protein engineering of biosensors: rational protein design, directed evolution, semirational design (a combination of the two mentioned before), and de novo design. Of the four methods, rational protein engineering and directed evolution (Fig. 40.2) are considered to be efficient in application (Eriksen et al., 2014).

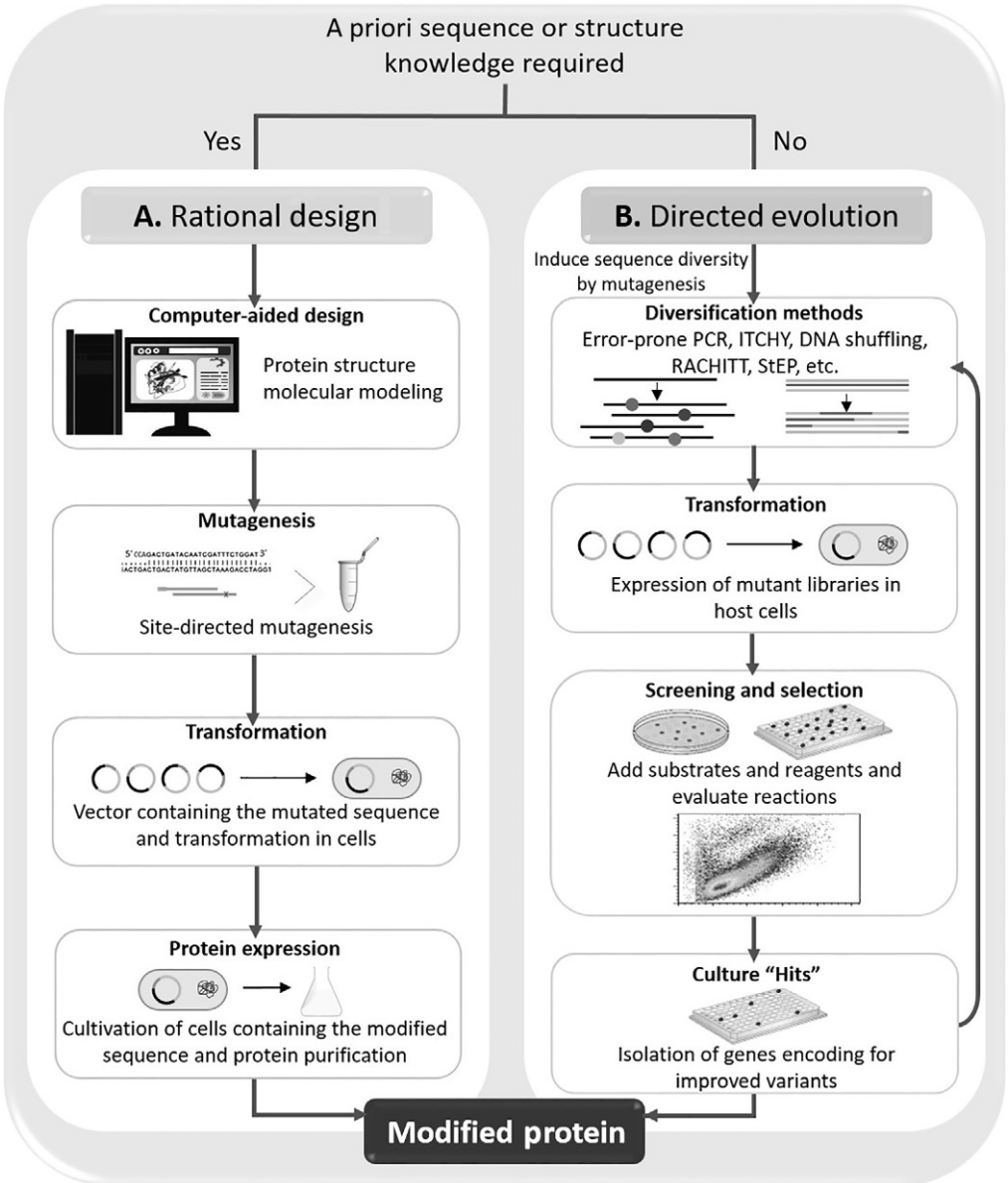

FIG. 40.2 Schematic diagram of rational protein design and directed evolution.

40.5.1.1 Rational Protein Design

Rational design needs prior information on a three-dimensional structure as well as the biophysical data and functions of the protein. It can be achieved either by single-point mutation, exchange of elements of secondary structure, exchange of whole domains, or by fusion of enzymes, creating hybrid enzymes (Nixon and Firestine, 2000; Nixon et al., 1998). Holland et al. (2012) improved the catalytic activity and stability of GOx by site-directed mutations in *Aspergillus niger*. They did this by combining the genetic elements responsible for the stability of *A. niger* and modifying its regions of catalytic activity with reference to *Penicullium amagasakiense*, which has a fourfold greater catalytic rate and sixfold superior substrate affinity. They reported four obtained mutants that demonstrated significantly higher catalytic activity and moderate improvement in stability than the parental *A. niger.* Other studies have reported the use of rational design to establish a new ligand to proteins. The specificity of biosensors based on proteins depends on the complex formed between the protein and the ligand (Bishop et al., 2000). Looger et al. (2003) redesigned computationally the ligand-binding site specificity of five members of the periplasmic binding protein (PBP) superfamily in *E. coli*, which binds to sugars or amino acids. The binding sites of the proteins were engineered to recognize trinitrotoluene (TNT), L-lactate, or serotonin and the engineered proteins showed higher detection of these compounds than sugars or amino acids. Nonetheless, Schreier et al. (2009) observed that there were false positive results when they experimentally validated this design, which highlights the importance of experimental studies to validate structure modification, ligand binding, etc. With respect to antibiotic biosensors, Tsang et al. (2015) developed a β-Lactamase-based fluorescent biosensor using site-directed mutagenesis, which offered a sensitive and rapid detection of cephalosporin. They employed the specific interaction between β-Lactamase and its cephalosporin substrates/inhibitors and replaced Tyr150 by serine, which impaired the catalytic activity of the protein and improved the fluorescence stability.

40.5.1.2 Directed Evolution

In directed evolution, experimental simulations using randomly distributed mutagenesis that represent natural genetic and/or protein evolution have been done to study the molecular diversity of the sequence of interest. By this method it is possible to improve protein structure, catalytic efficiency, binding, etc. Over time and through many cycles of mutagenesis and amplification of selected library members, the beneficial acquired characteristics will accumulate, enhancing the traits of the modified organism (Jäckel et al., 2008; Packer and Liu, 2015). Earlier, Sun et al. (2001) reported a 1.7-fold increase in substrate affinity (43 ± 2 against 57 ± 3 mM of the wild-type) and thermal stability of galactose oxidase in *E. coli* by using the directed evolution method. This method is extensively applied in the food industry, not only to obtain a higher yield and activity but also to replace the ligand for another compound of interest. Wu et al. (2017) used directed evolution in the effector-binding pocket of LacI to design a lactulose biosensor. They obtained a LacI mutant (LacI-L5) specifically induced by lactulose as an altered effector and observed the expression of a green fluorescent protein (GFP) at a concentration of 10 mM of lactulose, while the wild-type, LacI, showed no response.

40.6 CONCLUSION

It is evident that protein engineering is being implemented in increasing the range of targets and sensitivity by biosensors. With further advances in molecular biology and system biology, future studies will focus on the development of new biosensors to detect a wide array of harmful agents in foods with increased sensitivity. Therefore, this will aid in monitoring food quality, food safety during processing until consumption, and, most importantly, on issues related to global human health.

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CHAPTER

41

Application of Immobilized Enzymes in the Food Industry

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41.1 INTRODUCTION

Enzymes promote biochemical reactions in living systems. The role of enzymes in various biological processes, health issues, and the detection of disease has been extensively studied. They were used in food processing well before this extensive study of their nature (Brena and Francisc, 2006). They can catalyze reactions in a mild condition and have high substrate specificity with less by-product formation. The growing knowledge and technical improvement led to greater production and use of pure enzymes in various food and biotechnological applications (Homaei, 2016). Industrial applications of enzymes include food and beverage processing, animal feed, detergents, biosensors, wastewater treatment, pharmaceuticals, and, more recently, biofuels. But there problems with enzymes, including their stability, the high cost of isolation, and the difficulty in enzyme recovery from the reaction mixture at the end of the reaction. The problems of the free enzyme can be overcome by enzyme immobilization (Homaei et al., 2014). "Immobilized enzymes" refer to those enzymes that are confined in a certain space but their catalytic activities are retained (Brena and Francisc, 2006). The immobilized enzyme can be a free enzyme, a cell, an organelle, or a combination of these (Poul, 1984). In this immobilized form, the enzyme can be repeatedly and continuously used. The immobilized enzyme can be used in various industrial processes as well as in bioaffinity chromatography, biosensors, and various diagnoses. The applications of immobilized enzymes are summarized in Table 41.1.

41.2 TECHNIQUES OF IMMOBILIZATION

In the various literature, different protocols are reported for the immobilization of the enzyme. The enzymes are attached with the support due to various interactions such as physical adsorption, ionic bonds, and covalent bonds. The methods of enzyme immobilization can be

https://doi.org/10.1016/B978-0-12-813280-7.00041-4

TABLE 41.1 Application of Immobilized Enzymes in the Food Industry

Sl No.	Name of the Enzyme	Matrix used	Application	Reference
1.	Lipase	Silica	Ester synthesis to produce transfat free margarines and shortening	Aravindan et al. (2007)
2.	β-Galactosidase	Alumina, silica, gluteraldehyde, alginate	Removal of lactose from milk to avoid lactose crystallization, resulting in products with a mealy, sandy, or gritty texture	Grosová et al. (2008)
3.	Pectinase	Anion exchange resin	For clarification of fruit juice	Khan and Alzohairy (2010)
4.	Glucose isomerase	DEAE cellulose, polyacrylamide gel, alginate	Production of fructose syrup	Rhimi et al. (2007)
5.	Laccase	Silica gel	Convert lignin to cellulose and hemicellulose	Chiacchierini et al. (2004)
6.	Transglutaminase	Poly(lysy1)-α-casein	Modify viscoelastic properties, strengthen dough	Shi et al. (2011)
7.	Amino acid acylase	DEAE Sephadex	Hydrolyze D,L acyl amino acid to produce L amino acid	D'Souza (2002)
8.	Invertase	Calcium alginate	Production of invert syrup	Kotwal and Shankar (2009)

classified in various ways. They can be classified as either reversible or irreversible methods. They can be also classified on the basis of chemical reaction. All the immobilization methods have certain advantages and disadvantages. The choice of the proper method of immobilization depends on the enzyme and the carrier material characteristics (Brena et al., 2013). The different enzyme immobilization methods and their classifications are shown in Fig. 41.1. The enzyme immobilization may be the pure enzyme immobilization or the immobilization of the cell containing the enzyme. Classification of support is summarized in Table.41.2.

41.2.1 Binding to a Support by Physical, Ionic, or Covalent Bonding

These are very simple methods of enzyme immobilization. Typical supports for enzyme immobilization are polysaccharides, synthetic resins, biopolymers, or inorganic solids such as silica or zeolites (Sheldon and Pelt, 2013).

41.2.1.1 Physical Bonding

The easiest technique of enzyme immobilization is adsorption. It includes a reversible surface interaction between the carrier and enzyme, which is a weak force such as the Van der Waals forces, ionic bonds, or hydrogen bonding interactions, for example, amyloglucosidase

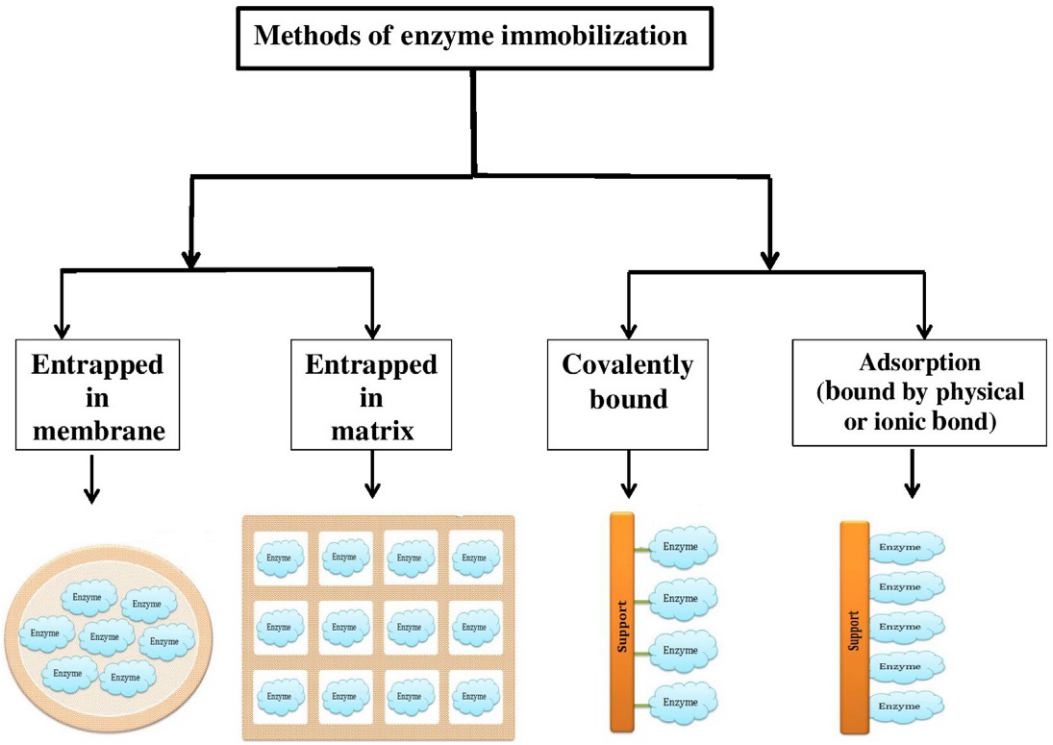

FIG. 41.1 Different methods of enzyme immobilization.

TABLE 41.2 Classification of Support (Brena et al., 2013)

Type of Support	Chemical Nature of the Support	Examples of Support
Organic (natural polymer)	Polysaccharides	Cellulose, dextrans, agar, agarose, chitin, alginate
	Proteins	Collagen, albumin
Organic (synthetic polymer)	Synthetic	Polystyrene
Organic (other polymer)	Mixed type	Polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl and allyl-polymers
Inorganic	Natural minerals	Bentonite, silica
	Processed materials	Glass (nonporous and controlled pore), metals, controlled pore metal oxides

on the agarose gel (Shuler and Kargi, 2017). This method is advantageous as it is simple, cheap, and causes little or no damage to the enzyme and carrier molecule. The method has the drawback that the product separation is not easy when desorption of the enzyme occurs from the support and the binding of the enzyme with the carrier is not very specific.

41.2.1.2 Ionic Bonding

The principle of protein ligand interaction used in the method of chromatography is the basis of enzyme immobilization by ionic bonding, for example, α-amylase on calcium phosphate (Shuler and Kargi, 2017). The method is advantageous as it is simple, cheap, and causes little or no damage to the enzyme and carrier molecule. The method has the drawback that the enzyme desorption occurs in the presence of a highly charged substrate and product.

41.2.1.3 Covalent Bonding

In covalent binding, the enzyme immobilization method is the strong bond formation that occurs between the functional groups on the enzyme and the carrier molecule. The enzyme molecule contains functional groups on its surface as amino groups (NH_2) of arginine or lysine, a carboxylic group (COOH) of glutamic acid or aspartic acid, a hydroxyl group (OH) of threonine or serine, and a sulfhydryl group (SH) of cysteine (Hassan et al., 2016), for example, α-amylase on DEAE cellulose or lactase on cellulose (Shuler and Kargi, 2017). The advantage of this method is that the attachment is not reversed by the change of pH and the ionic strength of the substrate. The disadvantage of this method is that the active sites of the enzyme molecule may be blocked during covalent bonding.

41.2.2 Entrapment of the Enzyme in Support

In the method of entrapment, enzyme inclusion occurs within a polymeric network. It retains the enzyme but substrate and products are allowed to pass through. The support acts as a barrier to mass transfer and has serious reaction kinetics implications. There are several major methods of entrapment such as ionotropic gelation of macromolecules with multivalent cations (e.g., alginate), temperature-induced gelation (e.g., agarose, gelatin), organic polymerization reaction by chemical/photochemical (e.g., polyacrylamide), and precipitation from an immiscible solvent (e.g., polystyrene), for example, α-amylase on a polyacrylamide gel or amyloglucosidase on a polyacrylamide gel (Shuler and Kargi, 2017). The advantages of this method are a wide application and very mild reaction conditions while the method is free from the risk of the blocking of the active sites of the enzyme. However, the disadvantages of this method are retardation of the enzymatic reaction due to diffusional control of the substrate and the product and the continuous loss of the enzyme through the pores of the matrix.

41.2.3 Cross-Linking of the Enzyme With Support

In this method of enzyme immobilization, the joining of the enzyme molecule with the support occurs by chemical bonding to form a three-dimensional structure. Gluteraldehyde is one of the most popular cross-linking agents that forms bond through its amino group whereas diamines form cross-linking through carboxyl groups (Poul, 1984). The advantage of this method is that the enzymes are strongly bound so there's a very little chance of enzyme

desorption. However, the disadvantage of this method is that the cross-linking may cause significant changes in the active site of the enzyme molecule.

41.3 APPLICATION OF IMMOBILIZED ENZYMES IN DIFFERENT FOOD INDUSTRIES

41.3.1 Dairy Industry

The enzyme β-galactosidase is also known as lactase. It is obtained from microorganisms, plants, and animals. It is used for the hydrolysis of the disaccharide sugar lactose present in milk and whey. A large number of people are lactose-intolerant and cannot consume the milk in which the lactose has been hydrolyzed by lactase. This problem of lactose intolerance is very common in India. Lactose hydrolysis increases the sweetness and solubility of the sugar. This biochemical reaction has future potential in the preparation of different dairy products. In a whey-based beverage, the whey in which lactose is hydrolyzed can be used as a component. It can also be fermented to produce ethanol or can be used as a leavening agent and feedstuff. In this way, the by-product, which is inexpensive, can be converted to a food ingredient that is highly nutritious and of good quality. In the immobilized form, the enzyme has the advantage of being used both in batch and continuous operations. Immobilization makes the enzyme thermostable and prevents the loss of enzyme activity. The enzyme is immobilized in various methods such as absorption, entrapment, and covalent binding on various supports (Panesar et al., 2010). The most commonly used lactases for immobilization are obtained from *E. coli* and *A. niger*. The lactase enzyme immobilized on Teflon stirring bars that are coated with a polymer polyisocyanate was stable up to pH 8.75. It can be used continuously for 137.6 h without appreciable losses in activity (Homaei, 2016). D-Tagatose is a monosaccharide naturally present in dairy products, but in small amounts. Its sweetness is comparable with sucrose at 92% but has only 38% of the calories. From galactose, it can be produced via isomerization using the L-arabinose isomerase enzyme in an immobilized form obtained from *Thermotoga neapolitana* (Walsh, 2007).

41.3.2 Brewing Industry

Fermented beverages such as beer require 6–7 days of fermentation time with large-scale fermentation and a large storage capacity. The immobilization technique is used in the brewing industry by the entrapment of yeast cells to increase their concentration; this reduces the process time. Immobilized yeast cells reduce processing time without affecting the product quality (Mahmoud and Helmy, 2009; Russel et al., 1997). In immobilized cell technology, beer is produced in less than 2 days. In the process of flavor maturation, the primary objective is the removal of two compounds and their precursors. Those compounds are vicinal diketones diacetyl and 2,3-pentanedione. The precursors of these compounds are a-aceto lactate and a-aceto hydroxyl butyrate. This process takes 3–4 weeks. When the immobilized yeast cells are used, the time period is reduced to 2 h. Yeast cells are immobilized on DEAE-cellulose. These immobilized cells are packed in a column reactor. The immobilized yeast cells are applied successfully for the controlled ethanol production of beers. This beer is low in alcohol content or is alcohol-free (Russel et al., 1996; Van Dieren, 1995; Walsh 2007).

41.3.3 Fruit and Vegetable Industries

For the clarification of fruit juice, the immobilized pectinases are used. The pectinases are applied in fruit juice for its clarification and depectinization. After pressing, the raw fruit juice is obtained in a very turbid form as it contains colloidal compounds that mainly contain pectin. Pectin causes a cloudiness of raw fruit juice. Therefore, the removal of the juice haziness is the clarification of fruit juice. It actually occurs by enzymatic hydrolysis of pectin with pectolytic enzymes. An excellent result was obtained using immobilized polygalacturonase for the clarification of pineapple juice. (Kohli et al., 2015). They are also used to remove the bitterness from citrus fruit juice. Citrus fruit contains naringin, the main bitter component, and limonin, responsible for the delayed bitterness of fruit juice. The naringinase enzyme used for immobilization is produced by *A. niger* and is immobilized on copolymers of styrene and maleic anhydride. This immobilized enzyme has been used for the hydrolysis of naringin (Puri et al., 2008).

41.3.4 Protein Modification

Several immobilized enzymes are used for the complete hydrolysis of the protein present in food. The protein is hydrolyzed for changing the digestibility of the protein. It is related to the quality of protein. For this purpose, the enzymes such as pepsin, trypsin, chymotrypsin, and intestinal mucosal peptidases are used in the immobilized form. Enzymatic hydrolysis is done to produce hydrolyzed casein that contains a high ratio of branched-chain amino acids to aromatic amino acids. This product can be used as a medical food for patients who are suffering from phenylketonuria, hepatic encephalophathies, and tyrosinemia. The protein hydrolysates from various foods such as milk, fish, corn, eggs, and cereals have several bioactive peptides (Walsh, 2007). Immobilized proteases are used for the limited proteolysis of food proteins that have enhanced functionality. β-Lactoglobulin hydrolysates are derived with immobilized trypsin by limited proteolysis. It produces a lower gel point and is gelled more rapidly than the native protein. The intrinsic viscosity is increased with the decrease in gelation temperature. As a result, stronger and more brittle gels were formed after heating. It is produced from whey protein by immobilized transglutaminase enzyme (Walsh, 2007).

41.3.5 Flavor Development

For the synthesis of fatty acid esters, the immobilized lipases are used. The flavor compounds are short chain fatty acids and alcohols such as methyl butyrate, butyl butyrate, isobutyl isobutyrate, and isoamyl isovalerate those are similar to pineapple or apple flavor, ethyl butyrate that is similar to pineapple or strawberry flavor, isoamyl acetate/butyrate that is similar to banana flavor. Immobilized lipases catalyze the synthesis of natural flavors under mild conditions. The immobilized lipase is used for the production of butyl butyrate from butyric acid and butanol. The process is esterification of an alcohol and a fatty acid in organic solutions (Kumar and Rao, 2004; Soares et al., 2005). Isoamyl butyrate and isoamyl alcohol are also produced in this process (Krishna et al., 1999). In the same way, off esterification isoamyl isovalerate and isoamyl alcohol are produced from isovaleric acid (Chowdary et al., 2000, 2002), isoamyl isobutyrate and isoamyl alcohol from isobutyric acid (Krishna et al., 2001), and

isobutyl isobutyrate and isobutyl alcohol from isobutyric acid (Hamsaveni et al., 2001; Walsh, 2007). From aspartic acid and phenylalanine, aspartame can be synthesized chemically but in this process an optical isomer is also produced that is bitter in taste. Thermolysin is an endopeptidase that catalyzes the hydrolysis of peptide bonds that contain hydrophobic amino acid residue. It can catalyze the reverse reaction also. Peptides are synthesized that are used as the precursor of aspartame, which is used as an artificial sweetener. The enzyme is obtained from the organism *Bacillus thermoproteolyticus*. It is commercially available as Thermolase. The enzymatic synthesis of aspartame has the advantage that it can recognize only the L isomer of the phenylalanine and β-carboxyl group of aspartate. So the process does not require any protection and deprotection to prevent the formation of the bitter isomer (Oyama et al., 1981; Walsh, 2007).

41.3.6 Cider Production

The process of cider transformation to apple juice is a complex process. It requires the combined activity of yeast and lactic acid bacteria for alcohol and malolactic fermentation, respectively (Willaert et al., 2005). For the coimmobilization process, *Saccharomyces bayanus* and *Leuconostoc oenos* cells are immobilized in the matrix of calcium alginate. It is used to perform alcoholic and malolactic fermentation simultaneously by the biocatalytic system in integrated form to produce cider from apple juice (Durieux et al., 2005).

41.3.7 Processing of Cocoa

Cocoa butter has a melting point of 37°C and contains palmitic and stearic acid. It melts in the mouth, thereby providing a cooling sensation. Immobilized lipase is used to produce a cocoa butter substitute; the technology was patented by Unilever in 1976. In the commercial application, immobilized lipase produced by *Rhizomucor miehei* is used, which makes the transesterification reaction. In this process, there will be a replacement of plamitic acid with stearic acid. It produces the desired stearic-oleic-stearic triglyceride (Aravindan et al., 2007).

41.3.8 Processing of Edible Fats and Oils

In the fat and oil industry, the immobilized lipase has great application. It is mainly used for the processing of fats or oils and flavor development. Immobilized lipase is preferred over a free enzyme as the stability and activity of the enzyme is improved by immobilization. In the immobilized form, the enzyme can be reused. Immobilization methods mostly employ noncovalent interactions. The immobilized lipase produced by *R. miehei* is used for the transesterification reaction in palm oil. The palmitic acid is replaced with stearic acid in this process. Immobilized lipases are obtained from various microbial sources such as *Geotrichum candidum, Humicola lanuginose, Candida cylindracea* AY30, and *Pseudomonas* sp. The immobilized lipase is used for the esterification of functionalized phenols. It is used in the synthesis of antioxidants, which are lipophilic in nature and used in sunflower oil. *Staphylococcus warneri* and *Staphylococcus xylosus* produce lipase, which is immobilized and used for flavor development (Sharma and Shamsher, 2014). Immobilized lipases are also used to produce transfat-free oils. Soy oil contains a good amount of unsaturated fatty acid but it has poor

oxidative stability. For that reason, it is partially hydrolyzed, which produces the transfat that has an adverse health effect. At a very low aqueous medium, interesterification can be done enzymatically. This reaction is very specific and mild but requires little downstream processing. The immobilized enzyme is used to produce zero or low transfat oil, shortening, and margarine from sources such as corn, sunflowers, and/or soy oil (Walsh, 2007).

41.3.9 Sugar Industry

In this sector of food processing, the major use of the immobilized enzyme is in the production of high fructose corn syrup (HFCS). In this process, the immobilized isomerase enzyme is used. Under ambient pH and temperature, immobilized enzymes produce HFCS. When the immobilized isomerase enzyme is used, fewer side products are formed with the higher concentration of fructose. The whole cell immobilization is mostly done by crosslinking the heat-treated cells with glutaraldehyde (Walsh, 2007). The enzyme isomaltulose synthase is used to convert sugar into isomaltulose (palatinose). The by-product of this reaction is trehalulose. The natural reducing sugar present in honey is isomaltulose. It is a low-calorie sugar. It has some characteristics that are advantageous compared with sucrose, including stability in acid solutions, promoting the growth of *Bifidobacteria* in the human intestine, and its noncariogenic property. In the production of isomaltulose, the immobilized *Erwinia rhaponica* cells were used. The immobilized cell reactors using any one of *E. rhaponica, Protaminobacter rubrum*, or *Serratia plymuthica* cells are used in the production of isomaltulose (Walsh, 2007).

41.3.10 Organic Acid

One of the important microbial products is organic acids, which are applied as food and medicine. The most important organic acid is citric acid, which is produced by *Aspergillus niger.* The disadvantage of fungal fermentations is that it increases the viscosity during growth, which leads to poor oxygen supply to the cells. So it is necessary to add large volumes of sterile air to aerate the cultures. It is possible to operate the fermenter without affecting the viscosity because growth is restricted in immobilized cells. In this way, immobilized cells are used to produce other organic acids such as gluconic acid, fumaric acid, malic acid, propionic acid, itaconic acid, gibberelic acid, succinic acid, and butyric acid (Gary and Sharma, 1992).

41.3.11 Food Supplements

Various food supplements are available in which the main ingredient is amino acids. They can be added solely or in combination. The most common products containing amino acid contain arginine, tryptophan, lysine, tyrosine, and glutamine. These products are recommended for the purpose of building up the body, sleep aids, fighting depression, etc. As a food and feed supplement, the L-amino acids are very important. Usually they are synthesized by a chemical method that produces both D and L-amino acids as racemic mixtures. After their acylation, D,L acyl amino acids are formed. The enzyme amino acylase is immobilized on DEAE-sephadex. The enzyme can selectively hydrolyze D,L-acyl amino acid, producing L-amino acids. The acylated racemic mixture is passed through a column containing the immobilized aminoacylase enzyme. Then from the unhydrolysed deacyl amino acid,

the free L-amino acids are separated. Thus the eluent from the column contains a mixture of L-amino acid and acetylated D-amino acid. L-Amino acid can be easily crystallized and separated from acetylated D-amino acid (Das, 2007). In this approach, a huge quantity of L-amino acids such as L-methionine, L-phenylalanine, L-tryptophan, and L-valine are produced throughout the world (Ghosh, 2011). Immobilized enzymes are also used in the production of nutraceuticals, which are food components having health benefits rather than nutritional value. The immobilization technique is used for the isolation and incorporation of nutraceuticals in ordinary foods to increase their medicinal value. Immobilization of lipase from *Candida antartica* and *Lactobacillus ruteri* was successfully applied for the isolation of nutraceuticals (Baianu et al., 2004). The immobilized lipase introduces conjugated linoleic acid (CLA) in dairy foods (Sakshi et al., 2015). Immobilized enzymes are used in the synthesis of xylo-, fructo-, isomalto-, and inulo-oligosaccharides. These are used in food as supplements. They act as soluble dietary fibers, which are prebiotics. They can stimulate the growth of probiotic microorganisms such as *Lactobacillus* sp. in the gut and *Bifidobacterium* sp. in the colon. These oligosaccharides have a degree of polymerization between 2 and 10 saccharides. The enzymes used in the production of functional oligosaccharides belong to two groups. The glycosidases can make the hydrolytic cleavage of glycosidic bonds. The glycosyl groups are transferred by the enzymes glycosyltransferases or transglycosylases. These are the enzymes produced by microorganisms. Immobilized purified enzymes or immobilized microbial cells containing the enzyme are used to produce functional oligosaccharides in conventional batch reactions (Walsh, 2007). Another functional oligosaccharide found in garlic, asparagus root, Jerusalem artichoke, dahlia tubers, and chicory roots is inulin. It contains linear β 1–2 linked fructose molecules. The fructose syrups or oligofructose are obtained as the yield when the inulin is hydrolyzed. Inulo oligosaccharides are produced with either the immobilized endo-inulinase enzyme or whole cells.

41.4 CONCLUSION

In the past few years, several studies have been done with a primary focus on the development of immobilized enzymes for future commercial use. Though the immobilized enzyme has several advantages in food processing, there are very few successful examples of immobilized enzymes in food processing. The immobilized glucose isomerase is used in the production of HFCS. The immobilized lipases are used in the production of diacylglycerols and transfree fats and/or oils. The main drawback of the immobilized enzyme system is its economics, which offset most of the other benefits of immobilized enzymes. Due to the changes made by food and drug regulations and the worldwide concern about chemical waste, the use of enzymes both in free and immobilized form may increase in the future. The cost of the immobilized enzyme process may decrease with the advances in microbial biodiversity, molecular biology, and genomics; this also may increase the use of immobilized enzymes in food processing sectors. Immobilized enzymes have to be very heat stable and must be able to work in a reliable optimized process system so the products will be cheap. But if in the market any cheap source of soluble enzyme is available and if other processes are well established, then the newly developed immobilized enzyme process cannot survive. In the development of processes using multienzyme systems, a significant future is expected.

It involves the cofactor regeneration in high value compound production. The bioprocess can be integrated with the downstream processing via the immobilized enzyme technology that may increase the productivity while minimizing the product recovery cost. Immobilized enzyme technology is also useful in nonaqueous enzymology, especially in the development of continuous bioreactors. Thus, there are rising possibilities in the field of immobilized enzymes. It is possible that in the future many applications will be replaced by immobilized systems. The new systems developed will become technically and commercially feasible.

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CHAPTER

42

Biosensors: An Enzyme-Based Biophysical Technique for the Detection of Foodborne Pathogens

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42.1 INTRODUCTION

Foodborne pathogens are microbes such as germs, fungi, and a number of other organisms that can cause disease to humans; they are transmitted by polluted food or water. In the last decade, there has been an increase in the number of diseases caused by foodborne pathogens that become important health issue in the world (Zhao et al., 2014). There are numerous foodborne pathogens that have been recognized for foodborne diseases such as *Escherichia coli* O157:H7, *Campylobacter, Salmonella, Listeria monocytogenes, Norovirus, Staphylococcus aureus, Clostridium perfringens,* and *Toxoplasma gondii* (Alocilja and Radke, 2003; Chemburu et al., 2005; Velusamy et al., 2010). Uncooked meat and vegetables as well as raw milk may be the basis of the microorganisms, which are transported through cross contamination of food preparation and the supply of foodstuffs. Some type of foods such as milk, cream, and meat must be restricted from pathogens, especially from *L. monocytogenes* and *S. aureus* (Poltronieri et al., 2014). The excessive incidences of foodborne diseases in many developing countries point to major underlying food safety problems. Therefore, it is important to detect foodborne pathogens in order to decrease the incidences of foodborne diseases (Zhao et al., 2014). There are several systematic programs such as good farming and industrial practices, threat analysis, critical control points, and food codes representing approaches that can significantly decrease the pathogenic microbes in foodstuffs (Jin et al., 2008; Mucchetti et al., 2008; Taylor, 2007). Old procedures for the detection of foodborne bacterial pathogens are a time-consuming process, taking so much time for initial outcomes and consuming more than 1 week to confirm the pathogenic microbes (Sharma and Mutharasan, 2013). Therefore, for food safety and the protection of the public health

https://doi.org/10.1016/B978-0-12-813280-7.00042-6

from infectious diseases, there is the need for more rapid detection methods for foodborne pathogens (Zhao et al., 2014). Recently, many researchers have concentrated on the progress of quick detection devices for foodborne pathogens. The development of specific biosensor methods that may detect foodborne pathogens in a small amount of time with sensitivity and selectivity in comparison to the old methods can possibly be used as stand-alone tools for onsite detection of pathogens. Accordingly, the objective of this chapter is to discuss the mechanisms and applications of enzyme-based biosensors for rapid detection and identification of foodborne pathogens, and to review some of the more recent and novel techniques used in the related food industry.

42.1.1 Biosensors

Biosensors are diagnostic devices that can change biological reactions into detectable and measurable signs. Fig. 42.1 shows block diagram of a biosensor. Biosensors have many uses in the fields of medication, food processing, and environmental safety. One of the most important applications of biosensors is finding foodborne pathogens. Biosensors that are used for the detection of foodborne pathogens mainly contain three elements: a living capture molecule, a technique for changing capture molecule-target interactions into a sign, and an information output system (Perumal and Hashim, 2014; Velusamy et al., 2010). Biosensors are simple to use as they do not need sample preenrichment and are dissimilar to nucleic acid-based and immunological techniques for the detection of foodborne pathogens. The main advantages of biosensors are real-time rapid detection, portability, ease of use, and multipathogen detection for both field and laboratory analyses.

42.1.2 Classification of Biosensors

Biosensors can be categorized by the type of biological receptor and the principle of operation of the transducer used. A bioreceptor can be classified according to the use of a cell, enzyme, antibody, nucleic acid, or biomimetic. Transducers can be classified according to optical, electrochemical, calorimetric, piezoelectric, and different combinations of the above methods. Fig. 42.2 demonstrates different biosensors used for the detection of food pathogens. However, in the present chapter we will discuss enzyme-based biosensors.

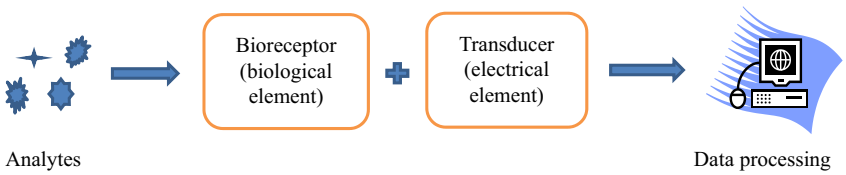

FIG. 42.1 Block diagram of biosensor.

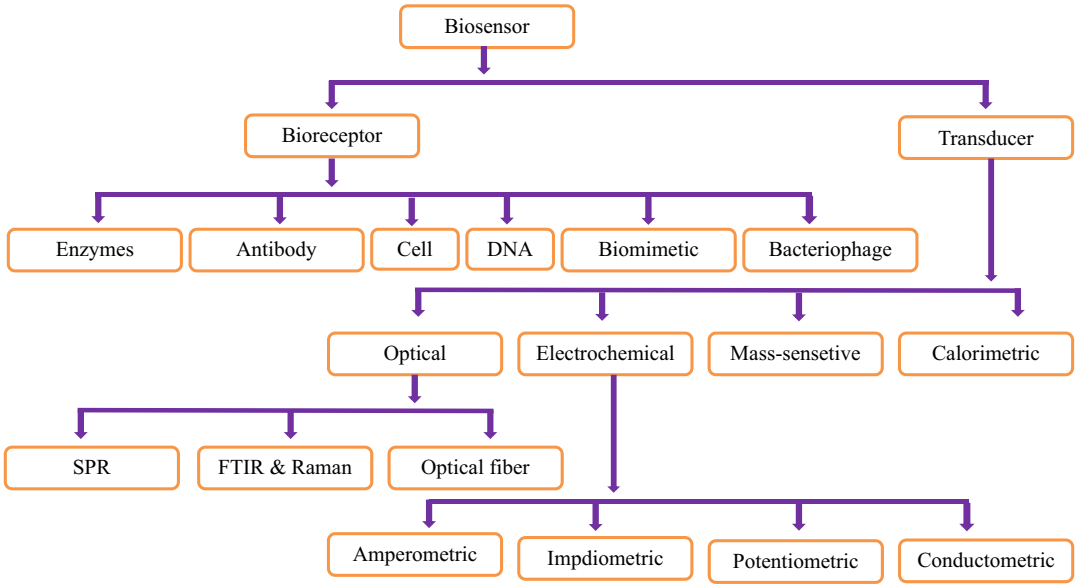

FIG. 42.2 Classification of biosensor.

42.2 ENZYME-BASED BIOSENSORS

Enzyme-based biosensors are analytical device that use enzymes either in the biorecognition process, in the transducing element, or in both of them. They can be classified in two major categories such as enzyme bioreceptor and enzyme transducer. They can be further classified into different types. Some examples of enzymes used in various types of biosensors are given in Table 42.1.

42.2.1 Enzyme-Based Bioreceptors

An enzyme is a compound molecule that works as a great catalytic agent to change substrates into products. Mostly, enzymes are proteins except ribonucleoprotien, in which RNA possesses the catalytic ability compared to protein. The biosensor uses enzymes as a bioreceptor and their mode of action, which involves oxidation, can be identified electrochemically (Kumar and Naalam, 2016). The transducer changes the signals into a computable response, such as potential, electric current, heat change, or absorption of light through electrochemical, thermal, or optical means. The selection of the enzyme is based on binding ability and the catalytic activity of enzymes (Vo-Dinh and Cullum, 2000). The main reason to use enzymes as biological receptors is the great number of reactions they catalyze, the probability of identifying a wide range of analytes, and the diverse transduction principles that can be applied to identify the analyte of interest (Maria and Maria, 2002). The biosensors use enzymes that are specific for the selected molecules. Some of the important enzymes used for the construction of biosensors are fructose dehydrogenase used for fructose (Trivedi et al., 2009), alcohol oxidase for alcohol (Hammerle et al., 2011), amino acid oxidase for amino acids (Lata et al.,

TABLE 42.1 Enzymes Used in Different Biosensors

Enzyme	Type of Biosensor	Reference
Luciferin and luciferase	ATP bioluminescence-biosensor	Eed et al. (2016)
Toluene orthomono oxygenase	Optical biosensor	Zhong et al. (2011)
Tyrosinase	Optical biosensor	Kumar et al. (2011)
Horseradish peroxidase, Tyrosinase	Bienzyme electrochemical biosensor	Yang et al. (2001)
Horseradish peroxidase	Electrochemical biosensor	Chemburu et al. (2005)
Alcohol oxidase	Electrochemical biosensor	Trivedi et al. (2009) and Hammerle et al. (2011)
Amino acid oxidase	Amperometric L-amino acid biosensor	Lata et al. (2013)
Glucose dehydrogenase	Amperometric glucose biosensor	Liang et al. (2013)
Urease	Silicon chip-based, light-addressable potentiometric sensor.	Dill et al. (1999)
Urease	Light-addressable potentiometric sensor	Gehring et al. (1998)
Esterase 2	Esterase 2-amplification-based DNA array sensor	Pohlman et al. (2009)
Horseradish peroxidase	HRP-amplification-based DNA multiwell sensor strips	Lagier et al. (2007)
Horseradish peroxidase	Impedance-based HRP-labeled immunosensor	Yu et al. (2006)
Cholesterol esterase and cholesterol oxidase	Cholesterol biosensor	Singh et al. (2005)
Fructose dehydrogenase	Amperometric fructose biosensor	Trivedi et al. (2009)
Urease	Fiberoptic spectrometer	Swati et al. (2010)
Toluene orthomono oxygenase	Enzymatic biosensor	Zhong et al. (2011)
Pumpkin urease	Fiberoptic biosensor	Prakash et al. (2008)
Horseradish peroxidase	Fiberoptic biosensor	Marquette et al. (2000)
Alcohol dehydrogenase	Fiberoptic spectrometer	Kudo et al. (2009)

2013), and glucose dehydrogenase for glucose (Liang et al., 2013). The lifespan of biosensor instruments is restricted by the stability of the enzyme in the system. There are five simple techniques of enzyme stabilization (immobilization): adsorption, microencapsulation, entrapment, cross-linking, and covalent bonding. In most of the biosensors, enzymes are working as a label rather than the real bioreceptor. Using enzymes as a label has extra benefits over fluorescently labeled and radiolabeled materials. Enzyme immunoassay substances are more constant and sensitive, so there are no health hazards. Numerous enzyme recognition techniques are visual, reducing the need for a costly and complex apparatus. It is also reported

that in immunosense systems enzymes can be used as labels for the recognition of foodborne pathogens. Chemburu et al. (2005) used the horseradish peroxidase (HRP) enzyme to label the antibody for the recognition of food pathogens in a sandwich immunoassay. Shen et al. (2014) also reported enzyme-based immunoassay for the discovery of food pathogens.

42.2.1.1 Enzyme-Based Antibody Biosensors

Antibodies are usually used as a bioreceptor in biosensors. An antibody is Y shaped immunoglobin (Ig) that is prepared by two heavy chains (H) and two light chains (L). Antibodies may be polyclonal, monoclonal, or recombinant, depending on their inequitable properties and the way they are generated (Pohanka, 2009; Wood, 2006). Polyclonal antibodies are groups of antibodies that have a different cellular source and therefore a different specificity. The development of monoclonal antibodies significantly improves the field of immunoassaying by providing a constant reliable basis of described antibodies. Antibodies are usually immobilized on a substrate, which can be the sensor surface (Lazcka et al., 2007). An antigen and an antigen-specific antibody interconnect by the lock and key fit technique (Vo-Dinh and Cullum, 2000). Antibodies have the unique property of the key that marks the immune sensors as a great analytical device and notes their capability to diagnose molecular configurations (Velusamy et al., 2010). Antibodies can be covalently bound in several ways to ensemble the purpose of a particular assay. Guntupalli et al. (2007) reported a magnetoelastic resonance biosensor that is immobilized with a polyclonal antibody for recognition of *Salmonella typhimurium*. Several immunological techniques also include labeled antibodies. Enzymes covalently combine to antibodies, provide unique specificity and sensitive means for detection. ELISA and ELFA are examples of enzyme-based antibody biosensors. Many researchers reported the enzyme label immunoassay for the detection of foodborne pathogens, as presented in Table 42.2.

The enzyme-linked immunosorbent assay (ELISA) is a biochemical method that combines an immunoassay with an enzymatic assay. It is a very frequently used immunological means for finding foodborne pathogens. Sandwich ELISA is the current form of ELISA, which contains two antibodies (Zhao et al., 2014). The first antibody binds to a solid medium and captures the antigen from enrichment cultures and the second antibody conjugated to an enzyme is used for recognition. The enzyme is able to generate a product quantifiable by a change in

TABLE 42.2 Applvation of Enzyme-Linked Immunoassays (ELISA) for the Detection of Foodborne Pathogens

Detection Technique	Analyte	Detection Limit	Food Sample	Reference
FNP ELISA	*Escherichia coli* O157:H7	6.8×10^{2} CFU/mL 6.8×10^{3} CFU/mL	Vegetables, milk Ground beef	Shen et al. (2014)
C ELISA	*Escherichia coli* O157:H7	6.8×10^{5} CFU/mL	Vegetables, milk, ground beef	Shen et al. (2014)
IMS ELISA	*Escherichia coli* O157:H7	6.8×10^{3} CFU/mL	Vegetables, milk, ground beef	Shen et al. (2014)
ELISA	*Salmonella*	1 CFU/25 g	Food sample	Bolton et al. al. (2000)
ELISA	*S. typhimurium*	10^{3} CFU/mL	Milk	Wu et al. (2014)

color (Zhang, 2013). In ELISA, normally three types of enzymes are used: alkaline phosphatase, HRP, and beta-galactosidase. An enzyme-linked fluorescence assay (ELFA) generates a fluorescence, which allows for the indirect measurement of the antigen present in the contaminant using spectrophotometry (Jasson et al., 2010). There are various enzyme immunoassays for the detection of foodborne pathogens and contaminants in foodstuffs (Jasson et al., 2010; Savoye et al., 2011). Shen et al. (2014) reported ELISA for finding *E. coli* O157:H7 in various foodstuffs. Crowley et al. (1999) used ELISA and amperometric detection for increasing the sensitivity of *L. monocytogenes* assays. Examples of enzyme-linked immunoassays used for pathogen detection are given in Table 42.2.

42.2.2 Enzyme-Based Transducer

The transducer is an important element of a biosensor that has a significant role in the signal detection process. It is a device that can convert different types of physical, chemical, or biological outcomes into an electric signal. In the last decade, different types of transduction techniques have been discovered for the detection of foodborne pathogens. Out of that, three techniques—optical, electrochemical, and calorimetric-based transducers—are the most common in practice. These three main classes are further divided into different subclasses.

42.2.2.1 Enzyme-Based Optical Biosensors

Optical biosensors are based on the principle of optical measurements such as reflection, refraction, absorbance, fluorescence, chemiluminescence, etc. An optical biosensor is a small diagnostic method containing a biological sensor coupled with an optical transducing system. Enzyme-based optical biosensors primarily involve enzymes as the transducing elements, which catalytically change analytes into products that can be oxidized at a working electrode and maintained at a particular potential. Optical biosensors have many advantages over other techniques due to their high specification, sensitivity, small size, low cost, and biodegradable electrodes. Optical biosensors can be classified into many types according to the technique used, including absorption, reflection, refraction, Raman, infrared, chemiluminescence, dispersion, fluorescence, and phosphorescence. In the past decade, different types of optical biosensors for the rapid detection of foodborne pathogens have been developed (Abbas et al., 2011) (Table 42.3). However, all the above types of optical biosensors need an appropriate spectrometer to record the spectrochemical properties of the analyte. Surface plasmon resonance and fluorescence are commonly employed techniques of optical detection, due to their sensitivity. Optical systems using fiberoptics are also used for finding pathogens in foodstuffs.

42.2.2.1.1 ENZYME-BASED FIBEROPTIC BIOSENSORS

Fiberoptic biosensors work on the phenomena of total internal reflection (TIR). In these biosensors, transduction is based on the fiberoptic technique. The transducer produces a signal proportional to the concentration of a biochemical, to which the bioreceptor responds. The optical fiber is formed by a core and a cladding with a refractive index n_1 and n_2, respectively. Core is more dense than cladding ($n_1 > n_2$). With a light incident at an angle greater than the critical angle ($\theta_c = \sin - 1[n_2/n_1]$), it is totally internally reflected, and therefore propagated through the optical fiber (Maria and Maria, 2002). In fiberoptic biosensors, light is

TABLE 42.3 Enzyme-Based Optical Biosensor for Food Pathogens

Detection Technique	Analyte	Detection Limit	Assay Time	Food Sample	Reference
Enzyme-based optical biosensor	*Escherichia coli* O157:H7	10–100 CFU/mL	2 h	–	Li and Su (2006)
Enzyme-based optical biosensor	*Listeria monocytogenes*	3.5×10^3 CFU/mL	–	Contaminated apple juice	Taylor et al. (2006)
Enzyme-based optical biosensor	*Campylobacter jejuni*	1.1×10^5 CFU/mL	–	Contaminated apple juice	Taylor et al. (2006)
Chemiluminescence enzyme immunoassay	*Escherichia coli* O157:H7	10^1–10^2/g	24 h	Ground beef	Kovacs and Rasky (2001)
Optical immunosensors	*Listeria monocytogenes* and *Salmonella*	10^3–10^9 CFU/mL and 10^6–10^9 CFU/mL	1 h	Food sample	Bhunia et al. (2004)
Enzyme-based fiberoptic biosensor	*Escherichia coli* O157:H7	10^3 CFU/mL	4 h	Ground beef	Ohk and Bhunia (2013)
ATP bioluminescence-sensing assay	*E. coli*	1.6×10^6 to 10^7 CFU/mL	24 h	White cheese	Eed et al. (2016)

transmitted through glass or plastic optical fibers. Fiberoptic biosensors combine the use of bioreceptors with optical fibers. They can be classified according to the bioreceptor used such as enzyme and antibody (Monk and Walt, 2004). Fiberoptic biosensors can be joined with various spectroscopic methods, for example, surface plasmon resonance, absorption, fluorescence, phosphorescence etc. Fiberoptic biosensors can be minimized and united for dissimilar target compounds. The key advantages of using fiberoptic biosensors are the sample analysis at outdoor sights and the significant role for field monitoring. Fiberoptic biosensors can also be used in bad environments and are resistant to electrical or magnetic effects. Therefore, they can be safer than electrochemical biosensors and can be minimized in size for a small cost.

The biological elements that are used in most fiberoptic biosensors are commonly enzymes. Because enzymes are usually proteins that convert a precise substrate into a product without being consumed in the reaction, they can definitely be used for uninterrupted biosensing of a particular compound (Eggins, 1996). Also, enzymes are very selective and sensitive compared with chemical compounds; they are also very fast acting in comparison with other biological elements. In fiberoptic biosensors, light transmission by a waveguide can be very sensitive to backgrounds, which makes the optical fibers outstanding. Evanescent wave fiberoptic biosensors can detect target analytes in minutes directly from complex samples using robust antibody-based assays; this improves the detection limit and speed (Lim, 2003). Enzymes are selected in optical fiber biosensors in such a way that the reactions couple the target analyte to make a basis of recognition (Narsaiah et al., 2011). Many researchers reported enzyme-based optical fiber biosensors for recognition of food pathogens. Prakash et al. (2008) used enzyme-based optical fiber cable for finding mercuric ions in water. Zhong et al. (2011) constructed an enzyme-based biosensor using a toluene orthomono oxygenase enzyme to

detect toluene. Kumar et al. (2011) used a tyrosinase enzyme-based optical biosensor to test tea quality. Ohk and Bhunia (2013) used enzyme-based fiberoptic biosensors for the detection of *E. coli* O157:H7 in ground beef at a detection limit of 10^3 CFU/mL. Liu et al. (2003) reported rapid detection of *E. coli* O157:H7 injected in ground beef, chicken carcasses, and lettuce samples with an immunomagnetic chemiluminescence fiberoptic biosensor.

42.2.2.1.2 ENZYME-BASED BIOLUMINESCENCE BIOSENSOR

A number of enzymes have the ability to radiate photons as a result of their reaction. This incidence is known as bioluminescence and can be used to identify the biological form of a cell. This method gives a result in a very small amount of time and can be applied for detection of food pathogens in the food production process (Jasson et al., 2010; Samkutty et al., 2001). All living cells have an ATP molecule that can be analyzed by processes of light emission produced by an enzymatic reaction between an enzyme and coenzyme complex (luciferin and luciferase). The amount of light produced is proportional to the concentration of ATP, and therefore the number of microbes in the sample. But at least 10^4 cells are necessary to produce an indication (Mandal et al., 2011). This method is extensively applied to find surface hygiene that comes into contact with food containing the organic filtrates and bacterial pollutants (Cunningham et al., 2011). Eed et al. (2016) used an ATP bioluminescence-sensing assay for *E. coli* at a detection limit of 1.6×10^6 to 10^7 CFU/mL for two food samples, namely white cheese and protein concentrate (animal feed), by using the enzymes luciferin and luciferase.

42.2.2.2 Enzyme-Based Electrochemical Biosensors

Electrochemical biosensors uses bioelectrochemical components as a transducing element. Although electrochemical biosensors use a variety of biorecognition elements such as antibodies, nucleic acids, cells, and microorganisms, they mostly use enzymes because enzymes have specific binding capabilities and biocatalytic activity (D'Orazio, 2003). Electrochemical sensing usually contains three electrodes: a reference electrode, a counter or auxiliary electrode, and a working electrode. The reference electrode, generally prepared by Ag/AgCl, is put at a distance from the reaction site in order to uphold a known and steady potential. The working electrode functions as the transducing element in the biochemical reaction, and the counter electrode starts a link to the electrolytic solution so that an electric current can be applied to the working electrode (Grieshaber et al., 2008). Electrochemical biosensors are based on the transduction mechanism, which can be used for the recognition of food pathogens. These biosensors can be categorized according to observed parameters such as electric current, potential, impedance, and conductance. These are amperometric, potentiometric, impedimetric, and conductometric biosensors. Out of these methods, enzymes are mostly used in amperometric, potentiometric, and conductometric biosensors. Some example of enzyme-based electrochemical biosensors for the detection of foodborne pathogens are listed in Table 42.4.

42.2.2.2.1 ENZYME-BASED AMPEROMETRIC BIOSENSOR

Amperometric biosensors normally depend on an enzyme structure that catalytically transforms electrochemical nonactive analytes into products that can be oxidized at a working electrode. The detection depends on the change in current as a function of applied potential. In the amperometric detection-based biosensor, the sensor potential is fixed at a value

TABLE 42.4 Enzyme-Based Electrochemical Biosensor for Detection of Food Pathogens

Detection Technique	Analyte	Detection Limit	Assay Time	Food Sample	Reference
Enzyme-based electrochemical biosensor	*Escherichia coli* O157:H7	50 cells/mL	30 min	contaminated milk and chicken extract	Chemburu et al. (2005)
Enzyme-based electrochemical biosensor	*Listeria monocytogenes*	10 cells/mL	30 min	contaminated milk and chicken extract	Chemburu et al. (2005)
Enzyme-based electrochemical biosensor	*Campylobacter jejuni*	50 cells/mL	30 min	contaminated milk and chicken extract	Chemburu et al. (2005)
Enzyme-based electrochemical biosensor	*Campylobacter jejuni*	10^3 CFU/mL	2–3 h	Culture and chicken carcass wash water	Che et al. (2001)
Conductometric biosensor	*Bacillus cereus*	35–88 CFU/mL	6 min	Lettuce, tomatoes, fried rice	Pal et al. (2008)
Amperiometric biosensor	*Escherichia coli* O157:H7	10^2 CFU/mL	–		Rao et al. (2005)
Enzyme-based amperiometric biosensor	*Staphylococcus, E. coli*	1 cell/100 mL	6–8 h	–	Neufeld et al. (2003)
Enzyme-based amperiometric biosensor	*S. aureus*	10^4–10^5 CFU/mL	–	–	Brooks et al. (1992)
Enzyme-based amperiometric biosensor	*S. typhimurium*	1.09×10^3 CFU/mL	2.5 h	Chicken carcase and Ground beef	Yang et al. (2001)
Enzyme-based amperiometric biosensor	*Escherichia coli* O157:H7	5000 cells/mL	25 min	–	Brewster and Mazenko (1998)
Enzyme-based electrochemical immunosensor	*Salmonella*	5.0 CFU/mL	–	–	Xiang et al. (2015)
Enzyme-based conductometric biosensor	*Escherichia coli* O157:H7, *Salmonella*	81 CFU/mL	10 min	Fresh food sample	Tahir and Alocilja, 2004)
Light-addressable potentiometric sensor	*Escherichia coli* O157:H7	2.5×10^4 cells/mL	30 min	Buffered saline	Gehring et al. (1998)
Electrochemical biosensor	*Escherichia coli* O157:H7	3 CFU/10 mL	–	Surface water	Zhang et al. (2009)

where analyte produces current. Thus the applied potential function is the driving force for the electron transfer reaction, and the produced current is a straight measurement of the rate of charge transfer. Neufeld et al. (2003) has reported an enzyme-based amperometric biosensor for *Staphylococcus* and *E. coli*, with a LOD (detection limit) of 1 cell/100 mL in 6–8 h. Brooks et al. (1992) reported an enzyme-linked amperometric technique for the detection of *S. aureus* with a LOD of 10^4–10^5 CFU/mL. Yang et al. (2001) used a HRP and tyrosinase bienzyme electrochemical biosensor coupled with an immunomagnetic separation method for finding *S. typhimurium* in chicken and ground beef with a LOD of 10^3 CFU/mL in an assay time of 2.5 h. Rishpon and Ivnitski (1997) reported an amperometric enzyme-channeling immunosensor for the detection of *S. aureus* in an assay time of 5–30 min.

42.2.2.2.2 ENZYME-BASED POTENTIOMETRIC BIOSENSOR

In potentiometric detection-based biosensors, an ion-selective electrode is used to transduce the biological reaction into an electrical signal. In this method, common pH electrodes are used to determine the changes in the concentration of ions. Hence a large amount of enzymatic reactions is involved in the release of hydrogen ions. The electrical potential between two electrodes is measured by a high impedance voltmeter. Some enzymes such as urease and alkaline phosphatases are used for labeling antibodies in a potentiometric immunosensor. These are capable of changing either the pH or ionic strength for the duration of bacteria recognition existing in foodstuffs (Kumar and Naalam, 2016). Potentiometric detection creates a logarithmic concentration response, therefore this method permits the recognition of extremely small changes in concentration. Light-addressable potentiometric (LAPs) biosensors are reported by various researchers for finding food pathogens (Gehring et al., 1998; Mackay et al., 1991). LAPs work on the joining of a transient photocurrent to an insulated n- or p-doped silicon thin layer in touching base with an electrolyte. The amount of the induced photocurrent is determined by applied potential to the silicon plate. It is possible to identify dissimilar physicochemical phenomena by applying different light sources with different spatial regions. Ercole et al. (2003) reported LAPs for detection of *E. coli* cells in vegetable foods. They reported that potentiometric biosensors are very sensitive and fast in comparison to traditional procedures while the concentration of 10cells/mL was detected in an assay time of 1.5 h. Singh et al. (2005) reported LAPs for the detection of *Yersinia pestis* and *Bacillus globigii* spores with a detection limit of 10 cells/spores per sample. A light-addressable potentiometric biosensor was used by Dill et al. (1997) for the detection of *S. typhimurium* at a level of 119 CFUs. Silicon chip-based light-addressable potentiometric biosensors were used for the detection of *S. typhimurium* at levels of 119 CFUs by Dill et al. (1999).

42.2.2.2.3 ENZYME-BASED CONDUCTOMETRIC BIOSENSOR

Conductometric biosensors are based on the correlation between conductance and a biological element. In this system, change in the ionic concentration provides change in electrical conductivity or electric current. In conductometric biosensors, two metal electrodes are placed at a distance and an AC voltage applied across the electrodes results in electric current flow. During a biorecognition process, changes in ionic concentration and conductance in metal electrodes are measured (Tahir and Alocilja, 2003). Conductometric biosensors are intensely related to enzymes. Due to an enzymatic reaction, the ionic strength and the conductivity of the solution between two electrodes are changed. Thus, conductometric

biosensors can be applied to read enzymatic reactions that produce changes in the concentration of charged species in a solution (Thevenot et al., 2001). Many researchers reported conductometric biosensors for recognition of food pathogens such as *E. coli* O157:H7 and *Salmonella* spp. Tahir and Alocilja (2003) reported that conductometric biosensors are precise, sensitive, small, and have a real-time detection device. A direct-charge transfer conductometric biosensor has been established for the recognition of *B. cereus* in various foodstuffs (Pal et al., 2008). They reported that due to speed, sensitivity, and ease of use, this biosensor is capable of rapid field-based analysis for the safety of the food supply. A conductometric biosensor is reported for the detection of *E. coli* O157:H7 and *Salmonella* in foods with an assay time of 10 min and a detection limit up to 81 CFU/mL (Tahir and Alocilja, 2004). Also, a bi-enzymatic conductometric biosensor was developed for heavy metal ions and pesticide recognition in water (Chouteau et al., 2005). Now it is possible to build multianalyte conductometric biosensors by the development of semiconductor technology and a biosensor combination with microelectronic devices, such as field effect transistor (FET) devices.

42.2.2.3 Enzyme-Based Calorimetric Biosensor

Calorimeter-based biosensors are assembled by the immobilization of biomolecules on temperature sensors. This type of biosensor works on the basic of the calorimetric principle and changes in temperature during the reaction between the biorecognition element and analytes. This change in temperature is proportional to the molar enthalpy and the number of molecules present in the process (Xie et al., 1999). The temperature is recorded by enzyme thermistors that contain an enzyme with the temperature sensor. When the analyte interacts with the enzyme, the heat of the reaction is measured, which is adjusted further against the analyte concentration (Majumdar et al., 2015). The key benefits of calorimetric-based biosensors are the steadiness, increased sensitivity, and small size (Ahmad et al., 2010). This type of biosensor can also be easily combined with microfluidic for increasing sensitivity (Zhang and Tadigadapa, 2004). Calorimetric biosensors can also be used in the food industry for finding foodborne pathogens (Kirchner et al., 2012; Ma et al., 1998; Maskow et al., 2012). Miranda et al. (2011) used enzyme-based calorimetric biosensors for the detection of *E. coli* with a LOD of 1×10^2 bacteria/mL. An enzyme-based calorimetric biosensor was also developed for finding *E. coli* O157:H7 with a LOD of 5 CFU/mL by Hossain et al. (2012).

42.3 ENZYME-BASED NANOMATERIALS BIOSENSORS

Nanomaterials such as carbon nanostructures, magnetic NPs, and quantum dots have a significant role in improving the action of biosensors in terms of a smaller detection limit, greater sensitivity, and a charge transfer that is quicker. NPs and graphene metals are used to raise the surface area and conductivity of the biosensor. Carbon nanostructures such as CNTs and graphene are significant resources in nanoscience. They have distinct electrical, physical, mechanical, and chemical properties to advance the performance (Hu et al., 2010). Metal NPs are used in biosensors to increase sensitivity and the current signal response time. A gold NPs-based biosensor for *E. coli* O157:H7 detection in foodstuffs is reported by Ali et al. (2014). Xiang et al. (2015) also used a gold nanoparticle sensor for the recognition of *Salmonella*. A hybrid colorimetric enzymatic nanocomposite biosensor reported by Miranda et al. (2011) was

used for the detection of *E. coli* in aqueous solutions. In this device, cationic nanoparticles containing quaternary amine head groups are electrostatically bound to enzyme β-galactosidase; the binding process is measured by a colorimetric mechanism. A paper-based colorimetric biosensor is reported for the detection of *E. coli* O157:H7, *Salmonella,* and L. *monocytogenes* in foodstuffs by determining the color change when an enzyme connected with the pathogen of interest reacts with a chromogenic substrate (Jokerst et al., 2012). Pal et al. (2007) reported a nanowire-labeled biosensor for identifying *Bacillus species* with a LOD of 10^2 CFU/mL in 6 min. An electrochemical DNA biosensor for *S. aureus* with a LOD of 3.23×10^{-14} mol/L was reported by Sun et al. (2015). A magnetic nanoparticle antibody-based biosensor for the detection of *E. coli* O157: H7 in ground beef with a detection limit 8.0×10^1 CFU/mL was reported (Varshney et al., 2005).

42.4 FUTURE PROSPECTS

The advantages of enzyme-based biosensors are their speed, sensitivity, selectivity, and small size, but the main problem could be the high cost of some equipment. Biosensors in the past have been technologically advanced in all fields but there is also a need to change the traditional methods of the biosensor in some fields, such as food safety. To accomplish these aims, we require the improvement of biosensors so that they are capable of identification in a direct way at very small levels, that is, pico molar to fem molar. For this objective, there is a growing concern in biosensors based on micro/nanotechnologies for a large improvement in sensitivity and small size (Branton et al., 2008). The use of nanostructured devices in biosensors replaces traditional sensors with the benefits of large sensitivity, diminished energy consumption, and small size. For this purpose, carbon-nanotube and graphene-based electrical tools are broadly used for greater performance of label-free biosensors. Semiconductor technologies can also be applied effectively in biosensors while field-effect transistor technologies can play a significant role. There are several practical problems for biosensor preparation and additional investigation is required for finding better options. Presently, there is a risk of bioterrorism, so we are in need of improved, quicker, consistent, precise, transportable, and cheaper biosensors.

42.5 CONCLUSION

Conventional foodborne pathogen detection methods are sensitive enough, but often take too much time, as long as 3–5 days, to give results. Therefore, novel techniques are required to overcome this limitation. In recent years, various techniques have been discovered for the rapid detection of food pathogens. However, these methods still require an upgrade in sensitivity, selectivity, or accuracy to be of any practical use. Foodborne pathogens commonly exist in very small quantities (<100 CFU/g) and are very difficult to find in the presence of millions of other bacteria. There is the probability of missing bacteria during the detection process. Therefore, there is a need to establish an identification method that is dependable, fast, precise, simple, sensitive, selective, and inexpensive. Biosensors have made unlimited advancements in the past decade. They are a dynamic instrument used in many areas of biological

science research and medical science as well as in environmental, food safety, and military applications. Biosensor techniques have received heightened interest because they provide a smaller detection limit with a small assay time at a relatively small price. Biosensor-based devices are easy to carry out without training and produce results in small-time detection with high sensitivity and selectivity. However, there is still the possibility of improving them in food matrix detection. This chapter summarized various types of enzyme-based biosensors for the detection of foodborne pathogens. Enzymes are used either as a biological element or transducer and played an important role in foodborne pathogen detection techniques. In summary, we can conclude that enzyme-based biosensors are a great tool for the rapid and automatic detection of foodborne pathogens. But there is still a great chance for further improvement in the near future.

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CHAPTER

43

Production of Food-Processing Enzymes From Recombinant Microorganisms

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43.1 INTRODUCTION

The application of enzymes for food processing is not new for this century. It has been employed globally and traditionally to produce bread, wine, vinegar, and many other processed food products. The utilization of barley malt for starch conversion in brewing is the best well-known example for an intensive application of enzymes for industrial purposes. Though enzyme usage was not justified in ancient times, now different enzymes are being used extensively and scientifically in different food industries such as baking (amylase, maltogenic α-amylase, xylanase, lipase, glucose oxidase, transglutaminase, novamyl, and mylase), confectionery (invertase and glucose isomerase), dairy (acid proteinase, neutral proteinase, lipase, lactase, aminopeptidase, catalase, transglutaminase, and peptidase), meat products (protease), beverages (pectinase, glucose oxidase, α-amylase, β-amylase, protease, pullulanase, naringinase, limoninase, aminopeptidases, A-acetolactate decarboxylase, glucose isomerase, and invertase) as well as oil seed processing for the extraction of vegetable oil and fat (cellulase, hemicellulase, pectinase, and protease). Apart from that, many enzymes are also being used in animal feed (phytase, xylanase, β-glucanase, galactosidase, and glucanase) to enhance digestibility and reduce the viscosity of the food (Fernandes, 2010; Kalia et al., 2001; Panesar, 2010; Singh et al., 2016).

According to a report from the United Nations Department of Economic and Social Affairs (UNDESA), the world population is predicted to grow from 6.9 billion to 9.1 billion by 2050. Consequentially, food demand would also increase drastically, that is, it is expected to increase by 70% (UNDESA, 2013). Thus, food quantity as well as quality needs to be a prime focus of concern across the globe. High consumption demand can only be achieved by increasing the production of agriculture along with processed food. Scaling of food production is facilitated

https://doi.org/10.1016/B978-0-12-813280-7.00043-8

by extensive application of enzymes in food industries for improvement, production, processing, and other related components such as flavor, aroma, color, texture, appearance, shelf life, nutritive value, etc. (Neidleman, 1984), which can solve the problem to some extent. These enzymes are also very important from an economic point of view. According to a recent report by Grand View Research, the global enzymes market is expected to reach $17.5 billion by 2024. This market includes the enzymes useful in many industries related to food and beverages, textiles, biofuel production, animal feed, cosmetics, and pharmaceuticals (Grand View Research, 2016). Among all, the food and beverage industries are the most dominating ones with the positive slope of demand as they are associated with the basic necessities of life. In another recent report by a leading international business research company, Freedonia, that market is supposed to reach a value of $2.3 billion by 2020 (Freedonia, 2016).

Food enzymes can be obtained from different sources, but microbial sources are the most promising and potential one, as will be discussed in this chapter. On the other hand, the implementation of recombinant DNA technology and process engineering makes these microbial sources more comfortable, flexible, and appropriate for industrial purposes at the commercial level. The building blocks of this designed chapter include technical and scientific involvement of microorganisms in enzyme production for food purposes; the methodology for the isolation of microbes harboring the desired food-processing enzyme; identification of the corresponding gene for an enzyme of interest; cloning; and expression in a suitable host followed by purification of an enzyme of interest. It also covers the state of the art in genetic engineering techniques, applied for the enhancement and improvement in production of microbial enzymes applicable in food industries. The last section of this chapter includes the potential involvement of regulatory bodies and their obligatory safety norms for the production and consumption of food-processing enzymes.

43.2 FOOD ENZYMES FROM MICROBIAL SOURCES

Enzymes obtained from microbial sources have gained much attention because this option generally meets industrial demands such as cost and time effectiveness, conventionality, flexibility in the process modification and optimization, high yield, and productivity. More than half of the industrial enzymes are made by yeasts and molds, with bacteria producing about 30%. Only 8% is produced by animals and 4% by plants (Demain and Vaishnav, 2009). The use of microbial enzymes in the food and feed industries was a revolutionary step. It was found that some enzymes obtained from microbial sources were similar in function to the proteases of the pancreas and papaya fruit, or to the amylases of malt. The industrial application of enzymes became possible due to the initial efforts of Jokichi Takamine (1894, 1914) as well as Boidin and Effront (1917), who cultivated enzymes from fungal and bacterial sources, respectively, at an industrial scale (Underkofler et al., 1958). These sources are economically favorable as their simple cultivation does not involve any constraints related to time and space as compared to other sources. With the help of efficient advanced sequencing technologies, the genomes of microbes are being sequenced and many new gene sequences encoding enzyme variants are being identified. Nevertheless, the implementation of recombinant DNA technologies also suppresses the limitations associated with the nonreachable or pathogenic/toxin-producing source of the enzyme and allows the production of the enzyme

of interest in a preferred microbial safe host. Enzymes from microorganisms that are difficult to grow or handle genetically are now being produced in industrial microorganisms such as species of *Aspergillus* and *Trichoderma*, and *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Bacillus licheniformis*. Many microbial enzymes have been approved by regulatory authorities for their intended usage for food-processing purposes. For example, Health Canada, the governmental department in Canada that is responsible for national public health, has approved a list of food enzymes along with their permitted sources (plant, animal, and microbes) and safe usages in food applications. Most of the permitted enzymes are of microbial origin (Table 43.1).

43.3 ADVANCED STRATEGIES TO PRODUCE FOOD-PROCESSING ENZYMES AT AN INDUSTRIAL SCALE

In the present scenario, the combination of genetic engineering applications, fermentation technology, and process optimization is being implemented with great effort to achieve the industrial-scale production of enzymes from microbial sourcea. The large-scale production depends on the type of microbial strain used, the appropriate growth conditions, and the size of the well-equipped fermenter (Olempska-Beer et al., 2006). Large proteins/enzymes (greater than 100 kDa) are usually expressed in eukaryotic systems while smaller ones (less than 30 kDa) are expressed in prokaryotic systems. Microbial productivity and yield can be increased by the use of multiple gene copies, strong promoters, and efficient signal sequences. Effective screening tools using antibiotic resistant genes or selective markers are being employed to screen genetically manipulated microorganisms. Heterologous expression offers the possibility of enzyme production with a high yield and a secretion of the product into the culture medium (extracellular secretion), which greatly favors industrial production by reducing extraction cost and time. On the other hand, the fusion of N-terminal/C-terminal peptide tags facilitates the purification of intracellular fusion proteins/enzymes. Apart from that, the implementation of protein engineering also enhances the performance of enzymes to be used for food purposes by increasing their stability, activity, and specificity (Liu et al., 2013).

43.4 CLONING, EXPRESSION, AND PURIFICATION OF ENZYMES

As discussed in the previous section, enzymes can be obtained from different sources. The basic steps are almost all the same, irrelevant of the source of selection (Fig. 43.1). First, the enzyme gene is amplified using gene-specific primers and genomic DNA as the template. The amplified gene fragment is being cloned in corresponding sites of a digested cloning vector (discussed below), followed by transformation into microbial cells (e.g., *Escherichia coli*). After that, the selection of transformants and the confirmation of cloning (through restriction digestion analysis) are carried out. Confirmed cloned gene fragments are subcloned into expression vectors, followed by transformation in suitable host cells. There are numerous examples where transmission of native bacterial or fungal enzyme genes was carried out into the eukaryotic or prokaryotic host for the enhancement or flexibility of production. In one research work, α-amylase producing *Bacilli* was isolated from a soil sample using starch

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin

A.01	α-Acetolactate decarboxylase	*Bacillus subtilis* ToC46 (pUW235)	Brewers' mash
			Distillers' mash
A.02	Aminopeptidase	*Lactococcus lactis*	Cheddar cheese; (naming the variety)
			Dairy-based flavoring preparations
			Hydrolyzed animal, milk, and vegetable protein
A.1	Amylase	*Aspergillus niger* var.*; Aspergillus oryzae* var.*; Bacillus amyloliquefaciens* var.*; Bacillus subtilis* var.*; Rhizopus oryzae* var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Bread; flour; Whole wheat flour
			Cider; Wine
			Chocolate syrups
			Distillers' mash
			Malt-flavored dried breakfast cereals
			Single-strength fruit juices
			Precooked (instant) breakfast cereals
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
			Unstandardized bakery products
			Plant-based beverages
			Infant cereal products
		Aspergillus niger STz18-9 (pHUda7)	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Distillers' mash
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose

		Bacillus amyloliquefaciens EBA 20 (pUBH2); *Bacillus licheniformis*; *Bacillus licheniformis* BML 592 (pAmyAmp); *Bacillus licheniformis* BML 730 (pAmyAmp); *Bacillus licheniformis* LA 57 (pDN1981); *Bacillus licheniformis* LAT8 (pLAT3); *Bacillus licheniformis* LiH 1159 (pLiH1108); *Bacillus licheniformis* LiH 1464 (pLiH1346); *Bacillus licheniformis* MOL2083 (pCA164-LE399); *Bacillus licheniformis* PL 1303 (pPL1117)	Distillers' mash Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose Brewers' mash
		Bacillus licheniformis 3253 (plCatH-3253); *Bacillus licheniformis* 3266 (plCatH-3266ori1); *Bacillus stearothermophilus*; *Bacillus subtilis* B1.109 (pCPC800)	Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose Distillers' mash Brewers' mash Bread; flour; Whole wheat flour Unstandardized bakery products
		Bacillus subtilis B1.109 (pCPC720) (ATCC 39, 705)	Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
		Bacillus licheniformis MDT06-228	Bread Unstandardized bakery products
		Bacillus licheniformis JS1252	Bread; flour; Whole wheat flour Unstandardized bakery products
		Pseudomonas fluorescence DC88	Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose Distillers' mash
		Bacillus subtilis NBA (DS 68703)	Bread; flour; Whole wheat flour Unstandardized bakery products
A.2	Amylase (maltogenic)	*Bacillus subtilis* BRG-1 (pBRG1); *Bacillus subtilis* DN1413 (pDN1413); *Bacillus subtilis* LFA 63 (pLFA63); *Bacillus subtilis* RB-147 (pRB147)	Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose Bread; flour; Whole wheat flour Unstandardized bakery products
		Bacillus subtilis BS154	Bread; flour; Whole wheat flour Unstandardized bakery products

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

A.3	Asparaginase	*Aspergillus niger* AGN7–41	Bread; flour; Whole wheat flour
			Unstandardized foods
		Aspergillus niger ASP72	Bread; flour; Whole wheat flour
			Unstandardized foods
		Aspergillus oryzae (pCaHj621/BECh2#10); *Bacillus subtilis* MOL2940	Bread; flour; Whole wheat flour
			Unstandardized foods
			Green Coffee
C.01	Carboxypeptidase	*Aspergillus niger* PEG-1	Cheddar cheese; Dairy based flavoring preparations; Mascarpone; (naming the variety) Cheese; Preserved meat (Division 14); Sausage
C.1	Catalase	*Aspergillus niger* var.; *Micrococcus lysodeikticu*	Soft drinks
			Liquid egg-white (liquid albumen), liquid whole egg, or liquid yolk, destined for drying
			Liquid whey treated with hydrogen peroxide in accordance with item H.1 of the List of Permitted Food Additives With Other Accepted Uses
C.2	Cellulase	*Aspergillus niger* var.	Distillers' mash Liquid coffee concentrate Natural flavor and color extractives; Spice extracts
		Rasamsonia emersonii (previous name: *Talaromyces emersonii*)	Brewers' mash
		Trichoderma longibrachiatum QM9414 (previous name: *Trichoderma reesei* QM9414)	Single-strength fruit juices
			Tea leaves for the production of tea solids
		Trichoderma longibrachiatum A83 (previous name: *Trichoderma reesei* A83)	Apple juice; Grape juice; Grapefruit juice; Lemon juice; Lime juice; (Naming the fruit) juice; Orange juice; Pineapple juice
			Bread; flour; Whole wheat flour
			Unstandardized bakery products

C.3	Chymosin (i) Chymosin A	*Escherichia coli* K-12, GE81 (pPFZ87A)	Cheddar cheese; (naming the variety) cheese; Cottage cheese; Cream cheese; Cream cheese with (naming the added ingredients); Cream cheese spread; Cream cheese spread with (naming the added ingredients); Sour cream
			Unstandardized milk-based dessert preparations
	(ii) Chymosin B	*Aspergillus niger* var. *awamori*, GCC0349 (pGAMpR); *Kluyveromyces marxianus* var. *lactis*, DS1182 (pKS105)	Cheddar cheese; (naming the variety) cheese; Cottage cheese; Cream cheese; Cream cheese with (naming the added ingredients); Cream cheese spread; Cream cheese spread with (naming the added ingredients); Sour cream
			Unstandardized milk-based dessert preparations
		Kluyveromyces lactis CIN	Cheddar cheese; (naming the variety) Cheese; Cottage cheese; Cream cheese; Cream cheese with (naming the added ingredients); Cream cheese spread; Cream cheese spread with (naming the added ingredients); Sour cream
			Unstandardized milk-based dessert preparations
			Kefir; Quark; Yogurt
	(iii) Chymosin	*Aspergillus niger* var. *awamori* (pCCEx3)	Cheddar cheese; (naming the variety) cheese Cottage cheese; Cream cheese; Cream cheese spread; Cream cheese spread with (naming the added ingredients); Cream cheese with (naming the added ingredients); Sour cream
			Unstandardized milk-based dessert preparations
C.4	Cyprosin	*Cynara cardunculus* L. var. *altilis DC.*	Cottage cheese; Cream cheese; Cream cheese with (naming the added ingredients); (naming the variety) Cheese
G.1	Glucoamylase (Amyloglucosidase; Maltase)	*Aspergillus niger* var.; *Aspergillus oryzae* var.; *Rhizopus oryzae* var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Bread; flour; Whole wheat flour
			Chocolate syrups
			Distillers' mash
			Precooked (instant) breakfast cereals
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
			Unstandardized bakery products

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

		Aspergillus niger 126-PE001-32	Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Breakfast cereals
			Infant cereal products
		Aspergillus niger	Apple juice; Banana juice; Grape juice; Grapefruit juice; Lemon juice; Lime juice or lime fruit juice; Orange juice; Pear juice; Pineapple juice
			Infant cereal products
		Aspergillus niger STz18-9 (pHUda7)	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Distillers' mash
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
		Rhizopus niveus var.	Distillers' mash
			Mash destined for vinegar manufacture
		Rhizopus delemar var. *multiplicisporus*	Brewers' mash
			Distillers' mash
			Mash destined for vinegar manufacture
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
G.2	Glucanase	*Aspergillus niger* var.*; Bacillus amyloliquefaciens* var.; *Bacillus subtilis* var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Corn for degermination
			Distillers' mash
			Mash destined for vinegar manufacture
			Unstandardized bakery products
		Disporotrichum dimorphosporum DXL-1; *Rasamsonia emersonii* (previous name: *Talaromyces emersonii*)	Brewers' mash
		Humicola insolens var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Distillers' mash
		Trichoderma reesei A83	Apple Juice; Grape juice; Grapefruit juice; Lemon juice; Lime juice; (Naming the fruit) juice; Orange juice; Pineapple juice

G.3	Glucose oxidase	*Aspergillus niger* ZGL528-72	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Aspergillus niger var.; *Aspergillus oryzae* Mtl-72 (pHUda107)	Soft drinks
			Liquid egg-white (liquid albumen), liquid whole egg, or liquid yolk, destined for drying
			Bread; flour; Whole wheat flour
			Unstandardized bakery products
G.4	Glucose Isomerase	*Actinoplanes missouriensis* var.; *Bacillus coagulans* var.; *Microbacterium arborescens* NRRL B-11022; *Streptomyces murinus* DSM 3252; *Streptomyces olivaceus* var.; *Streptomyces olivochromogenes* var.; *Streptomyces rubiginosus* ATCC No. 21,175; *Streptomyces rubiginosus* SYC 5406 (pSYC5239)	Glucose (glucose syrup) to be partially or completely isomerized to fructose
H.1	Hemicellulase	*Bacillus amyloliquefaciens* var.; *Bacillus subtilis* var.	Distillers' mash
			Liquid coffee concentrate
			Mash destined for vinegar manufacture
H.2	Hexose oxidase	*Hansenula polymorpha* (B13-HOX4-Mut45)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Milk, partly skimmed milk, skim milk, and sterilized milk, heat-treated to at least 100°C
			Part skim pizza mozzarella cheese; Pizza mozzarella cheese
I.01	Inulinase	*Aspergillus niger* var. *Tieghem*	Inulin
I.1	Invertase	*Aspergillus japonicus*	Sucrose used in the production of fructooligosaccharides
		Saccharomyces sp.	Unstandardized soft-centered and liquid-centered confectionery
			Unstandardized bakery products

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

L.1	Lactase	*Bacillus licheniformis* PP3930	Lactose-reducing enzyme preparations
			Milk destined for use in ice cream mix (naming the flavor) Malted milk; (naming the flavor) Milk; (naming the flavor) Partly skimmed milk; (naming the flavor) Partly skimmed milk with added milk solids; (naming the flavor) Skim milk; (naming the flavor) Skimmed milk with added milk solids
		Aspergillus niger var.; *Aspergillus oryzae* var.; *Kluyveromyces fragilis(Kluyveromyces marxianus* var. *marxianus*); *Kluyveromyces lactis (Kluyveromyces marxianus* var. *lactis*); *Saccharomyces* sp.	Lactose-reducing enzyme preparations
			Milk destined for use in ice cream mix
			Bread; flour; Whole wheat flour
			(naming the flavor) Malted milk; (naming the flavor) Milk; (naming the flavor) Partly skimmed milk; (naming the flavor) Partly skimmed milk with added milk solids; (naming the flavor) Skim milk; (naming the flavor) Skimmed milk with added milk solids
		Cell-free extracts from *Candida pseudotropicalis*	Milk destined for use in ice cream mix
			Yogurt
			Whey
			(naming the flavor) Malted milk; (naming the flavor) Milk; (naming the flavor) Partly skimmed milk; (naming the flavor) Partly skimmed milk with added milk solids; (naming the flavor) Skim milk; (naming the flavor) Skimmed milk with added milk solids
L.2	Lipase	*Aspergillus niger var.*; *Aspergillus oryzae* var.; *Rhizopus oryzae* var.	Dairy based flavoring preparations
			Dried egg-white (dried albumen); Liquid egg-white (liquid albumen)
			Cheddar cheese; (naming the variety) Cheese; Processed cheddar cheese
			Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Hydrolyzed animal, milk and vegetable protein

		Aspergillus niger (LFS-54)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Aspergillus niger (pCaHj600/MBin118#11)	Modified fats and oils
		Aspergillus oryzae (MLT-2) (pRML 787) (p3SR2); *Rhizomucor miehei* (Cooney and Emerson) (previous name*: Mucor miehei* (Cooney and Emerson)); *Rhizopus niveus*	Modified fats and oils
			Cheddar cheese; (naming the variety) Cheese
			Dairy based flavoring preparations
			Hydrolyzed animal, milk, and vegetable protein
		Aspergillus oryzae AI-11 (pBoel 960)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Modified fats and oils
		Aspergillus oryzae BECh2#3 (pCaHj559); *Aspergillus oryzae* (MStr115) (pMStr20)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Modified lecithin
			Unstandardized egg products
		Candida rugosa; *Mucor circinelloides f. circinelloides* (previous name: *Mucor javanicus*); *Penicillium roquefortii*	Dairy-based flavoring preparations
		Mucor circinelloides f. circinelloides (previous name: *Mucor javanicus*)	Dairy-based flavoring preparations
		Penicillium camembertii	Edible fats and oils
M1	Milk coagulating enzyme	*Mucor pusillus Lindt* by pure culture fermentation process or *Aspergillus oryzae* RET-1 (pBoel777)	Cheddar cheese; Cottage cheese; (naming the variety) Cheese; Sour cream
			Dairy-based flavoring preparations
			Hydrolyzed animal, milk, and vegetable protein
		Rhizomucor miehei (Cooney and Emerson) (previous name: *Mucor miehei* (Cooney and Emerson))	Cheddar cheese; Cottage cheese; (naming the variety) Cheese; Sour cream
			Dairy-based flavoring preparations
			Hydrolyzed animal, milk and vegetable protein
			Cheese analogues

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

		Endothia parasitica by pure culture fermentation processes	Emmentaler (Emmental, Swiss) Cheese
			Parmesan Cheese
			Romano Cheese
			Mozzarella (Scamorza) Cheese
			Part Skim Mozzarella (Part Skim Scamorza) Cheese
P.3	Pectinase	*Aspergillus niger* var.; *Rhizopus oryzae* var.	Cider; Wine
			Distillers' mash
			Single-strength fruit juices
			Natural flavor and color extractives
			Skins of citrus fruits destined for jam, marmalade, and candied fruit production
			Vegetable stock for use in soups
			Tea leaves for the production of tea solids
		Aspergillus oryzae Km-1-1 (pA2PEI)	Cider; Wine
			Single-strength fruit juices
			Unstandardized fruit and vegetable products
P.4	Pentosanase	*Aspergillus niger* var.; *Bacillus amyloliquefaciens* var.; *Bacillus subtilis* var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Corn for degermination
			Distillers' mash
			Mash destined for vinegar manufacture
			Unstandardized bakery products
			Bread; flour; Whole wheat flour
		Trichoderma reesei (QM9414)	Bread; flour; Whole wheat flour
			Distiller's mash
			Unstandardized bakery products

P.5A	Phospholipase	*Streptomyces violaceoruber*	Modified lecithin
			Unstandardized egg products
		Aspergillus oryzae (pPFJo142)	Cheddar cheese; (naming the variety) Cheese
		Aspergillus niger (PLA-54)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Unstandardized whole egg; unstandardized egg yolk
			Modified lecithin
P.5.1	Peroxidase	*Aspergillus niger* MOX-54	Liquid whey treated with hydrogen peroxide in accordance with item H.1 of the List of Permitted Food Additives With Other Accepted Uses
P.6	Protease	*Geobacillus stearothermophilus* TP7	Hydrolyzed animal, milk, and vegetable protein
			Hydrolyzed yeast
		Fusarium venenatum WTY939-8-3	Hydrolyzed animal, milk, and vegetable protein
		Aspergillus niger var.; *Aspergillus oryzae* var.; *Bacillus amyloliquefaciens* var.; *Bacillus subtilis* var.	Ale; Beer; Light beer; Malt liquor; Porter; Stout
			Bread; flour; Whole wheat flour
			Dairy-based flavoring preparations
			Distillers' mash
			Sausage casings
			Hydrolyzed animal, milk, and vegetable protein
			Industrial spray-dried cheese powder
			Cuts of prepared meat; Cuts of prepared poultry meat
			Meat tenderizing preparations
			Precooked (instant) breakfast cereals
			Unstandardized bakery products
		Micrococcus caseolyticus var.	(naming the variety) Cheese
		Bacillus licheniformis (Cx)	Hydrolyzed animal, milk, and vegetable protein

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

		Bacillus licheniformis S10-34zEK4	Hydrolyzed animal, milk, and vegetable protein
		Aspergillus melleus	Hydrolyzed animal, milk, and vegetable protein
		Aspergillus oryzae var.	Cheddar cheese; Cheddar cheese for processing (granular curd cheese; Stirred curd cheese; Washed curd cheese)
		Bacillus subtilis Raa1102	Bread; flour; Whole Wheat Flour
			Unstandardized bakery products
P.7	Pullulanase	*Bacillus acidopullulyticus* NCIB 11647; *Bacillus licheniformis* SE2-Pul-int211 (pUBCDEBR A11DNSI)	Bread; flour; Whole wheat flour Starch used in the production of dextrins, dextrose, fructose syrups and solids, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
			Unstandardized bakery products
		Bacillus licheniformis BMP 139 (pR11Amp)	Bread; flour; Whole wheat flour
			Brewers' mash
			Starch used in the production of dextrins, dextrose, fructose syrups and solids, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
			Unstandardized bakery products
		Bacillus subtilis B1-163 (pEB301)	Bread; flour; Whole wheat flour
			Brewers' mash
			Distillers' mash
			Starch used in the production of dextrins, dextrose, fructose syrups and solids, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose
			Unstandardized bakery products
		Bacillus subtilis RB121 (pDG268)	Brewers' mash
			Distillers' mash
			Starch used in the production of dextrins, dextrose, glucose (glucose syrup), glucose solids (dried glucose syrup), or maltose

T.01	Transglutaminase	*Streptoverticillium mobaraense* S-8112	Unstandardized prepared fish products
			Simulated meat products
			Unstandardized cheese products
			Unstandardized processed cheese products
			Unstandardized cream cheese products
			Yogurt
			Unstandardized frozen dairy desserts
			Bread; flour; Whole wheat flour
			Unstandardized bakery products
			Brawn; Headcheese; Meat by-product loaf; Meat loaf; (naming the prepared meat or prepared meat by-product) with (naming the nonmeat ingredients); Prepared meat (Division 14); Prepared meat by-product; Preserved meat (Division 14); Preserved meat by-product; Pumping pickle, cover pickle and dry cure employed in the curing of preserved meat or preserved meat by-product; Sausage
			Prepared poultry meat; Prepared poultry meat by-product; Preserved poultry meat; Preserved poultry meat by-product

Continued

TABLE 43.1 Permitted/Approved Enzymes From Microbial Origin—cont'd

U.1	Urease	*Lactobacillus fermentum*	Sake; Wine
X.1	Xylanase	*Aspergillus oryzae* Fa 1-1 (pA2X1TI)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Aspergillus oryzae JaL 339 (pJaL537); *Bacillus subtilis* DIDK 0115 (pUB110 OIS2)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Aspergillus niger (XYL-2)(pXYL3); *Bacillus subtilis* (XAS); *Trichoderma reesei* A83	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Bacillus licheniformis strain HyGe329	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Bacillus subtilis CF 307 (pJHPaprE-xynAss-BS3xylanase#1)	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Bacillus subtilis Gizα3508	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Bacillus subtilis RH 6000	Bread; flour; Whole wheat flour
			Unstandardized bakery products
		Disporotrichum dimorphosporum DXL-1; *Rasamsonia emersonii* (previous name: *Talaromyces emersonii)*	Brewers' mash

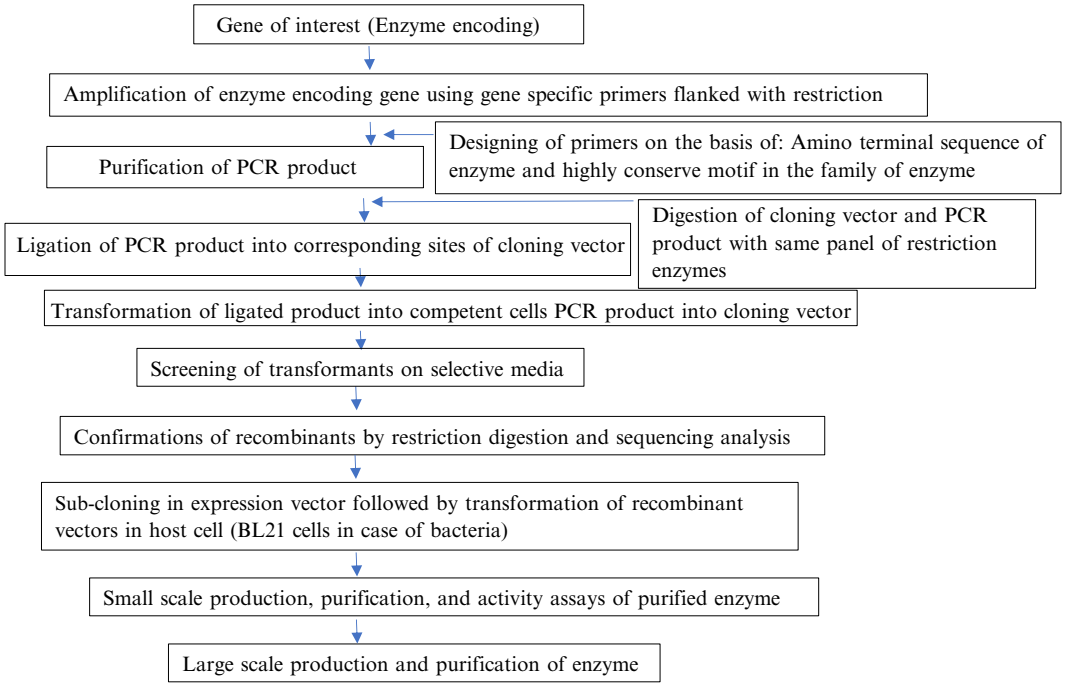

FIG. 43.1 Basic steps for cloning, expression, and purification of the enzyme gene.

selective media. The *amyE* (gene corresponding to amylaseE) gene was amplified using gene-specific primers and genomic DNA as the template. The PCR product is ligated into a plasmid (p316TDH3) using T4 ligase. This step was followed by transformation, selection, and confirmation of selection of recombinants, as described below. The recombinant plasmid was extracted by the midiprep method and transformed into *S. cerevisiae* competent cells. Recombinant yeast cells could grow on minimal media. For further confirmation of cloning, the recombinant plasmid was extracted from these colonies, followed by agarose gel electrophoresis. Confirmation was also verified by the colony PCR of yeast colonies using specific amyE primers (Afzal-Javan and Mobini-Dehkordi, 2013). Similar strategies have been used for the cloning and expression of the thermostable falactosidase fene from *Bacillus stearothermophilus* into *B. subtilis* (Chen et al., 2008).

After that, the expression of an enzyme is analyzed at a small scale, followed by purification studies. An activity of the purified enzyme is also checked before going for large-scale production.

In this section, the steps involved in enzyme production are briefly described, covering screening and isolation of enzyme-producing microbes, construction of a genomic library for screening of the best enzyme gene harboring clone, the cloning of the enzyme gene into a suitable vector, and its expression and purification in a sequential step-wise manner (Fig. 43.2). The descriptions of the steps involved are as follows:

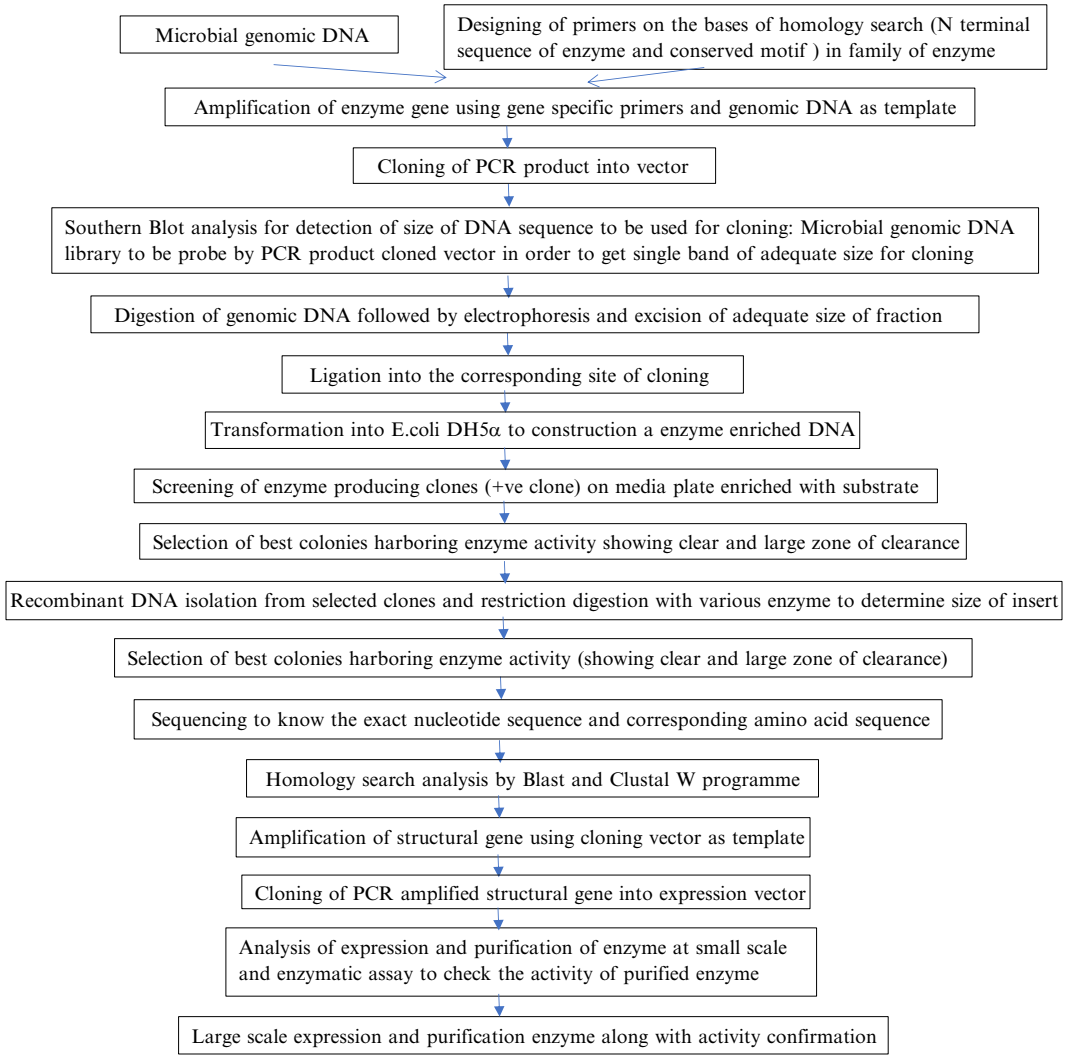

FIG. 43.2 Steps involved in enzyme production.

43.4.1 Isolation of Enzyme-Producing Microbes

The first step is to screen out enzyme-producing microbes from their natural habitat such as soil, water, root nodules, etc. For isolation of enzyme-producing microbes from a soil sample, a serially diluted soil sample is plated on selective media on which only specific microorganisms can grow. For example, for screening of amylase-producing bacteria, serially diluted samples are grown on a starch agar plate at 37°C for 24–48 h. The colonies that are able to synthesize amylase can only grow on selective media by utilizing starch as a carbon source. Individual plates were flooded with Gram's iodine to produce a deep blue-colored starch-iodine complex. The zone of clearance is where no blue color form reflects the presence of

the amylolytic strain in that area. After that, the potential isolates were identified by standard morphological and biochemical characterization (Ali et al., 2015; Gebreyohannes, 2015; Sen et al., 2014; Singh and Kumari, 2016). Similar screening strategies are also reported for different enzymes such as protease, β-galactosidase lipase, cellulase, etc. (Khannous et al., 2014).

43.4.2 Screening of the Clone-Harboring Enzyme-Encoding Gene From Genomic DNA Library

After screening and isolation of enzyme-producing microbes from different sources, the next task is to identify and isolate the DNA fragment/enzyme encoding gene from the ocean of the whole genome. For that, genomic DNA is isolated using the phenol-chloroform extraction protocol from the overnight grown culture of the best enzyme-producing microbial isolates (obtained by the procedure described in the previous step). Genomic DNA is digested with a restriction enzyme run on agarose gel. DNA fragments (2–3 kb range) will be cut and purified from the agarose gel, followed by cloning into the corresponding sites of the digested vector. Recombinants obtained after transformation are screened on selective media. The best enzyme-producing clone that shows a maximum and clear zone on selective media is considered for sequencing analysis. The obtained insert sequence is converted into a corresponding peptide sequence using available online software such as EMBOSS Transeq. A sequence similarity search is also performed using the Protein Blast bioinformatics program (Kanmani et al., 2015).

43.4.3 Amplification of the Enzyme-Encoding Gene

Gene-specific forward and reverse primers are designed for the amplification of the enzyme gene. The designing of primers is based on homology search. The flanking region of primers consists of restriction enzyme sites for cloning of amplified respective genes into a suitable vector. The genomic/chromosomal DNA of the best enzyme-producing clone (obtained by the procedure described in the previous step) will be taken as a template for the amplification of the target enzyme gene. Analysis and confirmation of amplification will be done by gel electrophoresis.

43.4.4 Cloning and Expression of Gene Interest Into a Suitable Cloning Vector

The amplified product will be purified and inserted in a cloning vector at the corresponding site. Nowadays, many cloning vectors are available commercially. These vectors should have the following features: (1) origin of replication, (2) multiple cloning sites (MCS) that contain a panel of multiple restriction enzyme sites, (3) antibiotic-resistant gene or selective marker for the selection of transformed recombinant cells, and (4) a reporter gene for the screening of successful clones by using features of these genes that allow the correct clone to be easily identified. For example, insertional inactivation of the β-galactosidase gene (reporter gene) by cloned fragments allows identification of recombinants by blue/white screening on IPTG and X-gal indicator plates. Nowadays, linearized vectors (e.g., pGEM-T and pGEM-T Easy Vectors) are also available with single T-overhangs (3′-terminal thymidine) at both ends for easy PCR cloning. The T-overhangs at the insertion site facilitate the ligation of PCR

products by preventing recircularization of the vector and also providing a compatible overhang for PCR products generated by thermostable *Taq* polymerases (pGEM-T and pGEM-T Easy Vectors system, Promega, 2015).

For cloning, the PCR product and vector are digested by the same panel of enzymes, followed by ligation and transformation of the ligated product into the host cell. Recombinant cells will be selected by plating of the transformation mixture on antibiotic-supplemented agar plates. To verify the correct orientation of the insert, the recombinant vector will be digested using suitable restriction enzymes. Apart from that, further confirmation is achieved by sequencing analysis. After that, the insert sequence is excised and subcloned into suitable expression vector, followed by transformation of recombinant expression vectors into the host cell (e.g., BL21 cells in the case of bacteria). Confirmation of subcloning is similar as described for cloning with cloning vectors. The expression vectors are designed to obtain maximum expression of the desired gene of interest. It should have the following features (1) MCS, (2) a strong and inducible promoter upstream of MCS, (3) strong transcription initiation and termination sequence, (4) a selective marker gene, (5) cleavable N and/or C-terminal fusion tags sequence (amino acid sequences) such as poly-histidines (6× His), glutathione-S-transferases (GST), maltose binding protein (MBP) strep-tag, etc., flanking or adjacent to the cloning site for improved expression, detection, solubility, and, more importantly, effective purification (Kosobokova et al., 2016), and (6) the addition of a protease cleavage site for the removal of fusion tags to obtain the enzyme in its native form. Nowadays, numerous commercial vectors are available that are designed to serve as cloning as well as expression vectors, as they have critical features of both cloning and expression vectors. The amplified gene of interest is cloned into these vectors. After the confirmation of correct cloning, the recombinant vector is transformed directly into an expression host system. The choice of these kinds of commercially available vectors such as pET SUMO (for prokaryotic system) minimizes the time span, cost, and intensive labor associated with cloning and expression of the gene (Champion pET SUMO Protein Expression System, Invitrogen, 2010). Some expression vectors for the eukaryotic system are described below.

43.4.4.1 Yeast Expression Vectors

Just like other expression vectors, eukaryotic expression vectors also have similar features such as the selective eukaryotic marker gene, a eukaryotic promoter sequence, an appropriate eukaryotic transcriptional and translation stop signals, etc. Most eukaryotic expression vectors are designed as a shuttle vector, that is they carry two types of ori and selective markers: one that is specific for the bacterial host and one for the eukaryotic host (yeast). Because initial genetic manipulations are being carried out in bacterial cells, all yeast vectors consist of sequences derived from a bacterial plasmid,that is, ori and antibiotic resistance markers, allowing subsequent propagation of the DNA and selection of transformants, respectively. The recombinant plasmid is transformed into yeast for heterologous expression of the protein/enzyme. The selection of recombinant transformed cells is based on auxotrophic selection rather than antibiotic selection. This type of selection employs special marker genes such as URA3, HIS3, LEU2, TRP1, MET15, etc., which encodes key enzymes involved in essential metabolic pathways. The auxotrophic mutant yeast strain, devoid of marker genes, is unable to grow on minimal media unless transformed with recombinant yeast vector carrying respective marker genes (Pronk, 2002; Mumberg et al., 1995).

The basic yeast vectors used for cloning and expression are YIp (yeast integrating plasmid), YRp (yeast replicating plasmid), YCp (yeast centromere plasmid), and YEp (yeast episomal plasmid). YIp vectors lack a yeast replication origin, so they propagate by integrating themselves into yeast chromosomes. YRp can autonomously replicate in the yeast host as it consists of an autonomously replicating sequence (ARS). The YCp (yeast centromere plasmid) vector consists of a norigin of replication as well as a centromere sequence; therefore, they are autonomously replicating and stably segregated plasmids. The YEp (yeast episomal plasmid) vector consists of a fragment from yeast 2 μ plasmid (natural yeast plasmid), propagated as high-copy-number, autonomously replicating, irregularly segregated plasmids (Stearns et al., 1990).

43.4.4.2 Pichia pastoris *Expression Vectors*

Pichia pastoris produces alcohol oxidase (AOX). But this enzyme has poor affinity for oxygen, which is naturally compensated for by expressing a large amount of enzyme by the corresponding two genes (AOX1 and AOX2). The AOX1 gene is regulated by the efficient methanol inducible promoter. This promoter has been used for the construction of a vector. A typical *P. pastoris* expression vector has the following features: (1) AOX1 promoter fragment, (2) AOX1 termination regions, (3) 3′ AOX1 region, (4) HIS4 marker gene, (5) ColE1 sequence and antibiotic-resistant gene for subcloning into *E. coli*, and (6) integration of the vector into the genome of the host by homologous recombination. Most strains of *P. pastoris* are histidinol-dehydrogenase deficient. That makes this system suitable for auxotrophic selection. Thus the transformants harboring the expression vector containing the HIS4 gene can be screened on minimal media. Some other genes involved in biosynthesis and metabolic pathways such as arg4-argininosuccinate lysate and ura3-orotidine 5′-phosphate decarboxylase are also exploited for auxotrophic selection-based screening methods (Macauley-Patrick et al., 2005; Spohner et al., 2015).

43.4.4.3 *Filamentous Fungi Expression Vectors*

Fungal expression vectors have generally been constructed on the backbone of bacterial plasmids. The expression vectors used to transform fungi for enzyme production include the following features: a strong constitutive promoter sequence from the host or a related species; MCS that allow the insertion of enzyme-encoding genes into vectors; upstream signal sequences to facilitate the secretion of the enzyme into extracellular space, downstream sequences for transcriptional termination and polyadenylation; and selectable marker(s) for screening of transformants (auxotrophic, resistance, or gain of function markers). The enzyme-encoding gene can be incorporated into chromosomes of the filamentous fungi via plasmids. Long-term genetic stability is achieved by tandem repeats (Van Gorcom and van den Hondel, 1988). Many commercial vectors are available for filamentous fungi. For example, pyrithiamine or pPTR vectors (*E. coli-Aspergillus* shuttle) provided by the Clotech company, contain an ampicillin resistance gene (AmpR) and a pyrithiamine resistance gene (ptrA) as the selection marker for *E. coli* and *Aspergillus*, respectively. Pyrithiamine is a pyridine analog and inhibitor of thiamine. MCS in pPTR vectors are located within the lacZ region. The insertion of the enzyme encoding sequence can be easily verified by blue/white screening on bacterial plates containing IPTG and X-Gal (Pyrithiamine Vectors, 2017, Clontech).

43.4.5 Extraction and Purification of Enzymes

Extraction protocols for enzymes are designed on the basis of the site of enzyme production in the microbial cell. For extraction of the intracellular enzyme, cells will be disrupted by mechanical (e.g., sonication) and enzymatic (e.g., lysozyme) methods, followed by centrifugation at high rpm. The obtained supernatant will be used as a sample for purification whereas the extracellular enzyme secreted in the media itself does not require any cell disruption method. The whole culture is centrifuged and the obtained supernatant will be used as a sample for purification (Kanmani et al., 2015). The obtained supernatant is precipitated by the ammonium sulfate precipitation method, followed by purification using the most efficient chromatographic separation techniques such as ion exchange, gel filtration, affinity chromatography, hydrophobic interaction chromatography, etc. The selection of techniques depends on the characteristics and properties of the enzyme to be purified, such as size (gel-filtration chromatography), charge (ion-exchange chromatography), and binding affinity (affinity chromatography) and hydrophobicity (hydrophobic interaction chromatography) (Asenjo and Andrews, 2009; Berg et al., 2002). One can purify the enzyme of interest from a complex mixture of unknown proteins/cell extract (sample) by choosing the most efficient and minimum number of purification techniques to achieve the best level of purity (e.g., 98%, 99.5%, or 99.9%). Different enzymes are being purified using these advanced strategies at the lab as well as at an industrial scale (Celińska et al., 2015; He et al., 2017; Kanmani et al., 2015; Yang et al., 2011).

43.5 HETEROLOGOUS EXPRESSION SYSTEM (HOST) FOR RECOMBINANT ENZYME PRODUCTION

The selection of the right expression system is also a critical decision. The factors that need to be considered for industrial purposes such as protein quality, functionality, production speed, yield, and, most importantly, cost are very much dependent on the selected expression system for recombinant enzyme production. In order to fulfill industrial demand with maximum stability, recombinant enzymes are cloned and expressed in the heterologous expression system/host. The heterologous expression system can broadly be categorized into the prokaryotic and eukaryotic host. The prokaryotic systems include bacteria and the eukaryotic systems include yeasts, filamentous fungi, insects, and mammalian cells. Working with bacteria, yeasts and filamentous fungi are generally easier than insects and mammalian cells, making them favorable for industrial purposes (Liu et al., 2013).

The microorganisms that are being used as the hosts for recombinant enzyme production are nonpathogenic, and reliable, as they have a long history of safe usage and have shown satisfactory results for industrial production. On the other hand, there are many other hosts even though they do not have any historical evidence of usage for food processing but are used as a successful host for the expression of the heterologous enzyme such as *E. coli* K-12, *F. venenatum,* and *Pseudomonas fluorescence* (Olempska-Beer et al., 2006). It has been reported that *E. coli* is the most widely used host for heterologous expression of industrially applicable lipase and phospholipase enzymes. *Basicllus* strains such as *B. licheniformis* and *B. amyloliquefaciens* have been successfully used for recombinant enzyme production. On the other hand, *P. pastoris* also has a significant role in the expression of these enzymes (25)

(Borrelli and Trono, 2015). Many enzymes that have food-based applications such as Laccases (beverage stabilization, uses in baking industry, and roles in the improvement of overall food quality) have also been expressed in the prokaryotic as well as eukaryotic systems. Though laccase naturally presents in bacteria, fungi, and yeast but overproduction with a reduced cost is required for many industries, where recombinant DNA technology plays a promising role for its large scale production. The gene-encoding laccase enzyme has been successfully cloned as well as heterologously expressed in the filamentous fungi *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Fewer examples of a few bacterial laccases have also been studied for large-scale production. The laccase gene has also been cloned and successfully expressed in the bacterial system (*E. coli* BL21(DE3)). The recombinant strain produced a high level of laccase compared to the wild type. Bacterial laccases have been found to be advantageous in terms of activity and stability as compared to fungal laccases (Wu et al., 2010). Direct comparison of different expression platforms—*Y. lipolytica*, *S. cerevisiae*, *P. pastoris*, *Arxula adeninivorans*, *Hansenula polymorpha*, *K. lactis*, and *Schizosaccharomyces* pombe—demonstrated that *Y. lipolytica* is characterized by several advantageous traits for heterologous protein expression in the other expression systems (Gellissen et al., 2005; Müller et al., 1998). In this section, the host that is used for the production of recombinant enzymes has been covered along with associated advantages and limitations of their usage.

43.5.1 *E. coli*

It is one of the most favorable and widely used hosts for the production of heterologous proteins, mainly nonglycosylated proteins. It's easy cultivation, rapid growth, and expression make it a favorable host for the production of enzymes. Moreover, its well-known genome can easily be modified while expression of the fusion protein can be manipulated with promoter control and plasmid copy number alteration (Demain and Vaishnav, 2009; Terpe, 2006). Some limitations are also present, such as high cell density that results in toxicity due to acetate formation, lack of posttranslational modification, refolding, etc. Proteins produced in the form of inclusion bodies are often inactive and insoluble (Rosano and Ceccarelli, 2014). However, many steps have been taken to make *E. coli* an efficient host, such as (i) use of efficient promoters to regulate expression; (ii) use of different host strains; (iii) lowering the growth temperature to maximize the synthesis of correctly folded heterologous proteins as well as to reduce undesired metabolite formation (Gadgil et al., 2005); (iv) coexpression of chaperones to increase the yield of properly folded recombinant proteins, although some negative side effects have been reported (Martínez-Alonso et al., 2010); and (v) secretion of proteins into the periplasmic space or into the medium by fusing a leader peptide at the N-terminus. Some other efforts such as the addition of a fusion partner; the expression of a fragment of the protein, in-vitro denaturation, refolding of the protein, etc., have also been documented, supporting *E. coli* for use as a favorable host for heterologous protein expression (Demain and Vaishnav, 2009).

43.5.2 *Bacillus*

It is an available host system for heterologous expression of enzymes such as proteases and amylases and is widely used in industry. It is also approved as a safe host by the US Food and Drug Administration (USFDA). The genomes of *B. subtilis* and *B. licheniformis* have been

sequenced and do not produce harmful exotoxins or endotoxins. They are also a favorable economical host as they are able to secrete heterologous protein/enzymes (extracellular) into the media. This ability eliminates the requirement of cell disruption methods for extraction of the enzyme from microbial cells, as required for extraction of an intracellular enzyme. This reduces the cost of downstream processing. One major limitation associated with Bacillus is the secretion of extracellular protease, which destroys the desired recombinant hetreologous proteins. However, the development of extracellular protease-deficient strains has resolved this problem to some extent (Demain and Vaishnav, 2009).

43.5.3 Yeast

Recombinant proteins or enzymes that necessitate the requirement of folding, posttranslational modifications, or glycosylation and are not able to express well in bacterial cells are being expressed in the yeast, single-celled eukaryotic fungal organisms. Glycosylation of proteins, disulfide bond formation, proper folding, etc., are the necessary features for an enzyme to become a functionally active enzyme. Apart from the safe history of usage (e.g., Baker's yeast) in the industry, it is also favorable for high yield, productivity, durability, and cost-effective production. The two most common yeast strains are *S. cerevisiae* and the methylotrophic. *P. pastoris* has been used for the production of recombinant proteins at an industrial scale. Nowadays, some nonconventional yeast host strains such as *Y. lipolytica* appear to be an attractive alternative for heterologous protein expression (Celińska et al., 2016). Initial efforts have been made to express *A. oryzae* α-amylase cDNA in a recombinant baker's yeast strain. The benefit of obtaining baking enzyme from the recombinant strain of yeast includes retarded bread firming, increased shelf life, and freshness of the bread. Initial cloning experiments were performed in pUC plasmid and after that, constructed recombinant yeast integrative and episomal plasmid were used to transform baker's yeast (Randez-Gil et al., 1995).

There are numerous examples where the enzyme gene of bacterial origin has been cloned in yeast strains in order to obtain a high yield. For example, thermostable α-amylase *B. amyloliquefaciens* and *B. stearothermophillus* were successfully cloned and expressed in *S. cerevisiae* (Adrio and Demain, 2010).

43.5.4 *Pichia pastoris*

This is the main expression system for heterologous proteins at an industrial scale as the yields obtained are high compared to other eukaryotic systems, including insect and mammalian cell lines. Second, it is also economical and less time consuming than other eukaryotic systems. This cost-effective system has been used for the expression/production of many economically important enzymes for the food industry, such as recombinant amylase (Parashar and Satyanarayana, 2017), phospholipase (Mueller et al., 2009), trypsin, and cold-adapted fish trypsin (CAFT) (Macouzet et al., 2005). Many studies are also continuing on a lab scale for cloning and expression of heterologous enzymes in *Pichia*. In a recent study, *P. pastoris* GS115 was used as a suitable host for expression of cDNA gene encoding a mature peptide of the mono- and diacylglycerol lipase (PcMdl) from *Penicillium cyclopium* PG37. Before this study, the expression/production of Mdl was only limited in a few genera such as *Aspergillus*, *Penicillium*, *Malassezia*, etc. (Tan et al., 2014).

43.5.5 Filamentous Fungi

Filamentous fungi such as *A. niger* and *A. oryzae* have a well-known and long history as a reliable source for enzyme production for food purposes. They are nonpathogenic and nontoxigenic, and are recognized as a safe host for enzyme production by regulatory authorities (USFDA). *A. niger* and *A. oryzae*, along with *A. awamori*, *Chrysosporium lucknowense,* and *A. chrysogenum,* are attractive hosts for recombinant protein production because of their ability to secrete very high yields of bioactive proteins (enzyme) into media (Adrio and Demain, 2010; Archer et al., 2008). It has been reported that it can produce recombinant protein at high levels (4.6 g L^{-1}). Recombinant molds are also one of the main sources of industrial enzyme production. The posttranslational processing is similar to mammalian cells, which is an another advantageous feature of filamentous fungi as a host (Adrio and Demain, 2010; Demain and Vaishnav, 2009; Nevalainen et al., 2005). An aspartic proteinase-encoding gene from *Rhizomucor miehei* has been cloned and expressed in *A. oryzae*. This heterologous enzyme is used in cheese production and has been approved by the USFDA. In the food industry, lipases are commonly used. Fungal lipases from *R. miehei*, *Thermomyces lanuginosus*, and *Fusarium oxysporum* are produced in *A. oryzae*. These recombinant lipases are currently used in the production of baked foods, fruit juices, desirable flavors in cheeses, and other food-based applications such as interesterification of fats and oils (Adrio and Demain, 2003).

43.6 SAFETY AND REGULATORY CONCERNS OF FOOD ENZYMES OR FOOD-PROCESSING ENZYMES

Because many industrial enzymes are used for food purposes, efficient legislative regulations should be very much required for the safety of consumers. A nonprofit European industry association, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), informs customers and other interested parties about the efficacy and safety aspects of enzyme products. As per the updated list (May 2015) of AMFEP, 243 microbial enzymes are manufactured for commercial purposes, out of which 225 are used in food applications. Out of those 225, at least 114 microbial enzymes were produced by recombinant microorganisms (AMFEP list of enzymes, updated, 2015). Because these enzymes are purified from microbial sources, toxic substances might be present in enzyme preparations/isolates. The toxic substances are basically bacterial toxins and mycotoxins, which might cause problems/risks related to the health of consumers. Safety legislation is also very much attentive regarding the allergenic properties of manufactured enzymes, as it is well known that enzymes are potent inhalative sensitizers. Apart from that, numerous uncharacterized extraneous substances/impurities of microbial/biological origin may also be present in the enzyme preparation, which is also a matter of prime concern while evaluating the safety of commercial enzyme products (Spök, 2006). Different countries follows different norms of safety regulation related to fundamental aspects, premarket approvals, parameters for safety regulations, evaluation, etc. The elements of safety evaluation include enzymes, strains, inhalative manufacturing process, safety studies, and exposure (Sewalt et al., 2016). In the United States, the commercialization of any food enzyme requires prior market authorization by the USFDA under the

Food, Drug, and Cosmetic (FDC) Act. Also, if used as food additives, they then need approval by the European Food Safety Authority (EFSA). These regulatory bodies ensure customers regarding the safe usage of enzymes for food purposes.

Food enzymes may either be classified as (i) GRAS (generally recognized as safe), (ii) non-GRAS, defined as food additives, or (iii) substances approved for use in food prior to Sept. 6, 1958, by the USFDA or the US Department of Agriculture. Enzyme preparations are considered as secondary direct food additives under Title 21 of the Code of Federal Regulations, Part 173 (21 CFR 173). Several enzyme preparations that have been approved as food additives are listed in 21 CFR 173. Enzyme preparations affirmed as GRAS are listed in 21 CFR 184 (Enzyme preparations used in food (partial list), USFDA, 2015).

According to the FDC Act, food additives that are non-GRAS need approval prior to marketing. In contrast, GRAS substances do not require approval or notification to the USFDA prior to marketing. GRAS status may be obtained either on the basis of the safe history of enzyme usage in food prior to 1958 or on the grounds of scientific procedures that require evidence related to safe and harmless usage. GRAS status may be either affirmed by the USFDA or determined independently by qualified experts. The regulatory status of substances affirmed as GRAS or food additives is established through a petition process. Though filing a petition and getting final approval takes many years, = without this obligatory approval, enzymes can't be used commercially. GMO-derived food processing enzymes can be introduced in the market through self-affirmation and GRAS notification/food petition. In the petition process for GRAS, the petitioner (company) would ask the USFDA for the affirmation of the product/enzyme as GRAS. As per Section 409(b)(1) of the FDC Act [21 U.S.C. 348(b)(1)], anyone can file a petition to obtain the regulatory status of a substance.

43.6.1 Petition for Enzyme Preparations

A petitioner files a petition for the consideration of a substance as GRAS by the USFDA. The statutory petition requirements for food additives are documented in Section 409(b)(2) of the FDC Act [21 U.S.C. 348(b)(2)]. It requires five basic pieces of information: the identity of the additive, the proposed and intended technical effect, the method of analysis for the additive in food, and full reports of all safety investigations of the prospective additive.

43.6.2 GRAS Notices for Enzyme Preparations

A substance can be considered as GRAS if its safety concern is justified either through scientific procedures (21 CFR 170.30(b)) or the substance has a history or experience of safe usage for food purposes prior to January 1958, (21 CFR 170.30(c)). Qualified experts will govern the evaluation of safety concerns. A voluntary notification program has replaced GRAS affirmation process by a USFDA proposal ((21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)) (available at: Federal Register Notice—the GRAS Proposal or the GPO website; search for 62 FR 18938)) in which a person may notify the USFDA of its determination that a substance is GRAS. In that case, the USFDA will allow a letter to the notifier, if the data and information provided by the notifier in the GRAS notice do not raise any safety concerns regarding the addition and usages of the substance.

An authorized letter also states that the agency has no objection regarding the notifier's conclusion about safety concerns. Thus the substance is safe and considered as GRAS under the intended conditions of use (Guidance for Industry, USFDA, 2010).

43.7 CONCLUSION

The prospects of industrial use of microbial enzymes for food purposes have increased greatly in the 21st century. The majority of food enzymes are applicable as processing aids and play a vital role in many food-related industries. Enzymes from microbial sources can cope with high industrial demand, facilitating extensive uses in the bakery, dairy, beverage, confectionery, fruit/vegetable, and meat-processing industries. With an application of genetic engineering, it is now possible to clone an enzyme-encoding gene and express in a suitable host, which supports the ease of use, high yield, and fewer time and cost constraints. The prokaryotic system (e.g., *Bacillus*) has been used for the expression of small proteins whereas the eukaryotic system (e.g., yeast, filamentous fungi, etc.) is used for enzymes that necessitate the requirement of posttranslation modification for functionality and high yield for industrial application. Advancement in recombinant DNA technology has geared up the process of heterologous protein production at a large scale. The safety concerns and health impacts of food enzymes from microbial origin are a prime focus in every country. The application of enzymes in food processing is monitored and regulated by food laws. Different regulatory bodies such as the USFDA, AMFEP, and EFSA, as discussed in this chapter, share legal enforceable responsibilities and provide guidelines for the safe preparation of food-processing enzymes that ensure consumers regarding safe usage.

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CHAPTER

44

Food Enzymes and Nanotechnology

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44.1 INTRODUCTION

Enzymes act as resourceful tools in diverse areas of science and technology. Enzymes find broad pertinence in the fields of medicine, chemistry, biochemistry, pharmaceutical science, food, and textiles due to their highly catalytic, selective, less toxic, and water-soluble attributes (Altinkayank et al., 2016). Generally, free enzymes particularly actuate reactions under mild experimental environments such as those of room temperature, neutral pH, and atmospheric pressure. The catalytic role of enzymes either extends to the whole of a substrate or to a part of it, say a functional group. In industrial applications, enzymes are favorably dominant over chemical catalysts for substrate specificity, lower toxicity, and the absence of undesirable products by the former (Jia et al., 2014). Irrespective of such exclusively exhibited traits, free enzymes counter issues connected with the degradation of their molecular structure at higher temperatures, acidic or basic pHs, and in presence of organic solvents (Mateo et al., 2007; Zhang et al., 2015). Complicating the issue further, they cannot be separated or retained from the reaction medium for reusability. Immobilization techniques are therefore adopted for countering these issues and finding probable solutions.

Immobilization implies restricting or limiting the movement of something by fixing it. For enzymes, it refers to confining or localizing them physically in a certain region of space, withholding their catalytic activities. However, immobilization confers an enzyme with certain alterations in structure, thereby changing its properties and activity (Netto et al., 2013). The sensitive nature of enzymes mandates the adoption of methods suitable for keeping and improving the enzyme's activity and stability under immobilization. Enzymes present strong affinities with metal ions, and on immobilization on a metal surface, they possess strong stability as well (Datta et al., 2012; Jia et al., 2014). Developments in enzyme immobilization have made possible the use of not only microsized materials, but also nanomaterials such as nanospheres, nanofibers, nanotubes, and nanopores. Support materials, whether microsized or nanosized, should have a large surface area, possess functional groups, and be water-insoluble and biocompatible (Ansari and Husain, 2012). Nanostructured materials possess a high surface area-to-volume ratio compared to conventional supports, providing higher immobilization

Enzymes in Food Biotechnology
https://doi.org/10.1016/B978-0-12-813280-7.00044-X

(Saallah et al., 2016). Most common immobilization is carried out reversibly and irreversibly, the former involving direct adsorption to the surface. Enzyme-surface interactions are promoted by hydrogen bonding, van der Waals forces, and hydrophobic interactions. This type of binding is also accomplished by electrostatic binding, affinity binding, and metal chelation. Irreversible immobilization involves attachment of the enzyme covalently to the surface, or entrapment within a matrix or microencapsulation. Irreversible immobilizations do prevent enzyme leaching during the course of reactions, but the supports binding the enzyme are not reusable and need to be discarded once the enzyme activity decays. Reversible immobilizations, on the other hand, allow the recovery of supports after enzyme inactivation (Ozturk et al., 2007; Sulek et al., 2010). To achieve the optimum immobilized enzyme, a sound understanding of support and enzyme characteristics is needed. The tridimensional conformation of enzymes, which is susceptible to change, forms a concern of importance. Another concern is the ampiphilic nature of proteins, which are hydrophobic in the interior and hydrophilic in the exterior. For such a molecule, the selection of support should consider the nature as well. For instance, when using a hydrophobic support, the fact that enzyme activity may be lost if the enzyme turns inside out should be addressed (Netto et al., 2013).

Recent developments in nanotechnology have furnished diverse options for enzyme immobilization in various nanoscaffolds (Husain, 2010). Nanostructures furnish better alternatives to conventional matrices by countering the low mass transfer rate, high diffusivity, and small surface area. The integration of enzymes as biocatalysts with nanomaterials produces nanobiocatalysts, which are a preferred option over chemical catalysts, considering the impacts of green technology (Agustian et al., 2016; Kim et al., 2008; Meyer et al., 2013). Such immobilizations have been reported in nanosized spheres, fibers, tubes, and similar structures (Martin and Kohli, 2003; Yim et al., 2003). Varying processes of immobilization, each possessing distinguished characteristics such as self-assembled monolayers, polymeric matrices, mesoporous materials, magnetic and nonmagnetic nanoparticles, gold nanoparticles, and thermally evaporated fatty lipid films have been developed (Bhat et al., 2003; Hung et al., 2006; Kim et al., 2005a; Sastry et al., 2002). The following sections discuss various methods of immobilizing enzymes at the nanoscale level and their applications.

44.2 IMMOBILIZATION SYSTEMS

44.2.1 Magnetic Nanoparticles

Nanoparticles are discrete entities possessing three dimensions of the order of 100 nm or less, and exhibiting properties exceptionally different from bulk materials. Magnetic nanoparticles consist of elements such as cobalt, iron, nickel, and their chemical compounds. However, for food applications, an important concern centering on them is their safety and toxicity. Therefore, in food systems, most commonly utilized nanoparticles include iron oxides, such as that of superparamagnetic Fe_3O_4 nanoparticles, owing to good biocompatibility and a lack of toxicity (Cao et al., 2012). Owing to typical sizes of 10–20 nm, they show better performance than bulk magnetic materials. This is primarily due to the emergence of paramagnetism, characterized by a large magnetic moment associated with the particles, making

them behave as giant paramagnetic atoms (Lu et al., 2007). They are well characterized by a large surface area and high mass transfer rate, making them suitable candidates for catalysis. Contrary to the magnetic materials that align their net magnetic domains as a response to an applied field, nanoparticles rotate their single vector to overcome anisotropy and consequent loss of paramagnetism (Frey et al., 2009). Magnetic nanoparticles are advantageous in that they can be recovered by applying a magnetic field (Netto et al., 2013). Because surface energies have an important role to play, surface interactions between magnetic particles and proteins need to be analyzed. The capacity for the proteins to be adsorbed depends on the activation of chemical bonds on both the particle and protein surface. Nanoporous materials as support show less enzyme loading but no diffusional limitations whereas porous materials suffer from diffusion of substrates; however, they do show high enzyme loading. Magnetic materials such as magnetite, maghemite, and some ferrites have been implicated for use in enzyme immobilization. Commonly employed methods for enzyme immobilization involve covalent bonding and physical adsorption. In covalent bonding, covalent bonds are created between chemical groups on the particles and chemical groups of enzymes. It does achieve strong enzyme binding preventing loss, but reduced enzyme activity. Physical adsorption is brought about by hydrogen bonding, the van der Waals force, and/or ionic bonding between nanoparticles and enzymes. However, with this method, enzyme retention on the surface is not maintained under changes in pH, ionic strength, or temperature (Zhu et al., 2009). Modification of magnetic nanoparticles with organic (e.g., lauric acid) and inorganic (e.g., SiO_2) materials is often carried out to attain desirable functionality. Coupling agents (e.g., glutaraldehyde) are also used for covalent linking of magnetic nanoparticles and enzymes, as they interact with functional groups of both. Such an enzyme is superior to that adsorbed without coupling agents (Mateo et al., 2006).

Various enzymes have been immobilized on different nanosystems. α-amylase, for instance, has been immobilized on carboxylated magnetic nanoparticles, aminated magnetic nanoparticles, cellulose-coated magnetic nanoparticles, and many more (Khan et al., 2012). Lipase immobilized on magnetic Fe_3O_4-chitosan was employed to synthesize ascorbyl palmitate using ascorbic acid and palmitic acid. After a 12 h reaction, conversion of 52% was achieved and the enzyme demonstrated enhanced reusability, easy recovery, and improved thermal stability. These considerations render immobilized lipase of potential use in industrial applications (Wang et al., 2015). Another system involving polydopamine-coated magnetic-chitin increased the optimum temperature and pH for starch hydrolysis as a result of multiple interactions between enzyme and surface. Dopamine self-polymerized on the surface of magnetic-chitin to immobilize the biomolecules (Sureshkumar and Lee, 2011). Lipase embedded on magnetic nanoparticles covered with chitosan lost only 12% of its activity after five batches of operation (Wu et al., 2009). Covalently attached lipase onto superparamagnetic Fe_3O_4 nanoparticles gave 90% of soybean transesterification and retained 70% of its initial activity even after four cycles (Xie and Ma, 2010). Immobilized pectinase on chitosan magnetic nanoparticles retained residual activity of 85% after seven successive cycles and could successfully be utilized for apple juice clarification. The juice clarification as estimated by turbidity reduction was 74% for the nanoparticle-stabilized enzyme against 70% for the free enzyme (Sojitra et al., 2017). Fungal α-amylase immobilized on magnetic nanoparticles was used as a biocatalyst

for the production of glucose from sweet potato starch. From the reaction kinetics, it was evident that the sweet potato starch had more affinity toward immobilized α-amylase. Because the enzyme could be easily recovered, it reduces the cost of production of starch hydrolysates (Baskar et al., 2015).

Pectinase, which finds wide usage in juice clarification, was immobilized in a multilayer assembly of polyelectrolyte/Fe_3O_4. A high surface area enabled retention of 80.2% of the free enzyme activity. Immobilized pectinase activity after 25 batch reactions was 73% against 30% for the free enzyme (Lei et al., 2007). Magnetiite nanoparticles obtained by solvothermal treatment of $FeCl_3$ were used to immobilize trypsin by covalent immobilization. Prior to immobilization, the nanoparticles were modified by tannic acid, as it is known to be cytocompatible. Casein digestion was carried out by both bound and free enzymes, for activity comparison. Efficient digestion of casein was observed with immobilized trypsin over the free enzyme that suffered autohydrolysis (Atacan et al., 2017). Silica-encapsulated magnetic nanoparticles stabilized glucose oxidase, which finds wide applications in food, beverages, textiles, and biotechnology. Immobilized enzyme retained 98% of its initial activity after 45 days and 90% of activity after 12 cycles of use. At elevated temperatures, the nanobiocatalyst was less labile and less sensitive to changes in pH (Ashtari et al., 2012).

Invertase is an enzyme-catalyzing hydrolysis of sucrose into glucose and fructose; these are, in turn, employed in food industries. It was covalently immobilized on silica-modified and polymer-grafted magnetic nanoparticles. Interactions between the polymer and enzyme bring about its conformational flexibility. The stability of the enzyme also increases due to the high density of the enzyme molecules on the polymer surface. This enzyme was characterized by an increased optimum temperature of activity, wide pH range, improved thermal stability and shelf life, and a meager loss of activity. This enzyme can be used, therefore, in continuous systems for enzymatic hydrolysis of sucrose (Bayramoglu et al., 2017). Nano zinc oxide was employed as a support for starch hydrolyzing enzyme diastase α-amylase. The enzyme adsorbed by electrostatic interaction with the functional groups on the support material. It possessed better heat resistance than the free enzyme and retained about 70% activity after even 30 days of storage (Antony et al., 2016).

Multipoint-immobilization involving enzyme stabilization via multiple covalent bonds with nanoparticles is also finding increased interest. Multipoint immobilization of chitosanase on amylose-coated magnetic nanoparticels was carried out by Kuroiwa et al. (2008). Multipoint covalent bonding gave 1.4–2.0 times more chitosanase immobilization over conventional adsorption. The enzyme stabilized this way was reusable, stable, and possessed good mass transfer properties (Cao et al., 2012). Magnetic nanoparticles are often coated with inert materials to prevent their aggregation, owing to dipole-dipole attractions and large surface area-to-volume ratio (Xie and Zang, 2016). Hydroxyapatite is one such inorganic material that acts as a composite support for magnetic nanoparticles, and the heterogenous catalyst thus formed synergizes the advantages of both materials. Hydroxyapatite-encapsulated γ-Fe_2O_3 forms an excellent magnetic support for constructing high-performance biocatalysts for many organic transformations (Sheykhan et al., 2011; Zhang et al., 2009). Hydroxyapatite nanoparticles were also seen to impart thermostability along with enhancing the activity of extracellularly isolated pectate lyase, which had an otherwise low native state activity. This enzyme was isolated from an attenuated strain of *Macrophomina phaseolina* that was functionally impaired, and was characterized by a feeble activity at 50°C and pH 5.6. The addition of

the nanoparticles increased activity by 27 times and half-life by 51 times at 90°C. Therefore, by imparting thermostability and restoration of activity, these nanoparticles could act as chaperones for enzymes intrinsically deficient in activity and stability (Dutta et al., 2013). Lipase immobilized onto aminopropyl-functionalized hydroxyapatite-encapsulated-γ-Fe_2O_3 nanoparticles was applied for carrying out interesterification of soybean oil with methyl stearate, to produce trans-free interesterified fat with desirable qualities. Immobilized lipase could effectively bind the stearoyl group to soybean tri-acylglycerols (TAG). Interesterification of soybean oil was also carried out with palm stearin, the former being composed mainly of unsaturated fatty acids and later having a significant content of palmitic acid. This blending produces structured lipids with a balanced combination of both saturated and unsaturated fatty acids. Moreover, interesterified products do not contain any trans fatty acid, as found with partially hydrogenated vegetable oils. The final products obtained exhibited lower slip melting points (SMPs) owing to the altered TAG composition. Such fats can be directly used as shortenings as they readily melt in the mouth without producing any waxy sensation (Xie and Zang, 2017). Similarly, Singh et al. (2016) immobilized α-amylase on iron oxide magnetic nanoparticles coated with gold and silica. This prevents irreversible aggregation and uncontrolled oxidation of the enzyme. In addition, the immobilized enzyme presented enhanced optimum temperature for activity as well as ease of recovery. The enzyme activity could also be maintained by 60% after 10 cycles of operation.

With an aim to serve multipurpose biocatalysis, an enzyme preparation rich in pectinase, xylanase, and cellulose was nanoimmobilized on iron oxide nanoparticles. This furnished a nanobiocatalyst that possessed both cellulose and hemicellulase activity, and retained 87% of pectinase, 69% of xylanase, and 58% of cellulase activity in the insoluble form. The enzyme conjugated to nanoparticles was stable when exposed to higher temperatures, and was reusable up to the fifth cycle, beyond which a slight decrease in activity came into view. High pectinase activity was reported even in organic solvents and chemical reagents compared to free enzymes. This enzyme system was found more effective for the clarification of pineapple and orange juice along with the production of bioethanol from wheat straw, than the free enzyme (Perwez et al., 2017). Similarly, Sojitra et al. (2016) reported a tri-enzyme magnetic nanobiocatalyst for fruit juice clarification. Simultaneous immobilization of α-amylase, pectinase, and cellulase was carried on amino-functionalized magnetic nanoparticles. The so-formed tri-enzyme catalyst showed more than a two-fold increase in half-life and a better tolerance toward low pH. A residual activity of 75% was retained after eight consecutive cycles of operation, and a reduction of turbidity of 41%, 46%, and 53% was attained with apple, grape, and pineapple juice. Multienzymatic systems are advantageous for their short cycle duration and enhanced production, which translate into economic viability (Pinelo et al., 2010).

For attaining nanoparticles with surface functionalities, surface modifying agents such as gold nanoparticles have been used as coating materials, providing biocompatibility and facile conditions for enzyme binding. Coupling gold with magnetic nanoparticles is advantageous over gold alone as it faces problems with recovery and isolation. Such a gold nanocomposite was used for the immobilization of papain. It is a protease widely used in food applications such as protein structural studies, peptide mapping, solubilization of membrane proteins, and production of glycoproteins from proteoglycans. Gold nanoparticles were chemisorbed on thiol-coated magnetic nanoparticles for the formation of magnetic gold nanocomposites. Papain is immobilized on magnetic gold nanoparticles through ionic or electrostatic

interaction. Immobilized papain retained catalytic activity over a wider pH range between 5 and 11, and its optimum activity was increased to 75°C compared to native enzyme with activity at 55°C. The shifting of activity for bound papain is attributed to the multipoint ionic interaction between the support and enzyme. Immobilized papain also exhibits higher activity over a broader temperature profile, due to conformational change brought about by its rigid binding on nanocomposite material. Moreover, the enzyme-hybrid nanocomposite retains 70% of its initial catalytic activity after five successful cycles of reuse, against a free enzyme that can be used only once (Sahoo et al., 2013). Maltogenase was immobilized on polyurethane (PU)-gold and silver nanoparticles. PU-silver NPs and PU-gold NPs were manufactured by the adsorption of metal nanoparticles on PU or by their synthesis within the bulk of PU. The immobilized enzyme was stable even after 25 days of storage and retained about 98.6% of its initial activity. PU modified with silver nanoparticles conferred more stability to the enzyme than PU alone (Kochane et al., 2017).

44.2.2 Nonmagnetic Nanoparticles

Silver nanoparticles coated with phytochemicals were employed as novel green supports for glucoamylase immobilization. The aqueous extract of *Fagonia indica* was used as the reducing and capping agent to produce silver nanoparticles from silver ions, the capping action attributed to the presence of flavonoids and polyphenolic compounds in the extract. Glucoamylase was bound by the establishment of ester linkages between its carboxyl groups and hydroxyl groups on the nanoparticle surface, aided by a coupling reaction. The immobilized enzyme was characterized by high pH and thermal stability compared to the free enzyme (Syed et al., 2016).

44.2.3 Organic-Inorganic Hybrid Nanoflowers

Nanoscale materials constructed for enzyme immobilization confer enhanced stability to free enzyme forms, but reduce their activities. It mainly occurs as a result of two-step immobilization involved with these materials. It is therefore necessary to develop a single-step immobilization for obtaining enzyme-embedded nanomaterials with enhanced catalytic activity (Ocsoy et al., 2015). Constructing enzymes into flower-shaped nanostructures enhances their activity and stability against environmental factors. In this regard, the production of hybrid nanoflowers from enzymes has been reported, which find use in various applications. In these novel structures, enzymes and metal ions act as organic and inorganic components, respectively, to constitute the hybrid nanoflowers. These are primarily manufactured through three steps involving nucleation, growth, and completion. The formation of nanoflowers could be explained, for instance, with the following example. In the initial stage, the copper phosphate primary crystals are formed from the reaction between copper ions and phosphate groups, followed by nucleation by binding to amine groups on protein (enzyme) backbone. Protein-primary crystals formed due to nucleation act as seed particles for the second stage and grow into separate nanopetals. In the final stage, the protein acts as a template for seed particles and nanopetals, and glues the nanopetals together for formation of large protein agglomerates. Metalloenzyme-incorporated hybrid nanoflowers (hNFs) such as with laccase, α-lactalbumin, and lipase have demonstrated higher catalytic activities and stabilities compared

to conventionally bound enzymes. The causes underlying the increased activity have been identified as (1) alleviation of mass-transfer limitations due to the high surface area of hNFs, (2) cooperative effects of nanoentrapped enzymes, and (3) favorable enzyme conformations within hNFs (Altinkayank et al., 2016). Employing this method, Wang et al. (2013) immobilized lipase in hNFs, which, after immobilization, effectively catalyzed the transformation of (*R*,*S*)-2-pentanol to (*S*)-2-pentanol. The selection of the enzyme and metal ions is essential to obtain enhanced catalytic activity. The enzymes acquire increased activity as a result of effector molecules binding to their active sites, favoring conformational changes in them. With this principle, $CaHPO_4$-α-amylase hybrid nanostructures were obtained that exhibited enhanced activities. Ca^{2+} ions acted as both entities for forming hybrid nanostructures and as effectors binding to inactive allosteric sites in α-amylase and thus generating active sites. Nanoflowers owing to many dispersive petals demonstrate higher activity than other nanostructures such as hexahedrons (Wang et al., 2013). These hNFs have found usage in proteomic analysis as well. α-chymotrypsin was immobilized in hNFs and was used for protein digestion. In the study, bovine serum albumin (BSA) and human serum albumin (HSA) were used as model proteins. Nanoflower enzymes exhibited higher proteolytic activity of 48% and 34% for BSA and HSA than a free enzyme (Yin et al., 2015). Similar immobilization has been carried out with α-acetolactate decarboxylase (ALDC) that prevents diacetyl formation during beer fermentation. This compound is responsible for an unpleasant buttery taste in beer at even smaller thresholds. α-acetolactate decarboxylase directly converts α-acetolactate into acetoin, which is flavorless, and consequently shortens the beer manufacturing time. However, the application of the free enzyme is restricted, owing to a lack of long-term stability, difficult recovery, and recycling. This problem is again countered by its immobilization in hybrid nanoflowers. ALDC is first constructed into enzyme-inorganic hybrid nanoflowers ($Ca_3(PO_4)_2$-ALDC) by coprecipitation onto $Ca_3(PO_4)_2$, followed by entrapping in alginate gel beads. The activity of the nanoflower enzyme increased with increasing concentration of ALDC. Increased activity of nanoflowers is attributed to the allosteric effect and morphology. The immobilized enzyme retained 98% of enzyme activity and was relatively stable and recyclable when entrapped in alginate. Diacetyl levels in conventionally aged beer were higher and took 15 days to drop to lower levels, compared to that subjected to immobilized ALDC in alginate microbeads, which took only 7 days for the diacetyl level to drop to a low value (Zhao et al., 2017). Similarly, glucoamylase nanoflowers were assembled by mixing an aqueous solution of copper sulphate with PBS containing glucoamylase. Glucoamylase activity was assessed by maltodextrin hydrolysis. An enhanced activity recovery of 204% and a two-fold improvement of thermal stability were observed with hybrid nanoflowers containing enzyme over its free form. After even eight successive cycles, the residual activity was measured to be 70%, and after 25 days of storage, the residual activity could be retained at 91% (Nadar et al., 2016).

44.2.4 Nanofibers

Nanomaterials such as fibers have gained increased importance owing to the unique properties they exhibit. Among them, electrospun nanofibers are preferable and simple as they are long, have a uniform diameter, and could be diversified in composition, thus enabling their use in biocatalysts (Kim et al., 2006). Nanofibers manufactured by electrospinning show a high surface area, higher porosity, and interconnectivity, which enable their usage across

a wide range of applications from drug delivery to water filtration systems (Huang et al., 2016; Sundarrajan et al., 2014). Nanofiber-enzyme composites exhibit improved activity over native enzymes by three orders of magnitude (Hwang and Gu, 2013; Kim et al., 2005a, b). For usage in biocatalysis, electrospun nanofibers act as supports for enzyme immobilization, and the enzyme could be immobilized by various techniques. One such method involves layer-by-layer (LBL) deposition of materials providing strong noncovalent integration and proper assemblage of oppositely charged molecules, giving rise to ordered nanoscale structures possessing remarkable functionalities (Xiang et al., 2012). Along this pattern, Huang et al. (2017) immobilized naringinase applying a LBL self-assembly comprised by alternately coating positively charged naringinase and negatively charged alginate on negatively charged cellulose acetate nanofibers. Naringinase is an enzyme widely applied for removing naringin, a bitter component of fresh grapefruit juice, and rendering it sweet. Immobilized naringinase tolerates a lower pH and a higher temperature; it is also more stable than the free enzyme (Busto et al., 2007). Immobilized naringinase exhibited an increase in activity with increasing multilayers, which implied more enzyme loading resulted in more catalytic efficiency. This enzyme was implicated for debittering grapefruit juice, and it was evident that the major components responsible for bitterness were hydrolyzed into components with considerably less bitterness (Huang et al., 2017).

44.2.5 Nanosheets

With an increase in the application of carbon family materials, various ways of producing functional nanomaterials from them have come up. Among these materials, graphene is used in enzyme immobilization owing to its explicit electronic, optical, thermal, and mechanical properties. It is composed of a dense layer of carbon atoms intertwined in a two-dimensional dense structure somewhat forming a honeycomb lattice (Novoselov, 2004). Besides finding applications in drug/gene delivery, graphene forms an ideal matrix for immobilization in that it retains the native state for enzyme activity. Functionalized graphene synthesized by thermal exfoliation of graphite oxide was used to immobilize α-galactosidase obtained from the white chickpea (*Cicer arietinum*). Graphite oxide was oxidized and then heated rapidly under an Ar atmosphere, followed by tube heating it further to 1050°C and then cooling to room temperature. Enzyme immobilization was carried out by using cysteamine and glutaraldehyde as the cross-linking agent. Glutaraldehyde containing the –CHO group binds to the $–NH_2$ group of cysteamine while the other –CHO group is attached to the enzyme through the amino group on the lysine residue. The immobilized enzyme exhibited higher thermal stability than the soluble enzyme. It also retained 60% of its activity after 10 successive runs, and was relatively stable (Singh et al., 2014). Laccase, which finds wide applications in the production of ethanol and the clarification of wine and tea, was obtained from *Aspergillus oryzae,* and subsequently bound on grapheme nanosheets by covalent bonding and physical adsorption. The immobilized enzyme was characterized by an increased pH range of activity and operating temperature. Contrary to physically adsorbed enzyme, which lost its activity after the second reaction cycle, the covalently bound enzyme could retain about 80% of its activity after six cycles of operation (Skoronski et al., 2017). α-galactosidase immobilized on graphene nanosheets was applied for the hydrolysis of raffinose family oligosaccharides (RFOs) present in soybeans; these cause flatulence when accumulated in the lower intestine.

The immobilized enzyme showed 75% hydrolysis compared to the soluble enzyme (81%), owing to diffusional limitation. However, it retained its stability and reusability, a feature not shown by the soluble enzyme (Singh et al., 2014).

44.2.6 Nanotubes

Among nanomaterials for enzyme immobilization, carbon nanotubes (CNTs) have gained increased attention due to their highly specific surface and their remarkable structural, mechanical, electrical, and chemical properties. Two main types of CNTs—single walled and multiwalled—have been used (Homaei and Samari, 2017). Enzyme immobilization on CNTs has recently emerged as a technique to enhance enzyme activity as well as stability, especially when subjected to denaturing environments. They provide a high surface area, higher enzyme loading, and lower mass transfer resistance (Saifuddin et al., 2013; Silva et al., 2014). Enzyme binding to nanotubes could be carried out by both covalent and noncovalent methods, among which the noncovalent method preserves the native enzyme conformation (Calvaresi and Zerbetto, 2013; Gao and Kyratzis, 2008). Mukhopadhyay et al. (2015a) obtained pectate lyase from a psychrophile and supplemented with calcium hydroxyapatite nanoparticles (NP-PL), which act as a calcium substitute for cationic activation. Further entrapment of the enzyme was done in single-walled nanotubes (SWNT). Thus immobilized, pectate lyase presented enhanced activity and stability at both 4°C and 80°C. The activity retention of this enzyme was observed even after repeated freeze-thaw cycles. Because these SWNT show a distinctive ability to activate and stabilize cold active enzymes at temperatures beyond their optimum, they form prospective candidates for processes involving low temperature bioconversions running parallel to those at high temperature. Similar immobilization was carried out for imparting psychrostability and enhanced activity to a psychrophilic laccase. However, copper-oxide nanoparticles were used as the cationic activator before entrapment in SWNT (Mukhopadhyay et al., 2015b). Similarly, glucoamylase immobilized on magnetic CNTs showed improved optimum temperature (55–60°C) against the free enzyme (55°C) (Zhao et al., 2011). Immobilized α-amylase has also exhibited increased substrate affinity of about 2.2 times more than the free enzyme, and the specific activity of 85% was maintained after 10 reuses (Uygun et al., 2012). Laccase immobilized on multiwalled carbon nanotubes (MWCNTs) maintained its catalytic performance up to nine cycles of operation and even was stable for a longer period of time over the free laccase, which is less stable (Tavares et al., 2015). Lipase from *Pseudomonas fluorescens*, covalently immobilized on MWCNTs, exhibited a 10-fold increase in activity, increased thermal stability to 80°C, and provided considerable reusability (eight cycles). This biocatalyst could be used for the efficient kinetic resolution of racemic compounds (Dwivedee et al., 2017). MWCNTs are composed of several graphite layers surrounding a central tube, and are characterized by easier dispersability and low cost. Papain noncovalently immobilized on MWCNTs exhibited improved thermal, pH, and recycling stability. Immobilization on MWCNTs did not hinder mass transfer and was able to protect the active conformation of the enzyme from getting damaged. These MWCNT-enzyme complexes also show stability under harsh conditions and thus form attractive choices in biocatalysis (Homaei and Samari, 2017).

Lipase obtained from *Candida rugosa* was immobilized on oxidized MWCNTs by employing activating agents for the promotion of covalent bonding of the enzyme. It was also

immobilized by adsorption onto the MWCNTs, by which the attached amount was two times less than that attained by covalent bonding. The lipolytic activity of the enzyme under conditions of covalent bonding was three times higher than with adsorption. Oxidation of MWCNT surface is seen to bring a slight decrease in enzyme loading. However, subsequent activation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) not only promoted covalent bonding, but increased the loading capacity significantly in addition to improving the enzyme activity. EDC acts as a coupling agent and NHS stabilizes and enhances coupling efficiency (Hazani et al., 2003; Prlainovic et al., 2016).

44.3 APPLICATION OF NANOIMMOBILIZED ENZYMES AS BIOSENSORS FOR FOOD SAFETY

Biosensing technology is influencing all sectors such as pharmaceuticals, the environment, healthcare, and food. In food, where safety is an unavoidable concern, the monitoring of nutrients and contaminants is a key issue calling for simple, rapid, and accurate analysis (Rotariu et al., 2016). Biosensors as analytical devices integrate biocomponents and bioreceptors such as enzymes, tissues, nucleic acids, and aptamers with a suitable transducing system such as electrochemical, optical, or thermal for the detection of chemical compounds. An electrical signal is generated on the interaction of target molecule and the biocomponent, which could be measured and then recorded. The incontestable advantages over conventional detection such as selectivity, fast analysis, low cost, portability, and direct detection independent of sample preparation make these biosensors the choices of preference. Biosensors also meet the specific needs for food analysis such as fast analysis and field-testing for varying parameters. In food, the analysis mainly centers on testing the compound of nutritional interest, contaminants, origin of the food itself, counterfeiting, and adulteration (Turner et al., 1987). Enzymatic biosensors represent the class of electrochemical biosensors used for food analysis. Substrates are detected either from their conversion products obtained by enzymatic catalysis or by the detection of enzyme inhibitors. Enzymes mainly employed for substrate detection include oxidases, peroxidases, and dehydrogenases while the compounds of detection include hydrogen peroxide and nicotinamide adenine dinucleotide in a reduced form (NADH). Oxidases have been used for developing enzyme biosensors that can determine the presence of biogenic amines in food formed by microbial decarboxylation of amino acids (Kivirand and Rinken, 2011). Similarly, L-lactic acid detection in tomatoes and infant food as indicators of freshness is done by biosensors based on L-lactate oxidase (Rassaei et al., 2013). L-lysine detection is carried out using lysine oxidase forming an important tool in food quality control (Narang et al., 2015). Table 44.1 enlists other similar applications of enzyme biosensors.

In recent years, functional nanomaterials have been used in biosensing for improving responses of the transducer and for immobilizing bioreceptors. In this context, immobilization nanomatrices discussed in the preceding sections have proven to be favorable candidates. For instance, MWCNTs and paraffin were used to construct a paste electrode for the immobilization of laccase, which could detect 4-aminophenol used as an enzyme substrate (Oliveira et al., 2014). For the detection of organophosphate neurotoxins, a biosensor based on cationic and anionic layers of CNTs modified with biopolymers was designed, immobilizing organophosphorous hydrolase. The designed biosensor had a high sensitivity and a stable

TABLE 44.1 Applications of Enzyme Biosensors

Enzyme	Mode of Action	Application	Reference
Laccase	Oxidation of substrate	Detection of monophenols, chloro- and alkyl-phenols or aromatic amines	Kim et al., 2016[a]; Li et al., 2014[b]
Peroxidase	Oxidation of substrate	Detection of phenols, chloro-phenols, alkyl-phenols	Chekin et al., 2015[c]; Fernandez et al., 2013[d]
Tyrosinase	Oxidation of substrate	Detection of tyramine, bisphenol-A	Apetrei and Apetrei, 2015[e]; Kochana et al., 2015[f]
Nitrite reductase	Direct electron transfer	Determination of nitrite	Nikolelis et al., 2013[g]
β-Lactamase	Resistance of bacteria to antibiotics	Determination of penicillin G	Prado et al., 2015[h]
Acetylcholine esterase Butyrylcholine esterase	Enzyme inhibition	Detection of organophosphorous and carbamates pesticides, aflatoxin	Puiu et al., 2012[i]
Peroxidase	Enzyme inhibition	Detection of cyanide	Attar et al., 2015[j]

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electrochemical response (Kirsch et al., 2012). Similarly, MWCNTs immobilizing acetylcholine esterase (AChE) formed a biosensor for organophosphate pesticides, based on the detection of thiocholine (Zamfir et al., 2011). Xanthine oxidase immobilized on a composite material comprised by MWCNT and a copolymer acted as a biosensor for xanthine detection in fish. MWCNTs conferred increased sensitivity to the biosensor over the copolymer alone as the immobilization matrix (Dervisevic et al., 2015a, b). Glutamate dehydrogenase immobilized in MWCNT formed a glutamate biosensor that could detect as low as 3 μM of monosodium glutamate in food (Hughes et al., 2015).

Pesticide detection in food products also forms a prime concern in food safety. In this context, a bi-enzymatic biosensor reported by Oliveira et al. (2014) was used for the detection of carbamate pesticides in citrus fruits. The biosensor was constructed by electrodeposition of laccase and tyrosinase in gold nanoparticle-chitosan composite film on a grapheme-doped

electrode. Gold nanoparticles (AuNPs) immobilizing AChE could effectively detect carbamates such as carbofuran, methomyl, and carbaryl in fruit and vegetable samples with a limit between 2 and 236 nM (Kestwal et al., 2015). Attar et al. (2015) designed a biosensor for the detection of cyanide based on the inhibition of horseradish peroxidase. Immobilization on AuNPs relatively improved the analytical performance of detection with a limit of detection as low as 0.03 μM. Thus immobilized enzymes as biosensors find applications in ensuring food safety, exhibiting advantages over conventional analytical tools.

44.4 CONCLUSIONS

Recent interest in nanomaterials and their wide applicability have made possible their usage as matrices for enzyme immobilization, primarily to counter the loss of enzyme activity and to maintain their reusability and recyclability. Nanoscaffolds such as nanoparticles, nanotubes, nanosheets, and nanoflowers have emerged as efficient immobilization matrices for enzymes, enabling their applications as biocatalysts to be preferred over chemical catalysts. Such enzymatic biocatalysts are also used as sensors in ensuring food safety and quality. For instance, they can be used in the detection of pesticides and their residue in food, biogenic amine formation in food, the presence of xanthine for identification of fish freshness, and so on. In the manufacture of these biosensors as well, nanoimmobilization has served as a tool enabling sensitive detection of such substrates.

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CHAPTER

45

Application of Nanobiocatalysts on Food Waste

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45.1 INTRODUCTION

Waste materials obtained from raw food sources have long been considered valuable ingredients for the production of biofuels (Ravindran and Jaiswal, 2016; Zhang et al., 2016). A variety of methods have been employed for the degradation or conversion of food waste into materials that can be exploited for energy generation (Pham et al., 2015; Yin et al., 2016). In recent years, in addition to bioenergy production, the value of food waste is being reassessed based on the presence of bioactive compounds (Sanford et al., 2016; Vodnar et al., 2017). The growing acceptance of natural bioactive substances as neutraceuticals, medicines, and cosmetics has fueled greater research into the extraction methods of such substances (Asif et al., 2016; Secmeler and Ustendag, 2017). Conventional methods for obtaining bioactive compounds from natural sources involve thermomechanical treatment followed by extraction with organic solvents (Ajila et al., 2007; Stalikas, 2007; Wrolstad et al., 2004). In addition to being energy-intensive, such processes are not environmentally friendly. Considering that the extraction of natural products involves the disruption of cell membranes and walls, a plethora of techniques have been explored as greener alternatives to conventional methods. Notable processes include pulsed electric field, ultrasonics, use of supercritical CO_2, and biological catalyst facilitated extraction (Cai et al., 2016; Diaz-Reinoso et al., 2006; Kammerer et al., 2005; Putnik et al., 2017). In particular, the enzyme-assisted extraction of bioactive substances from natural sources has been the focus of intense research owing to several lateral advantages offered by the process (Chen et al., 2010; Moore et al., 2006). Enzymes that catalyze the degradation of cell wall polysaccharides not only facilitate release of the entrapped pigments and natural products, but also produce fermentable sugars that are useful for biofuel production. The application of enzymes for the extraction of bioactive compounds is different in scope

https://doi.org/10.1016/B978-0-12-813280-7.00045-1

compared to the wider usage of enzymes as additives and biotransformation agents in the food industry. Enzymes have been used for the extraction of natural products from a wide variety of sources that include the skin or peel of fruits and vegetables that are otherwise consigned to waste. The use of free enzymes is fraught with well-known problems such as loss of stability and challenges in separation from reactions. The immobilization of enzymes on nanoparticles has sought to address these concerns, in addition to enhancing the efficiency of the biological catalysts. In this chapter, we review various facets of the use of nanobiocatalysts (NBCs) for the production of biofuel precursors and the extraction of bioactive compounds from food waste.

45.2 ENZYMES USED FOR EXTRACTION OF NATURAL PRODUCTS

The breakdown of plant cell walls for facilitating the release of natural products that may be trapped within is best affected by the use of hydrolytic enzymes that target membrane polysaccharides. The rigid cell walls of fruits and vegetables contribute to their shape and are primarily constituted of pectin, cellulose, and hemicellulose. The use of enzymes for the extraction of natural products from fruits and vegetables has been used from two different perspectives: (1) hydrolysis of pectin and cellulose, facilitating the release of juices and other trapped molecules and (2) altering the viscosity of extracts by disrupting specific polysaccharides present therein. The use of cellulolytic enzymes for releasing sugars from cellulose has been investigated for a long time (Kitts and Underkofler, 1954). Cellulases refer to an entire set of enzymes that are separately capable of degrading cellulose to different extents. The principle components of cellulases are *endo*-glucanase, *exo*-glucanase, and β-glucosidases (Bhat, 2000; Bhat and Bhat, 1997). These are mostly produced in combination by microbes and fungi and exhibit synergistic action. In fact, attributing specific chemical transformations of cellulose to these enzymes is overly simplistic and problematic (Wood and McCrae, 1979). Hemi-cellulases are another class of cellulolytic enzymes that have been widely used in conjunction with cellulases (Wong and Saddler, 1993). Hemicellulases mainly comprise endo- and exo-xylanases, galactanases, and xyloglucanases. In contrast to endo- and exo-glucanases that result in the breakdown of cellulose chain length, β-glucosidase hydrolyzes cellobiose, which otherwise blocks the action of the glucanases (Wood and McCrae, 1979). This function of β-glucosidase has also been used to assist in the cellulosic production of ethanol via conversion of cellobiose into glucose.

A combination of cellulases, hemicellulases, and pectinases has been explored at length as macerating enzymes for the extraction of juices from fruits. The contribution of pectinases toward the breakdown of pectin in the cell walls of fruits is an important contributor to the performance of macerating enzymes (Grassin and Fauquembergue, 1996). Macerating enzymes have been shown to improve the yield and characteristics of juice, nectar, or puree extracts from fruits (Galante et al., 1998; Grassin and Fauquembergue, 1996). These effects are attributed to the facilitation of improved cell wall hydrolysis of the fruit pulp mash with consequent lowering of viscosity and infusion of flavor in the extracts. Various combinations of cellulases, hemicellulases, and pectinases have been used for specific extraction profiles. For example, the use of polygalacturonase with low cellulase results in the production of high viscosity purees from fruits (Uhlig, 1998). In contrast, the use of polygalacturonase

with pectin transeliminase and hemicellulase leads to significant fruit juice clarification (Grassin and Fauquembergue, 1996). Enzymatic treatment of fruits and vegetables affects the pigments and small natural products that are trapped in the peel or flesh therein. For example, the pigments present in grapes and berries can be stabilized in optimum levels of sugar. Excessive polysaccharide hydrolysis accompanying the disruption of fruit or vegetable peel could lead to loss of the pigments, ultimately hampering the extraction process (Grassin and Coutel, 2009). The use of pectinases and to a lesser extent cellulases can result in adequate levels of pigments to be released. At the same time, high acidity and phenolic content are known to inhibit pectinases. The use of recombinant pectinases that can withstand these factors therefore has attractive potential in the food industry. Cellulolytic enzymes have also found application in the extraction of oils such as olive oil. An attractive ratio of saturated/unsaturated fatty acids in olive oil as well as the presence of antioxidants such as phenolic compounds and carotenoids have led to its popularity and demand across the world (Manna et al., 1999; Visioli and Galli, 1998). Enzyme-assisted extraction of olive oils has emerged as one of the most efficient methods of accessing residual oils from the source that are otherwise left untouched by mechanical extraction processes. The combination of pectinases, cellulases, and hemicellulases have been found to be effective in the extraction of oil from olives (Galante et al., 1998, 1993). Commercial enzyme preparations that facilitate olive oil extraction contain specific proportions of these enzymes (Ranalli and De Mattia, 1997). Variation in the pectinase and cellulase content can result in different amounts of polyphenols and other small molecules being released, ultimately rendering the extracted oil with a unique set of physicochemical properties. In fact, the use of enzymes in olive oil processing has been suggested as significantly lowering the extent of environmental pollution due to more effective extraction of oily fractions from the source (Ranalli et al., 2004).

45.3 APPLICATION OF NANOPARTICLE-IMMOBILIZED ENZYMES ON FOOD WASTE

Enzyme immobilization on solid supports has emerged as a robust strategy for enhancing their catalytic performance. Nanoparticle-immobilized enzymes exhibit several advantages when compared to the use of bare enzymes, across a variety of applications. Optimal immobilization strategies are recognized as being crucial for lowering diffusional limitations on enzyme performance. The nanoscale material employed for enzyme immobilization is said to be effective in sheltering the immobilized biological catalysts, albeit suboptimal conjugation strategies may lead to enzyme deactivation due to deformation of the enzyme active site (Datta et al., 2012; Rodrigues et al., 2013). Nanoparticle-immobilized enzymes are rendered with enhanced activity, and the term NBCs has been used in recognition of the syncretic combination of traits of the conjugated moieties (Dutta et al., 2013; Johnson et al., 2014). NBCs have been developed toward a range of industrial applications covering sensors, biofuel production, and extraction methods (Misson et al., 2015). Robust and reusable NBCs have especially found favor in applications involving food waste. The overall paradigm of the use of NBCs on food waste is depicted in Fig. 45.1. Cellulolytic and pectinolytic enzyme-based NBCs have been examined for their ability to hydrolyze plant biomass, aid in biofuel production, and release entrapped substances. In the following sections, we examine key aspects behind the development and application of such NBCs.

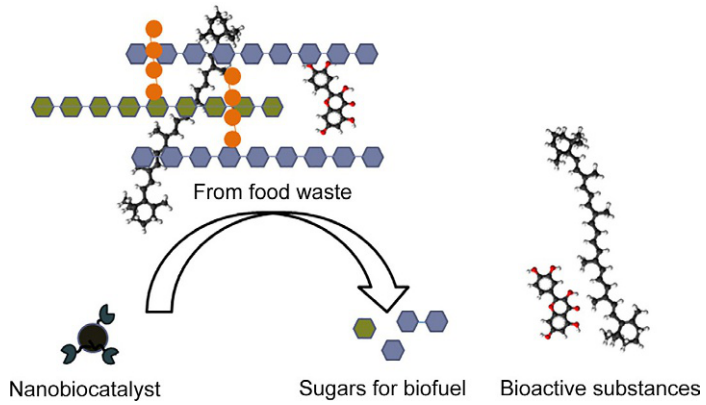

FIG. 45.1 Paradigm for application of nanobiocatalysts on food waste.

45.3.1 Conjugation Strategies for Developing Effective NBCs

The strategies used for the immobilization of enzymes on nanomaterials depend largely on the purported function of the resulting NBCs. The availability of specific chemical functionalities on the nanomaterial surface dictates the conjugation chemistry to be used for the attachment of enzymes. For example, metal nanoparticles such as gold and magnetic iron oxide are suited for the attachment of thiolate and carboxylic acid functionalities on their surface. These functionalities can be connected with the side chain functional groups of amino acids on enzymes using suitable cross-linking agents. In fact, the availability or ease of introduction of functional groups on nanoparticles is an important parameter in NBC preparation.

Polymer nanoparticles are the most potent carriers in this regard, owing to the scope of introducing diverse functional groups (Wang et al., 2009). In addition to the ease of attachment of enzymes, polymer nanocrystals are capable of supporting greater biocatalyst loading, ultimately leading to improved performance of NBCs (Neri et al., 2011). Polymer matrices coated on silica or metal oxide nanoparticles have been found to overcome the drawback of poor strength of polymer carriers alone (Hong et al., 2008; Mahmoud et al., 2009). In contrast to polymer nanocrystals, silica and metal oxide nanoparticles are somewhat restricted in terms of the functional groups available for the covalent attachment of enzymes. Carbodiimide and N-hydroxysuccinimide (NHS) have been used for direct attachment of biological catalysts onto silica surfaces (Guo et al., 2012; Yang et al., 2010). Limited control on the protection of the enzyme active site during chemical conjugation has often led to unpredictable changes in the activity of enzymes that are directly attached to the nanoparticle surface. Several strategies have been used to counter this challenge, including the use of cross-linking agents such as glutaraldehyde and epichlorohydrin (Zhang et al., 2011). The use of cross-linking groups or multifunctional agents has been especially useful for the immobilization of enzymes on gold and magnetic iron oxide nanoparticles (Jordan et al., 2011; Li et al., 2007). In recent times, carbon nanoparticles have emerged as an important carrier due to their diverse and robust morphologies. While the attachment of enzymes on carbon-based nanocarriers has mostly exploited their hydrophobic surface through physical adsorption, covalent linkages have also been developed along similar lines to those described previously for silica and metal oxide

nanoparticles (Alonso-Lomillo et al., 2007; Ji et al., 2010). The variety of methods employed for the immobilization of enzymes on nanoparticle supports reflects the optimization of several related factors, such as (a) availability of functional groups on the nanoparticle support; (b) stability of enzymes under the conjugation strategy adopted; (c) enzyme activity upon immobilization; and (d) applications targeted by the NBCs. The nanocarriers that have found the most favor in applications of NBCs on food waste include gold and magnetic iron oxide, thereby highlighting the importance of using strong, recyclable, and reusable materials for applications in the domain.

45.3.2 Outcome of NBC Action on Food Waste

The dominant application of enzymes in the food industry has been in the extraction and clarification of juices and oils (as outlined in Section 2.0, Enzymes Used for Extraction of Natural Products). Enzymes are also used for adding flavor and texture as well as for the transformation of biomolecules in food sources into other bioactive substances. Such substances include a variety of bioactive peptides from milk, functional carbohydrates as prebiotics with unique nutritional properties, and health-functional lipids (Hughes et al., 2007; Kristensen et al., 2005; Pihlanto-Leppala, 2001). In fact, the majority of enzymes used in free form or as NBCs in the food industry involve application on food sources or products. The application of NBCs on food waste has only emerged in the past few years and is currently based on two principle paradigms, illustrated in Scheme 1: (1) biofuel production and (2) extraction of bioactive substances. Enormous worldwide strain on fossil fuel resources has led to intense research into biobased sources of energy that are collectively termed biofuels. The use of immobilized cellulolytic enzymes for bioethanol production has been studied using several nanomaterials and conjugation strategies (Vaz et al., 2016). Silica and magnetic nanoparticles (MNPs) are among the most favored nanomaterials in this regard, owing to their robustness and reusability (Chang et al., 2011; Soozanipour et al., 2015). In addition to improving the stability of the cellulolytic enzymes, the NBC-mediated conversion of cellulosic and lignocellulosic biomass into glucose could be fulfilled at significantly greater conversion efficiencies compared to the use of free enzymes. The conversion of cellulosic substrates into ethanol has been facilitated by the incorporation of simultaneous saccharification and fermentation procedures (Lee et al., 2012). The substantive improvement in immobilized enzyme activity has been observed across different nanomaterial platforms (Dhiman et al., 2013; Yan et al., 2012). Nevertheless, the complexity of lignocellulosic biomass in agricultural waste presents a challenge for effective harvesting of glucose for subsequent fermentation into bioethanol. NBCs have been developed for the effective hydrolysis of agricultural waste toward bioethanol production. These NBCs used for this purpose include MNPs and MnO_2, primarily owing to their robustness and reusability (Alftren and Hobley, 2014; Cherian et al., 2015). Notably, the enhancement in the efficiency of immobilized cellulases may not be uniform and in some cases may even be comparable to the use of free enzymes (Alftren and Hobley, 2014). Nevertheless, the economics of biomass-derived ethanol become extremely attractive when reusability, stability, and conditions of use of NBCs are factored in. These advantages are also evident from the use of NBCs in the transformation of waste grease to biodiesel. MNP-immobilized lipases have been used for the efficient and reusable esterification and transesterification of waste grease into fatty acid methyl esters (FAMEs).

The application of NBCs on food waste with the goal of extracting specific natural products builds on the established use of free enzymes in the food industry. An unprecedented increase in global food waste has led to the emergence of an associated economy. In particular, processes that enable either the efficient extraction of bioactive substances or the transformation of components into valuable products are likely to drive this unique economy of the 21st century (Galanakis, 2012). Food waste has been suggested as an important resource for providing access to a range of chemical substances, in addition to its potential in energy production (Lin et al., 2013). The application of cellulolytic enzymes on natural sources has been found to release entrenched bioactive natural products such as pigments, polyphenols, vitamins, and other small molecules (Grohmann and Baldwin, 1992; Wilkins et al., 2007). In particular, the peel or skin and seeds of many fruits and vegetables contain attractive quantities of these bioactive substances (Fournand et al., 2006; Goodwin, 1988; Prieur et al., 1994). A combination of cellulolytic enzymes such as cellulase, pectinase, and xylanase has been found to facilitate the efficient release of polyphenol quercetin from onion skin waste (Choi et al., 2015). Quercetin is a prominent bioflavonoid that has been suggested as an effective agent in the reduction of various metabolic disorders and cardiovascular diseases (David et al., 2016). Interestingly, the use of cellulolytic enzymes for the disruption of the cell wall structure of onion skin waste was also effective in producing sugars that would be useful for bioethanol production (Choi et al., 2015). A combination of pectinase, cellulose, and tannase has been used for releasing polyphenol proanthocyanidins from grape seeds and skins (Fernandez et al., 2015). The enzyme-mediated approach was found to lower the degree of polymerization of the polyphenol in addition to effecting enhanced release, which is attractive toward the neutraceutical potential of the substances (Fernandez et al., 2015). NBC-assisted extraction of pigments from orange peels has revealed a correlation between the extent of peel hydrolysis and pigment extraction (Kumar et al., 2016). Further, reusability of the MNP-immobilized enzymes has been demonstrated across several extraction cycles (Kumar et al., 2016). MNP-immobilized β-galactosidase has been used for the preparation of the galacto-oligosaccharides lactulose and lactosucrose from dairy waste (Liu et al., 2012). Lactulose and lactosucrose are valuable ingredients in the food processing industry, and the NBC-mediated approach has been expanded for their continuous synthesis (Song et al., 2012).

45.4 CONCLUSION

The syncretic combination of nanomaterials and biological catalysts has introduced revolutionary ideas and possibilities for the food industry. As an appreciation of resource crunch and need for sustainable development across the globe, food waste has emerged as an area of immense potential for application of NBCs. Interestingly, NBCs can fulfill distinctly different requirements of energy and biochemical production in a complementary fashion. While challenges pertaining to the scalability of NBC application on food waste are real, intensive research continues to break new scientific ground in an effort to surpass those challenges. The wider industrial application of NBCs on food waste is likely to emerge as an attractive economy in the coming years.

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CHAPTER

46

Food Enzymes From Extreme Environments: Sources and Bioprocessing

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46.1 INTRODUCTION

Since 1907 when the first commercial enzyme was pioneered, enzymes have continued to play a significant role in the development of several products in the health, fine chemicals, and fast-moving consumer goods (FMCG) sectors (Talens-Perales et al., 2016). In simple terms, enzymes are responsible for speeding up the rate of breaking, assembling, and transforming biological compounds from one form to another. Enzymes are an attractive "green" class of biocatalysts. They are naturally occurring, nontoxic safe for the environment (biodegradable), operate under mild conditions, have high specificity for substrates, and are suitable for the catalysis of many different reactions (Haertlé, 2016; Talens-Perales et al., 2016). The global enzyme market is expected to reach $7.2 billion by 2020 (Freedonia Inc., 2016). This growth in enzyme demand has been served by the fact that several research advances have been made in the bioprocessing of enzymes (including expression, fermentation, and recovery methods) that enhance the commercial scale production of enzymes (Haertlé, 2016). In the food industry, enzymes assist in the creation of safe foods (gluten-free, lactose-free, reduced acrylamide, reduced transfats), aid in improving the bioavailability and bioaccessibility of food nutrients (as in predigested foods such as some infant formulae), and play a huge role in improving food functionalities such as texture, flavor, and bioactive properties. In addition to enzymes from conventional sources, the bioprocessing of extremozymes (enzymes produced by organisms living under extreme conditions) has the potential to expand the application scope of enzymes and therefore further increase the market value of enzymes. Extremozymes

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https://doi.org/10.1016/B978-0-12-813280-7.00046-3

have potential application in food for a number of reasons: (a) extremozymes are hardy enzymes that can survive other-than-usual food processing conditions, (b) extremozymes are more suitable for substrates whose solubility is enhanced only under extreme conditions, (c) extremozymes may allow in situ catalysis during food processing, for example, breaking down acrylamide during the baking of food, (d) extremozymes are more suitable for use in foods that require aging under extreme conditions (high salt, low temperature, etc.), and (e) the use of extremozymes helps control microbial contamination by microorganisms that grow under normal conditions. This chapter presents an overview on sources and characteristics of extremozymes as well as a discussion on techniques and methodologies for the bioprocessing of these enzymes. The chapter concludes with applications of extremozymes in foods, using the processing of edible oil as a case study.

46.2 EXTREMOZYMES FROM UNUSUAL ENVIRONMENTS

Extremozymes are enzymes that can function under extreme conditions of temperature, pressure, pH, and alkalinity. They are derived from extremophilic microorganisms (extremophiles) that are metabolically active and grow under extreme conditions (Elleuche et al., 2014). Extremophiles thrive in ecological niches such as deep-sea hydrothermal vents, hot springs, solfataric fields, shallow marine boiling water, heated sea floor volcanoes, hot lakes, coal and copper mines, and some environments contaminated with nuclear waste (Olusesan et al., 2011a; Raddadi et al., 2015). There are different classes of extremophiles, including thermophiles (high-temperature tolerant), psychrophiles (low-temperature tolerant), acidophiles (acidic-condition tolerant), alkalophiles (alkali-condition tolerant), halophiles (high salt concentration tolerant), piezophiles (high-pressure tolerant), radiophiles (high-radiation tolerant), and microaerophiles (low-oxygen tolerant) (Akanbi et al., 2010; Deming, 2002; Demirjian et al., 2001; Elleuche et al., 2014; Olusesan et al., 2009, 2011a,b; Zhang et al., 2009). Some of these microorganisms may exhibit more than one extreme property such as a combination of high temperature and alkaline or acid tolerance. Those that fall into these categories include haloalkalophiles, thermoalkalophiles, thermoacidophiles, and haloacidophiles, among others. Table 46.1 shows the types and properties of enzymes expressed from some extremophiles and their extreme habitats. Extremophiles produce enzymes with unique properties that are important for food and industrial applications. In this chapter, we will focus on food enzymes from selected extreme environments.

46.2.1 Hot Springs

Hot springs are natural thermal springs produced by geothermally heated groundwater. They harbor populations of microorganisms that can be a source of commercially important bioactive compounds such as enzymes, sugars, and antibiotics (Satyanarayana et al., 2005). Phylogenetic, physiological, and ecological studies have shown the abundant diversity of thermophilic extremophiles inhabiting hot springs around the world in locations such as Japan, Malaysia, New Zealand, Iceland, China, United States, Mexico, and India (Akanbi et al., 2010; Tobler and Benning, 2011; Castro-Ochoa et al., 2005; Kumar et al., 2014). Some of these hot springs have been reported to have temperatures exceeding 100°C (Margesin et al., 2008).

TABLE 46.1 Enzymes From Extremophiles and Their Extreme Habitats

Extremophile	Habitat	Microorganisms	Enzymes	References
Thermophile	Hot springs Hydrothermal vents Acidic geothermal spring Solfataric fields Shallow marine boiling-water Heated sea floor volcano	*Bacillus subtilis* NS 8, *Bacillus* sp., *Geobacillus* sp., *Thermus aquaticus*, *Pyrococcus woesei*, *Thermoplasma acidophilum*, *Anoxybacillus flavitermus*	Lipase, proteases, gelatinase, amylase, glucoamylase, xylanase, pullulanase, glucosidase, cyclodextrin glycosyltransferase	Olusesan et al., 2011a; Gugliandolo et al., 2012; Febriani et al., 2013; Bertoldo and Antranikian, 2002; Verma et al., 2014; Muñoz et al., 2015; Akanbi et al., 2010; Bin et al., 2012
Psychrophile	Antarctic lake water Arctic marine sediment Deep cold Pacific Ocean waters Chinese Yellow Sea	*Candida Antarctica*, *Clostridium* sp., *Flavobacterium* YS-80, *Anoxybacillus* species	Lipase, protease, amylase	Zhang et al., 2011; De María et al., 2005; Dube et al., 2001; Poli et al., 2006
Acidophile	Acidic geothermal spring Solfataric fields Hot springs Coal and copper mines	*Bacillus* species *Sulfolobus solfataricus* *Acidiphilium angustum* *Metallspaera prunae*	Amylases, proteases, glucoamylases, cellulases, endonuclease	Asoodeh et al., 2010; Limauro et al., 2001; Sharma et al., 2012
Alkaliphile	Hydrothermal vents Marine sediments Hot spring	*Bacillus* sp. MLA64, *Bacillus subtilis* DR8806, *Geobacillus* sp. *Saccharopolyspora* sp. *Anoxybacillus* sp.	Lipase, proteases, gelatinase, amylase, glucoamylopullunase	Gugliandolo et al., 2012; Chakraborty et al., 2011; Chai et al., 2012; Asoodeh and Lagzian, 2012; Lagzian and Asoodeh, 2012
Halophile	Hydrothermal vents Salt lakes	*Bacillus* sp., *Geobacillus* sp., archaeal strains	Lipase, protease, esterase, amylase,	Gugliandolo et al., 2012; Ozcan et al., 2009
Piezophile	Hydrothermal vents	*Pyrococcus* sp.	DNA polymerase	Neves et al., 2005
Radiophile	Antarctic valley Waste water treatment plant	*Deinococcus radiodurans*	Lipase	Liao et al., 2010; Shao et al., 2014
Microaerophile	Hot springs	*Sulfurihydrogenibium azorense*	Azoreductase	Sandhya et al., 2005; Aguiar et al., 2004

Thermophiles found in these environments are generally classified into three categories based on their cardinal growth temperatures: thermophiles (35–70°C), extreme thermophiles (55–80°C), and hyperthermophiles (75–113°C) (Stetter, 1999). Thermophiles grow fastest at temperatures above 50°C (Olusesan et al., 2011a) and these are temperatures where small biomolecules decompose. However, thermophiles produce special proteins known as "chaperonins," which are thermostable and resistant to denaturation and proteolysis. Proteins of thermophiles, denatured at high temperature are refolded by the chaperonins, thus restoring their native form and function (Paiardini et al., 2003). Also, proteins of thermophiles have

increased surface charge and less exposed thermostable amino acids. Thus, increased ionic interaction and hydrogen bonds, increased hydrophobicity, decreased flexibility, and smaller surface loops confer stability on the thermophilic protein (Kumar et al., 2014; Paiardini et al., 2003; Sterner and Liebl, 2001). Hot springs are the most reported sources of extremozymes and intensive research is still being performed, aimed at the isolation of extremozymes from these environments (Table 46.1). Notable microbial enzymes isolated from hot springs include lipases (Akanbi et al., 2010; Olusesan et al., 2011b), amylases (Baysal et al., 2003; Chen et al., 2005) proteases (Chen et al., 2006; Panda et al., 2013), esterase (Chen et al., 2005), pullulanase (Chai et al., 2012; Messaoud et al., 2002), glucosidase (Coleri et al., 2009; Schröder et al., 2014), cyclodextrin glycosyltransferase (Ballschmiter et al., 2005; Charensakdi et al., 2007), xylanase (Bin et al., 2012; Bataillon et al., 2000), and cellulases (Acharya and Chaudhary, 2012a,b). These enzymes are known to be more catalytically active and stable than plant and animal enzymes. Besides, they are favored sources for industrial enzymes due to easy availability and a fast growth rate (Singh et al., 2016). Apart from being highly thermostable, enzymes derived from hot springs show significant pH stability (Olusesan et al., 2011b) because pH values as low as 1.5 and as high as 9.0 have been reported for a number of hot springs (Nozaki et al., 2007; Zhang et al., 2008).

46.2.2 Hydrothermal Vents

Like hot spring, hydrothermal vents have drawn a great deal of attention because of their unique geological structures, complex physicochemical characteristics, and diverse biotic communities (Anderson et al., 2014; Sun et al., 2015). Because hydrothermal vents form at locations where seawater meets magma, temperatures can be as high as 250°C within this region and mainly hyperthermophiles can thrive there (Hannington et al., 2001; Jolivet et al., 2004). Most microorganisms isolated from hydrothermal vents produce extracellularly a number of important enzymes. For instance, *Bacillus stearothermophilus* DSM 22 and *B. thermodenitrificans* DSM 465 isolated from a shallow marine hydrothermal vent in Italy are good producers of esterases, lipases, α-glucosidase, and alkaline phosphatase (Caccamo et al., 2000). Also, alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase were produced by a gammaproteobacterium strain M41^T isolated from the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge (Gartner et al., 2008). Several other studies have reported the isolation of microbial α-amylase, α-glucosidase, alkaline and acidic phosphatase, and pullulanase from hydrothermal vents (Haki and Rakshit, 2003; Lévêque et al., 2000; Wery et al., 2001; Zappa et al., 2001). Interestingly, the majority of these enzymes are extracellular, requiring fewer extraction and purification processes. Besides, they are highly thermostable with optimal temperature stability of between 85 and 110°C (Lévêque et al., 2000), making them more useful for a broad range of food and biotechnological applications.

46.2.3 Saline Lakes

Saline lakes are found on all continents and in most countries. They range in salinity from just above the salt content of seawater (greater than 3.2% salts) to hypersaline lakes (greater than 20% salts) (Litchfield, 2011). A general characteristic of saline lakes is that although rivers

may flow into them, they have no outflow and as the water in the lakes evaporates, the lakes become more salty (Litchfield, 2011). As such, mainly halophilic extremophiles can survive in this environment. For instance, 231 moderately halophilic bacterial strains capable of growing optimally in media with 5%–15% of salt, and 49 extremely halophilic bacteria requiring 20%–25% of salt for optimal growth, were isolated from Howz Soltan playa, a hypersaline lake in the central desert zone of Iran (Rohban et al., 2009). Most of these strains produced extracellular hydrolytic enzymes, including lipases, amylases, proteases, inulinases, xylanases, cellulases, and pullinases (Rohban et al., 2009). More than 150 lipase-producing bacterial strains were isolated from lake Bogoria in Kenya. These strains could tolerate up to 10% (w/v) salinity (Vargas et al., 2004). Also, a previous study found that three extremely halophilic bacteria (genera *Salicola, Salinibacter*, and *Pseudomonas*) capable of producing amylases, proteases, and lipases were isolated in two hypersaline habitats of south Spain (De Lourdes Moreno et al., 2009). Also, nine extremely haloarchaeal strains isolated from the Aran-Bidgol hypersaline lake in the central desert of Iran could produce amylase, lipase, inulinase, pullulanase, protease, cellulase, chitinase, and xylanase, respectively (Kakhki et al., 2011). Two bacterial strains (MCC $P1^T$ and MCC P2) capable of growing at 15°C and producing esterase, alkaline and acidic phosphatase, α-glucosidase, leucine arylamidase, valine arylamidase, phosphohydrolase, and glucosaminidase were isolated from a high-altitude saline lake in the Western Himalayas of India (Polkade et al., 2015). These enzymes could potentially be used under high salt and low temperature conditions. Also, enzymes from halophiles are known to show optimal activities under low water activity, high temperature, and in the presence of organic solvents, thus making them useful in aqueous/organic and in nonaqueous media (Gupta et al., 2016; Marhuenda-Egea and Bonete, 2002). These enzymes have been commercialized in the food, baking, feed, chemical, pharmaceutical, paper and pulp, detergent, and leather industries (Marhuenda-Egea and Bonete, 2002; Oren, 2010; Sánchez-Porro et al., 2003). They have also been reported to be useful for fish and soy sauce preparations as well as for the treatment of saline waste and oilfield wastewaters (Gupta et al., 2016; Oren, 2010; Sánchez-Porro et al., 2003).

46.2.4 Antarctic/Polar Regions

The extreme conditions of the Antarctica and the diversity of its ecosystems have received great attention over the years. Different microorganisms have been discovered in terrestrial Antarctica and studied for their abilities to produce bioactive compounds (Gesheva, 2010; Gesheva and Negoita, 2012; Lo Giudice et al., 2007). Fungal assemblages including *Penicillium, Alternaria, Cladosporium, Phoma, Verticillium, Phialophora, Candida*, and *Rhodotorulla* were isolated from soil samples near Casey Station, one of three permanent bases and research outposts in Antarctica managed by the Australian Antarctic Division (Gesheva, 2010). Most of these strains produced extracellular enzymes including α-amylase, cellulase, and protease (Gesheva, 2010). Krishnan et al. (2011) screened 28 psychrophilic and psychrotolerant fungi obtained from the soils collected from Fildes Peninsula, King George Island, Antarctica, for extracellular hydrolase enzymes. Their results showed that at 4°C, the isolates could produce amylase, cellulase, and protease (Krishnan et al., 2011). Several studies have shown that microbial enzymes including protease, amylase, cellulase, pectinase, xylanase, chitinase, and keratinase have been isolated from the Antarctic region (Duncan et al., 2008; Krishnan et al., 2011).

Candida antarctica, a basidiomycetous yeast isolated from the lake Vanda in Antarctica, produces two distinct lipases known as CAL-A and CAL-B (De María et al., 2005). These enzymes are among the most widely used for biocatalytic reactions. They catalyze the hydrolysis of fats (Akanbi and Barrow, 2017; Fernández-Lorente et al., 2012; Olusesan et al., 2011a) and because they are compatible with an array of solvents, they are able to catalyze a range of reactions including, esterification, trans-esterification, acidolysis, aminolysis, and resolution of racemic mixtures (Akanbi and Barrow, 2015, 2017; Mathesh et al., 2016; Reetz, 2002; Villeneuve et al., 2000). Interestingly, both CAL-A and CAL-B are highly thermostable despite being isolated from the coldest region on earth. CAL-A has been reported to be very active at temperatures above 90°C and is considered the most thermostable lipase (De María et al., 2005). Therefore, most enzymes derived from the Antarctic possess unique qualities that make them suitable for a broad range of food and biotechnological applications.

46.2.5 Other Extreme Sources

Other extreme sources of proteases, lipases, β-galactosidase, α-galactosidase, α-amylase, and esterase include hydrocarbon contaminated soil, superficial saline soils, acidic creek and pools, acid mine drainage, brine pools, industrial wastewaters, refinery and exploration sites, mud sediments, and sewage treatment plants (Cai et al., 2009; De Almeida et al., 2013; Ferrer et al., 2007; Hu et al., 2007; Khannous et al., 2014; Patil and Chaudhari, 2013; Yalçın et al., 2014). Most of these enzymes have extreme properties. For instance, esterase and α-galactosidase, a new member of the glycosyl hydrolase family, were produced by the *Ferroplasma acidiphilum* strain Y (DSM 12658), a ferrous iron-oxidizing, acidophilic, and mesophilic archaeon isolated from acid mine drainage and acidic pools (Ferrer et al., 2005; Golyshina et al., 2006). The optimum pH of the purified α-galactosidase was between 2.4 and 3.5, although it retained more than 92% and 74% of its activity at pH 2.0 and 1.5, respectively. Meanwhile, the optimum pH of the purified esterase was 1.5. Both enzymes were also found to be highly stable at 50°C (Ferrer et al., 2005; Golyshina et al., 2006), suggesting their suitability for use under highly acidic conditions. Also, an extracellular, thermostable, alkaline, and solvent-tolerant protease was produced by *Bacillus circulans* isolated from hydrocarbon (gasoline and diesel) contaminated soil (Patil and Chaudhari, 2013).

46.3 DOWNSTREAM PROCESSING OF EXTREMOZYMES

Downstream processing comprises all the processes or operations required to extract or purify proteins and other bioactive compounds from natural sources such as plant, animals, or microorganisms. Operations involved in downstream processing in biotechnology research seem to be expensive in terms of cost and time; however, the final products of the operations are of high quality and standards. To this end, there is no amount of time or resources invested in these processes that can be considered wasted. The aim of carrying out most downstream operations is to obtain products high in quality and purity. Hence, most of the downstream processes involve product screening, isolation, and purification (Abdel-Fattah and Gaballa, 2008; Muffler and Ulber, 2005; Olusesan et al., 2011a, b). These techniques include a lot of more other subtechniques (depending on the source of the proteins being

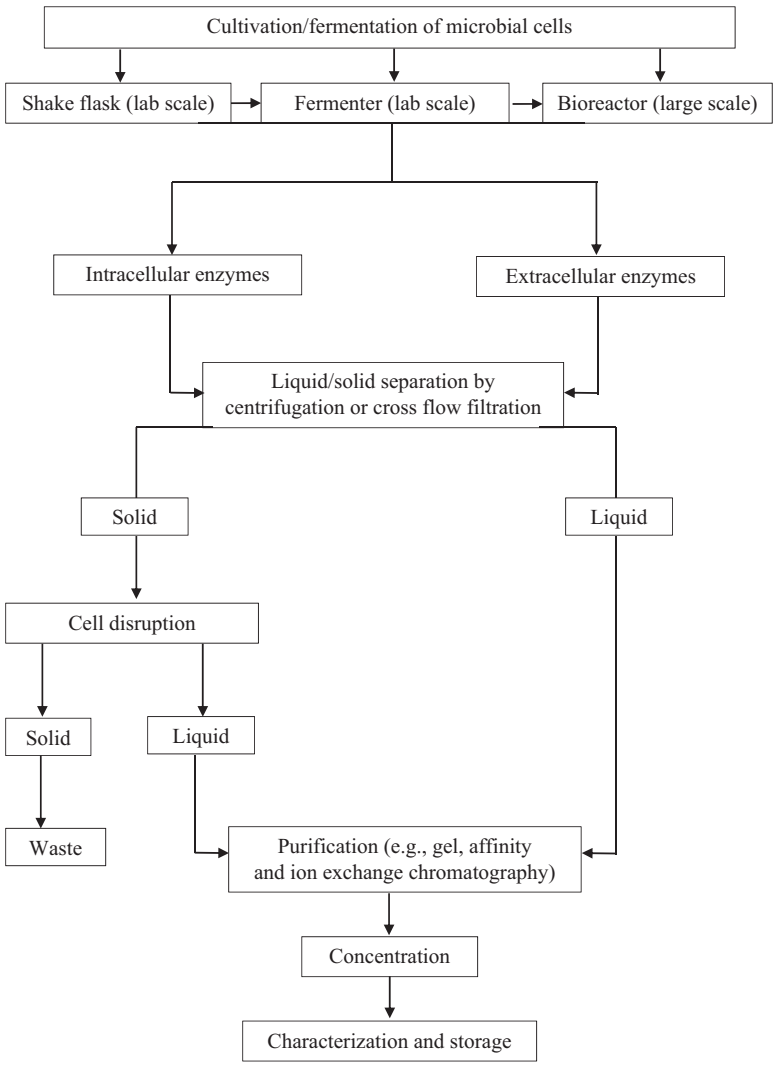

FIG. 46.1 Typical downstream processing of enzymes from microbial sources.

processed) that are very vital to achieving the downstream objectives. Fig. 46.1 shows some of the processes involved in a typical downstream processing of protein from microbial sources.

46.3.1 Enzyme Isolation

46.3.1.1 Intracellular Enzyme Isolation

Studies have shown that some intracellular enzymes including α-glucosidase, α-galactosidase, β-galactosidase, lipases, and proteases have been isolated from extremophiles (Ferrer et al., 2005, 2007; Fuciños et al., 2005; Kocabıyık and Erdem, 2002) and for

optimized recovery, cell disruption is necessary. Several cell disruption methods including freezing and thawing, sonication, homogenization, bead beating, and the use of a lysis buffer have been reported (Kikani et al., 2017). The effectiveness of combining two or more of these methods in protein extraction from microbial cells has been studied (Fuciños et al., 2005). However, only sonication ensured total release of intracellular proteins from microbial cells (Dominguez et al., 2004; Ewis et al., 2004; Fuciños et al., 2005). Besides, it has the advantage of being able to disrupt the cells at relatively low temperatures, leading to less thermal protein damage (Gerde et al., 2012). For instance, the cell suspension of a thermostable lipase and esterase producing *Thermus thermophilus* strain in Tris/HCl buffer, EDTA, and NaCl solution was sonicated at 0°C in two cycles of 2 min (Fuciños et al., 2005). In another study, frozen cells of thermophilic bacterial strains isolated from 42 hot springs in Turkey were disrupted by sonication for the extraction of α-glucosidase. Crude enzyme activity up to 13.5 U/g was obtained after centrifugation and washing of cell debris with 0.85% NaCl (Coleri et al., 2008). The genes of enzyme-producing strains have also been extracted by sonication and expressed in *Escherichia coli*. The genes of intracellular and cell bound carboxylesterase and three α-galactosidases from *F. acidiphilum* isolated from acid mine drainage and acidic pools (Ferrer et al., 2005; Golyshina et al., 2006) have been cloned and expressed in *E. coli*. The expressed cells (in *E. coli*) were incubated at 37°C in a shaking water bath for 2 h and sonicated on ice for 15 min. After purification, higher enzyme activities were recorded.

46.3.1.2 Extracellular Enzyme Isolation

Extracellular enzymes or exoenzymes are those secreted outside the cell to perform specific functions. Exoenzymes produced by extremophiles play important roles in allowing them to interact with their extreme environments. Because these enzymes are secreted, microbial culture conditions can be optimized in the laboratory for their continuous expression. Besides, they require fewer extraction and purification processes compared to intracellular enzymes that often require expensive cell disruption processes (Agyei and Danquah, 2012; Agyei et al., 2012). In one study, extracellular lipase was produced by *Bacillus subtilis* NS 8 isolated from a Malaysian hot spring (Olusesan et al., 2011a). The strain was cultured in nutrient broth, after which cells from the cultures were removed by centrifugation. A lipase solution was obtained by passing the cell-free supernatant through Whatman filter paper and a 0.22 μm filter membrane, then concentrated to 10-fold by ultrafiltration using a PLGC UF membrane with a nominal exclusion limit of 10 kDa at 4°C under 2.0 bars of nitrogen pressure (Olusesan et al., 2011a). Optimum lipase production of 5.67 U/mL was obtained from the strain when a combination of 3% olive oil (carbon source), 2% peptone (nitrogen source), 0.2% magnesium sulfate heptahydrate (mineral source), an agitation rate of 200 rpm, an incubation temperature, and a pH of 50°C and 7.5, respectively was used. Lipase production was further carried out inside a 2 L bioreactor, which yielded an enzyme activity of 14.5 U/mL after 15 h of incubation (Olusesan et al., 2011a). An extracellular protease-producing *Thermoanaerobacter keratinophilus* was isolated from heated vents on the island of São Miguel in the Azores (Riessen and Antranikian, 2001). After cultivation, cells were removed by centrifugation and the enzyme (in a sodium phosphate buffer) was concentrated 50-fold by ultrafiltration using a 10 kDa cut-off membrane. An enzyme activity of 17.5 U/mg was obtained (Riessen and Antranikian, 2001).

46.3.2 Enzyme Production in Bioreactors

There are several conditions that affect enzyme production by microorganisms in laboratory conditions. These include medium pH, temperature, medium composition, and agitation. Most of these factors are not easily controlled using conventional lab-scale shake flask systems. It has been reported that without a balance between aeration and agitation, microbial cell growth and microbial enzyme production will be greatly hindered (Puthli et al., 2006). Interestingly, these conditions can be controlled and balanced in a bioreactor while also enhancing enzyme yield (Olusesan et al., 2011a). Several extremozymes have been produced in batch, repeated batch, fed batch, and continuous fermentation processes (Treichel et al., 2010). Extracellular lipase production by a *Bacillus* strain isolated from a Spanish hot spring was investigated at the bioreactor scale and careful attention was paid to the effects of aeration and agitation rates. Results showed that there was a significant increase in cell concentration and lipase production in the 5 L stirred tank bioreactor (Deive et al., 2009). The production of *Rhodotorula mucilaginosa* extracellular lipase was optimized in a 1.5 L stirred tank bioreactor with a working volume of 1 L. Maximum lipase activity of 72 U/mL was obtained when the correct balance was established between aeration and agitation (Potumarthi et al., 2008). A significant improvement in alkaline protease production by *Bacillus mojavensis* was achieved in a 14 L bioreactor with an enzyme yield of 2389 U/mL compared to 558 U/mL in the shake flask cultures (Beg et al., 2003). This was a significant improvement in yield of up to a 4.2-fold increase. Scaled-up production of other extremozymes in bioreactors has also been reported (Dominguez et al., 2005; Poli et al., 2009).

46.3.3 Purification and Characterization of Extremozymes

Several microbial enzymes have been extensively purified and characterized in terms of their activity, stability, and reusability. The ability of extremozymes to tolerate a broad range of pH, temperature, solvents, metal ions, and chelating agents has been reported (Akanbi et al., 2012; Olusesan et al., 2011b; Ozcan et al., 2009; Patil and Chaudhari, 2013; Sharma et al., 2001; Zappa et al., 2001). Methods of enzyme purification include precipitation, ultrafiltration, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography (Asoodeh and Lagzian, 2012; Bataillon et al., 2000; Cai et al., 2009; Olusesan et al., 2011b). Because microbial enzymes can be both extracellular and intracellular, the fermentation process is always accompanied by sonication (for intracellular enzymes) and cell removal from the culture broth by centrifugation and filtration (Saxena et al., 2003)

46.3.3.1 Purification of Extremozymes

Cross-flow membrane filtration has been used in the downstream processing of lipases for cell removal and concentration of the supernatant of the spent media containing lipases (Saxena et al., 2003). Lipase from *Pseudomonas fluorescens* was concentrated and partially purified using two ultrafiltration capillary membranes, namely polyacrylonitrile and polysulfone with a 10 kDa molecular weight cut-off (Saxena et al., 2003). The molecular weight cut-off can also be effectively used to separate crude enzymes based on their molecular weights. For instance, serine alkaline protease was separated from the high molecular weight neutral protease and amylase using a crossflow ultrafiltration system with 30 and 10 kDa polysulfone

membranes, respectively (Takaç et al., 2000). Often, a single purification step is not enough to ensure proper purification of enzymes; as such, a combination of methods may be required. Chromatographic techniques such as ion exchange and gel filtration are the most frequently used purification methods. They have been combined with ultrafiltration, ammonium sulfate precipitation, and other techniques for efficient enzyme purification (Saxena et al., 2003). A lipase from *B. subtilis* NS 8 was purified 500-fold to homogeneity with using a three-step procedure. Concentration using a Millipore PLGC UF membrane (10 kDa) cut-off was followed by ion exchange chromatography (DEAE-Toyopearl 650M) and gel filtration (Sephadex G-75) (Olusesan et al., 2011b). A thermostable lipase produced by a thermophilic *Bacillus* sp. J33 was purified 175-fold by ammonium sulfate and phenyl Sepahrose column chromatography (Nawani and Kaur, 2000). A moderately thermostable xylanase produced by the *Bacillus* sp. strain SPS-0 was purified to homogeneity by ammonium sulfate precipitation, anion exchange, gel filtration, and affinity chromatography. Its molecular weight was found to be 99 kDa by SDS-PAGE (Bataillon et al., 2000).

46.3.3.2 Characterization of Purified Extremozymes

Microbial enzymes are very diverse in their catalytic properties. Purified microbial enzymes have been characterized in terms of their activity and stability with respect to pH, temperature, solvent, and metal ions (Sharma et al., 2001). The optimum pH and temperature for a purified thermostable lipase from *B. subtilis* NS 8 were 7.0 and 60°C, respectively (Olusesan et al., 2011b). The purified enzyme was stable between pH 7.0–9.0 and temperature stability was between 40 and 70°C. Half-lives of the enzymes were 273.38 min at 60°C, 51.04 min at 70°C, and 41.58 min at 80°C. Its decimal reduction (*D*-values) times at 60, 70, and 80°C were 788.7, 169.6, and 138.2 min, respectively. The activity of the enzyme was slightly enhanced by Mg^{2+} but significantly inhibited by Fe^{2+}, Cu^{2+}, Zn^{2+}, Na^{+}, and K^{+} (Olusesan et al., 2011b). The optimum and pH for thermostable xylanase isolated from the *Bacillus* sp. strain SPS-0 were 75°C and 6.0, respectively (Bataillon et al., 2000). The enzyme was stable up to 70°C for 4 h at pH 6.0 in the presence of xylane. Metal ions such as Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, and Na^{+} did not affect the activity of the enzyme, but mercury (Hg^{2+}) ions completely inhibited it. β-Mercaptoethanol and dithiothreitol stimulated xylanase activity while EDTA and urea did not affect it, but *N*-bromosuccinimide and 5.5′-dithio-bis(2-nitrobenzoic acid) remarkably inhibited it (Bataillon et al., 2000). Other extremozymes that have been purified and characterized include amylase (Asoodeh et al., 2010; Chakraborty et al., 2011), protease (Akel et al., 2009), phosphatase (Zappa et al., 2001), and glucoamylopullulanase (Asoodeh and Lagzian, 2012).

46.4 IMMOBILIZATION OF EXTREMOZYMES FOR FOOD-GRADE APPLICATIONS

Food application of free (nonimmobilized) enzymes is often hampered by difficult recovery and reusability. Immobilization helps overcome these problems. It improves the reusability and stability while reducing the cost-price of enzymes. It also facilitates efficient enzyme recovery, thereby minimizing or eliminating protein contamination of the product (Akanbi and Barrow, 2017; Sheldon, 2007). Although several materials have been used to successfully immobilize enzymes, nontoxic and biocompatible materials are needed to immobilize

enzymes for food applications. There are several lipase immobilization methods, including adsorption on hydrophobic supports, covalent bonding to functional groups, use of nanomaterials, immobilization by gel entrapment, and microencapsulation in semipermeable membranes, among others (Adlercreutz, 2013; Mathesh et al., 2016; Sheldon, 2007). Some of these methods involve the use of food-grade materials for immobilization of enzymes from extreme environments. An extremozyme, *C. antarctica* lipase (CAL-B), has been immobilized on chitin and chitosan (Silva et al., 2012). These biodegradable natural polyaminosaccharides (chitin and chitosan) are obtained at relatively low cost from shells of shellfish and have been used to produce value-added food products (Krajewska, 2004; Orrego et al., 2010). The immobilized CAL-B was found to be more thermostable than its liquid and commercially available immobilized (Novozym 435) forms. Chitosan has also been used to immobilize microbial dextranase isolated from hydrothermal springs. This immobilized enzyme was found to be highly stable at 80°C (Shahid et al., 2017). Dextranase has been used to hydrolyze dextrans (undesirable compounds) in sugar mill juices (Jiménez, 2009) and has also been used for the synthesis of valuable probiotic oligosaccharides (Patel and Goyal, 2011). Concentrates of omega-3 fatty acids have been prepared using lipases. However, to sell these products as food or nutritional supplements in North America, there is a requirement that both the enzyme and the immobilization matrix must be food-grade (Kralovec et al., 2010). Food-grade polymeric support (Amberlite FPX-66) was used to immobilize CAL-B and the immobilized enzyme was highly thermostable with multiple reuse of up to 80 repeats. The enzyme was used to prepare pure concentrates of omega-3 fatty acids (Kralovec et al., 2010). Other enzyme immobilization carriers suitable as food-grade resins have been reported for immobilizing enzymes from extreme environments (Akanbi and Barrow, 2017; Basso et al., 2013).

46.5 FOOD APPLICATIONS OF EXTREMOZYMES: CASE STUDY ON LIPID PROCESSING

Edible fat and oil processing involves recovery/extraction, refining, conversion, and stabilization (Johnson, 2002). Oil recovery from plant or animal tissue involves high-pressure pressing and extraction using solvents such as hexane (Johnson, 2002). Oil refining involves removal of contaminants such as pigments, free fatty acids, and phosphatides (Zhang et al., 2015). After refining, lipid classes of oil can be converted into different forms by hydrolysis or transesterification because food application of each lipid class differs (Akanbi et al., 2013; Akanbi and Barrow, 2015). These lipid classes include triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acid (FFA). Stabilization is necessary because oils are highly susceptible to oxidation. Besides, lipid peroxidation lowers the nutritive value of food, deteriorates the taste and flavor of food, and can trigger cardiovascular diseases. Lipid stabilization often involves the addition of acceptable antioxidants; however other stabilization methods such as spray drying and microencapsulation have been reported (Xia et al., 2017).

46.5.1 Enzyme Application in Oil Recovery/Extraction

Cell-wall degrading enzymes have helped in oil extraction from some seeds and fruits. The enzymes involved in degrading cell walls are mainly pectinases, cellulases, and hemicellulases.

It has been reported that cellulases, hemicellulases, and pectinases affected oil and protein extraction yields as well as dissolution of nonlipid materials (Zhang et al., 2015). The addition of 2% (w/w) pectinase to a fine meal of sunflower seeds prior to solvent extraction led to a higher oil release and a quick extraction (Perez et al., 2013). This enzyme-assisted reaction was carried out at 50°C (Perez et al., 2013). It has been reported that a combination of cell-wall degrading enzymes and hexane can be used simultaneously for oil extraction. These suggest that only thermostable and solvent-tolerant enzymes can be used for these processes. Therefore, several carbohydrases and proteases from extreme environments can be suitably used for oil extraction from seeds and fruits (Acharya and Chaudhary, 2012a,b; Duncan et al., 2008; Kakhki et al., 2011; Krishnan et al., 2011; Rohban et al., 2009). Another enzyme-catalyzed oil extraction method is known as enzyme-assisted aqueous oil extraction. In this oil extraction method, water containing selected enzymes forms the extraction medium used for incubating the ground oilseeds (Latif and Anwar, 2009; Yusoff et al., 2015). This method proves to be environmentally friendly as it involves no hazardous chemical such as hexane and may allow the recovery of high quality protein for human consumption (Latif and Anwar, 2009; Yusoff et al., 2015). However, there are major drawbacks with this method. Extracted oil is highly susceptible to oxidation because of water involvement. Also, because the extracted oil forms an emulsion with water, further extraction steps are going to be needed. Hydrolysis of oil (TAG) into DAG, MAG, and FFA is likely in case there are lipases or esterases in the oilseeds. Lastly, extraction yield has been found to be lower (Yusoff et al., 2015)

46.5.2 Enzymes in Oil Refining

Refining ensures the removal of impurities from edible oils. While some of these impurities can be easily removed using solvents, others are lipophilic and difficult to remove. Examples of these are phospholipids. Although some phospholipids are generally desirable in oil (such as those in krill oil), others can form gummy precipitate and therefore need to be removed from oil by degumming. Enzymatic degumming of vegetable oils using phospholipases has been widely reported. Phospholipases A1 and A2 are often used to remove one of the fatty acids from phospholipids to produce lysophospholipids, which can then be easily removed by water as part of the degumming process (Zhang et al., 2015). Studies are beginning to show that other types of phospholipases such as B, C, and D can be used for enzymatic degumming. These enzymes can be used to completely hydrolyze phospholipids and release FFA, thereby reducing gum volumes (De María et al., 2007; Zhang et al., 2015). Commercial phospholipases A1, A2, B, C, and D are still very expensive. Those from extreme environments can be viable alternatives. For instance, a highly thermostable phospholipase was isolated from the Jae Sawn hot spring in Thailand. The enzyme retained more than 50% of its maximal activity at temperatures between 50 and 75°C with optimal activity at 70°C and was stable at this temperature for 2 h (Tirawongsaroj et al., 2008). Also, *Grimontia hollisae* isolated from tidal flat sediments on the Korean west coast produced phospholipase A (Lee et al., 2012). These microbial phospholipases can be used for oil degumming.

46.5.3 Enzyme Application in Oil Conversion/Modification

Generally, oils and fats are comprised mainly of triacylglycerols (TAGs). Oil modification can be carried out through lipase-catalyzed reactions. Lipases can be used to break down

fats (hydrolysis) as well as couple fats (synthesis). Hydrolysis of fats and oils occurs at the lipid-water interface while synthesis (or fat coupling) occurs in the presence of solvents. These reactions are shown below.

(i) *Hydrolysis*
$RCOOR' + H_2O \leftrightarrow RCOOH + R'OH$ (Akanbi et al., 2013)

(ii) *Synthesis*

(a) Esterification
$RCOOH + R'OH \leftrightarrow RCOOR' + H_2O$ (Stergiou et al., 2013)

(b) Interesterification
$RCOOR' + R''COOR \leftrightarrow RCOOR'' + R'COOR'$ (Soumanou et al., 2013)

(c) Alcoholysis
$RCOOR' + R''OH \leftrightarrow RCOOR'' + R'OH$ (Valverde et al., 2014)

(d) Acidolysis
$RCOOR' + R''COOH \rightarrow R''COOR + RCOOH$ (Akanbi and Barrow, 2015)

(e) Aminolysis
$RCOOR' + R''NH_2 \leftrightarrow RCONHR + R'OH$ (Kumar et al., 2016)

Lipase-catalyzed hydrolysis of oils results in a combination of triacylglycerols (TAGs), free fatty acids (FFAs), diacylglycerols (DAGs), and monoacylglycerols (MAGs) (Fig. 46.2). Each of the lipid classes has been widely used in the food industry. For instance, MAGs have been used in bakery products, margarines, dairy products, and confectionaries because of their emulsifying, stabilizing, and conditioning properties (Damstrup et al., 2005; Feltes et al., 2013). It was found that 0.5% MAG is sufficient to obtain a stable emulsion (Dunford and Dunford, 2004). MAGs are used as emulsifiers in spreads or ice creams and to reinforce gluten structure in bread. MAGs are also used in pastas to obtain a firmer, less sticky pasta during cooking (Feltes et al., 2013). DAGs have been used as emulsifiers and as food additives. Studies have shown that when foods containing DAGs, such as muffins, crackers, cookies, and cereal bars, were administered to overweight patients, reduction in weight and body fat were noticeable (Maki et al., 2002). Furthermore, TAG and FFA forms of omega-3 fatty acids are available as nutritional supplements in Australian, American, and European markets. Lipases from extreme environments have been widely used for hydrolysis of fats and oils. For instance, highly thermostable lipase from *C. antarctica* (CAL-A) was used to produce concentrates of omega-3 fatty acids by partial hydrolysis of anchovy and tuna oils. These concentrates are comprised of TAG, DAG, and MAG that are rich in eicosapentaenoic (EPA), docosahexaenoic (DHA), and docosapentaenoic acid (DPA) (Akanbi and Barrow, 2017).

Lipases have also been used for the synthesis of structure lipids. For instance, transesterification of vegetable oils and omega-3 fatty acids has been carried out using lipases from extreme environments. Capric acid and EPA have been successfully incorporated into borage oil by *C. antarctica* lipase B (Akoh and Moussata, 1998). EPA-FFA was incorporated into emu oil using *Thermomyces lanuginosus* and *Rhizomucor miehei* lipases. These lipase-catalyzed acidolysis reactions led to more than 23% EPA incorporation into emu oil (Akanbi and Barrow, 2015). EPA- and DHA-enriched acyglycerols by alcoholysis of tuna and sardine oils have been produced. Lipases from *T. lanuginosus* and *Alcaligenes* sp. were used for the reactions (Valverde et al., 2014). Other thermostable and solvent tolerant lipases capable of carrying out the aforementioned reactions have been isolated from extreme environments (Abdel-Fattah and Gaballa, 2008; Akanbi et al., 2010, 2012; Castro-Ochoa et al., 2005; De Almeida et al., 2013;

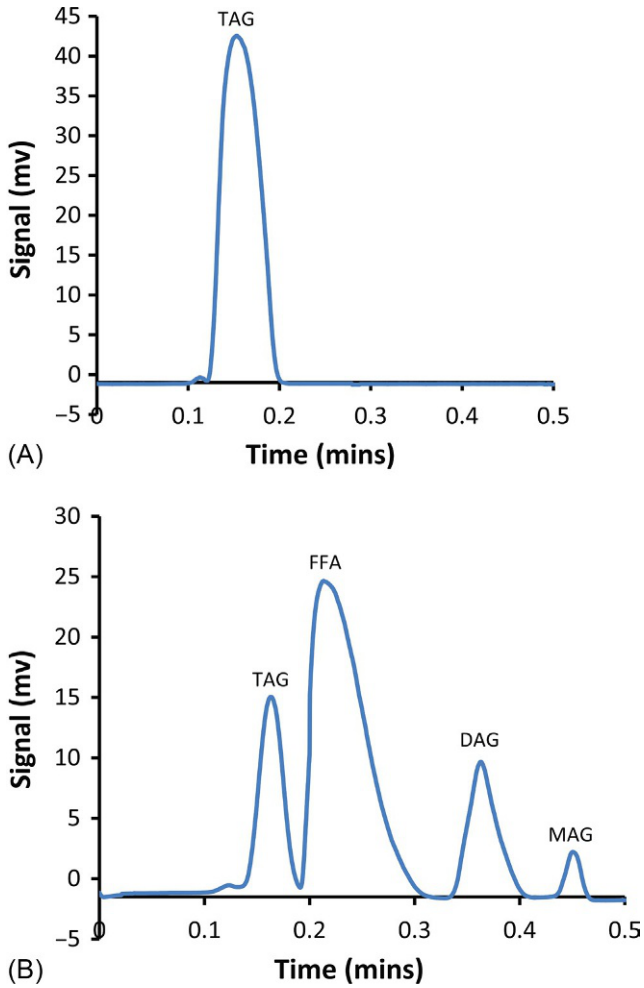

FIG. 46.2 Capillary chromatography (Iatroscan) profile of anchovy oil (A) before and (B) after hydrolysis by *Thermomyces lanuginosus* lipase for 24h at 40°C and pH 7.0. *Reprinted with permission from Akanbi, T.O., Adcock, J.L,. Barrow, C.J., 2013. Selective concentration of EPA and DHA using Thermomyces lanuginosus lipase is due to fatty acid selectivity and not regioselectivity. J. Food Chem. 138 (1), 615–620. Copyright (2001).*

De María et al., 2005; Deive et al., 2009; Febriani et al., 2013; Gupta et al., 2004; Hiol et al., 2000; Nawani and Kaur, 2000; Olusesan et al., 2009, 2011a; Potumarthi et al., 2008; Shao et al., 2014; Sharma et al., 2001; Vargas et al., 2004).

46.5.4 Enzyme Application in Oil Stabilization

Direct use of enzymes to stabilize oils and fats has not been directly reported. However, enzymes have been used to synthesize antioxidants for oil stabilization. Lipases from extreme environments have been used to synthesize very potent antioxidants for oil stabilization.

For instance, *C. antarctica* lipase B has been used to synthesize fatty acid ascorbyl esters for oil stabilization (Viklund et al., 2003). The process involves conjugating fatty acid with ascorbic acids via the lipase-catalyzed esterification reaction (Viklund et al., 2003). These conjugates are more suitable for stabilizing oil than the hydrophilic ascorbic acid because of their high hydrophilicity. Lipases have also been used to synthesize conjugates of fatty acids and polyphenols. For instance, hydroxytyrosol has been conjugated with saturated and unsaturated fatty acids using extremophilic lipases. These conjugates were found to be highly efficient in stabilizing fish oil (Medina et al., 2009; Pereira-Caro et al., 2009). Other potent phenolic fatty esters have been enzymatically synthesized using polyphenols such as tyrosol (Pande and Akoh, 2016), quercetin-3-*O*-glucoside (Warnakulasuriya and Rupasinghe, 2016), isoquercetin (Vavříková et al., 2016), hydroxycinnamic acid (Schär et al., 2017), ferrulic acid (Tan and Shahidi, 2011), and *p*-coumaric acid (Wang and Shahidi, 2013).

46.6 CONCLUSION

Enzymes play a huge role in food processing. Not only do enzymes help in the generation of desirable functional properties (e.g., foams from proteins) in foods, they are also responsible for generating safe foods (e.g., lactose free milk) and biologically active functional foods (e.g., bioactive peptides or omega-3 concentrated oils). The development of safe, healthy, and sensorial-pleasing foods in a sustainable manner has always been a priority for the 21st century consumer. As such, the exploration of alternative sources of enzymes for developing food products is imperative. Due to their ability to catalyze biochemical processes under extreme conditions, extremozymes are a suitable class of enzymes whose properties can be explored, leading to potential exploitation in the food industry. Extremozymes have the potential to assist in the release of nutritional/bioactive compounds from difficult-to-digest materials that are often generated as wastes or by-products in the food industry. These waste materials are expensive to dispose of and a menace to the environment. Extremozymes have the potential to assist in the valorization of waste because these enzymes can function at extreme conditions and they are required for the solubilization of some of these wastes and by-products. Extremozymes may therefore be useful for (a) the production of glucose from cellulose in wood chips; (b) the release of bioactive peptides from keratinous materials such as horns, hoofs, and feathers; and (c) the in situ degradation of polyacrylamide in foods during baking, etc. Most food and industrial applications of enzymes require their repeated reuse and easy separation from reaction mixtures, hence the need for immobilization. Enzyme immobilization often provides the additional advantage of an increase in the catalytic activities and stability compared to the nonimmobilized ones. Therefore, novel techniques for the expression, purification, and immobilization of extremozymes that meet the needs of cost, scalability, and sustainability should be further investigated.

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CHAPTER

47

Psychrophilic Enzymes: Potential Biocatalysts for Food Processing

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47.1 INTRODUCTION

Enzymes have been used since ancient times, especially in beverages and food processing. Food enzymes favor a wide range for processing food and food products, including texturing and flavoring. Enzymes are used as food additives, ingredients, and processing aids during food processing that enhances the nutritional value, flavor, taste, and appearance while helping in preservation. Enzymes are used for the production of cheese and dairy products; to improve the quality of bakery products; to improve wine color and clarity; and to reduce the sulfur content, enhance the filterability, and improve the flavor of the final food products (Sarmiento et al., 2015). Products that are developed from a bioprocess, hybrid chemical, or biocatalytic process are increasing daily in the markets. Some of the enzymes have been approved by the European Commission (EC) for use as food additives; however, there are enzymes that are used only for processing aids that will not be a part of the finished product. Enzymes cover the market by a growth of CAGR 5.1% during 2013–18, reaching $17 billion business, which was $12 billion in 2011 (Dewan, 2014). To avoid undesirable side reactions in food ingredients due to high temperatures during food processing, cold active enzymes can be used. In the food industry, the current trend is to substitute the high temperature process with a low temperature to avoid substrates spoilage, preserve flavors, avoid undesirable reactions, and increase the economic and environmental advantages (Horikoshi, 1999; Pulicherla et al., 2013). In this chapter, we discuss the importance of cold active enzymes as well as their adaptation, production, and applications, along with recent developments.

47.2 PSYCHROPHILIC ENZYMES

Low temperatures are experienced in more than 75% of our planet, and a wide variety of microorganisms have adapted naturally to survive in these conditions. They are called psychrophilic microorganisms, which can grow, multiply, and sustain metabolic activities at low temperatures (Sarmiento et al., 2015). Living organisms surviving at low temperatures adopt different mechanisms to overcome those difficult situations, such as low enzyme rates, altered transport systems, and decreased membrane fluidity (D'Amico et al., 2006). They produce more unsaturated and methyl-branched fatty acids and shorter acyl-chain fatty acids for increasing membrane fluidity (Chintalapati et al., 2004; Russell et al., 1998). For cellular functions, they produce cold shock proteins that help in protein folding while increasing membrane fluidity and restraining ice formation (Phadtare, 2004). Apart from all these mechanisms, they produce cold active enzymes (psychrophilic enzymes) that are highly flexible, maintain high specific activity, and are capable of performing catalytic processes at low temperatures ranging from 0 to 10°C. These enzymes have the potential to carry out biotechnological applications and are also more suitable for food processing. Therefore huge energy savings as well as environmental advantages are possible during food processing.

47.3 COLD ADAPTATION OF ENZYMES

At low temperatures, the mean kinetic energy available for a reaction is inadequate to overcome the energy barrier for activation catalysis. This results in low enzyme activity. Protein also gets denatured at low temperatures due to fewer water molecules associated with it (Karan et al., 2012). Therefore, these enzymes or protein molecules have some special adaptations and flexible characteristics when compared with thermophillic and mesophillic enzymes (Siddiqui and Cavicchioli, 2006). The adaptation and functioning of cold active enzymes to low temperatures has been observed based on crystal structures, homologous models, and comparative genomic studies (Spiwok et al., 2007). Mainly, the adaptation depends on protein stability and folding. However, its physical basis of impact on activity at low temperatures remains vague. The reduced number of salt bridges, the lesser number of the [Arg/(Arg + Lys)] ratio, the lower number of buried nonpolar residues, the higher number of uncovered nonpolar residues and glutamine residues, the increased number of glycine in the loop, and a lesser number of proline are the special features observed in cold active enzymes (Saunders et al., 2003). An inverse relationship between activity and stability of the cold active enzymes has been observed in earlier studies (Zavodszky et al., 1998). Retaining high conformational flexibility makes the enzymes active at low temperatures; however, it reduces their stability. A number of studies have been conducted to understand the relationship between the activity and stability of cold active enzymes (Beadle and Shoichet, 2002; Olufsen et al., 2005).

47.4 PSYCHROPHILIC ENZYMES IN FOOD PROCESSING

In recent years, mesophilic enzymes have replaced psychrophilic enzymes in the food industry due to the additional benefits. Under optimal conditions, the amount of enzymes

needed for reactions is less when cold active enzymes are used. This helps in huge energy savings and costs for a particular reaction. The enzymes can be inactivated by increasing the temperature of the reaction mixture to stop the reaction. This helps in using the enzyme for a particular period in a reaction such as meat tenderizing to avoid the unfavorable byproducts in a reaction at a higher temperature (Sarmiento et al., 2015). These enzymes have the flexibility to a variety of environmental conditions such as substrates, temperature, and pH or reaction conditions. This makes them novel for performing innovative reactions by using different substrates and cofactors and replacing mesophillic enzymes in the wine and beverages industries, animal feed supplements, cheese manufacturing, etc. Even bioremediation of food industry waste, which contains large amount of hydrocarbon, oil, lipids, and proteins, can be achieved by cold active enzymes such as catalases, oxidases, and peroxidases. Some of these enzymes are used for biotransformation and biosensors. Psychrophilic enzymes have a wide variety of applications in the food industry (Table 47.1). They are used in processing animal feed for the improvement of digestibility and assimilation

TABLE 47.1 Cold Active Enzymes and Their Application in the Food Industry

Enzyme	Application	References
Invertase and lysozymes	Stabilizers and preservatives	Sarmiento et al., 2015; Zhou et al., 2016
Lactases	Lactose-free milk products	Hoyoux et al., 2001
Lipase, protease, phytase, glucanases, xylanase	Animal feed improvement of digestibility improvement of assimilation	Sarmiento et al., 2015
Protease	removal of hemicellulosic material from feed	Wang et al., 2010
Chitinase	Meat tenderizing	Yu et al., 2011
α-Amylase, glucoamylase	Single-cell protein from shellfish waste	Kuddus et al., 2012
Pectinases	Starch hydrolysis Fermentation of beer and wine, breadmaking, and fruit juice processing	Novoshape (Novozymes), Pectinase 62L (Biocatalysts), Lallzyme (Lallemand)
α-Amylase, xylanase	Cheese ripening	Alquati et al., 2002
β-Galactosidase	Dough fermentation, bakery products Lactose-free milk products Removal of lactose from milk, conversion of lactose in whey into glucose and galactose in dairy industry	Ghosh et al., 2013
Feruloyl esterase	Wine and beverage stabilization food fermentation	Esteban-Torres et al., 2014

(Collins et al., 2005; Hatti-Kaul et al., 2005; Tutino et al., 2009; Ueda et al., 2010) and the removal of hemicelluloses from the feed (Wang et al., 2010), meat tenderization (Dahiya et al., 2006), production of single-cell proteins from shell fish waste (Gerday et al., 2000), the hydrolysis of starch (Collins et al., 2005; Nakagawa et al., 2004), the clarification of wine, fruit, and vegetable juices (Truong et al., 2001), wine and beverage stabilization, cheese ripening (Collins et al., 2005; Gerday et al., 2000), the production of bakery items, dough fermentation, the removal of lactose from milk and milk products, the conversion of whey lactose glucose and galactose (Ghosh et al., 2013) in dairy industries (Kunamneni et al., 2008), the production of vanillin as a food precursor, etc. Invertase and lysozymes are used as stabilizers and preservatives, respectively, which are approved by the EC as food additives. Some enzymes are added as food ingredients to increase nutritional value and some are added as processing aids that do not have any role in the final product. For the production of lactose-free milk products, lactases are used (Mateo et al., 2004). For getting definite characteristics for food enzymes such as α-amylase, peptide hydrolases, lipases, and catalases are used, which are deactivated at the final stage.

47.4.1 Amylases

Earlier thermophillic and mesophillic amylases were used in food industries, and recently these have been replaced by psychrophillic amylases because of their advantages. They play an important role in the breakdown of complex sugars into simpler ones while helping in the fermentation of wine and beer, bread making, and fruit juice processing. Cold active α-amylase was first isolated from *Alteromonas haloplanktis* and expressed in *Escherichia coli* (Feller et al., 1998). Later, cold active amylases from *Microbacterium foliorum* GA2 were isolated from the Gangothri glacier (Kuddus et al., 2012) and *Zunongwangia profunda* from marine bacterium (Qin et al., 2014). Alpha amylases isolated from *Bacillus licheniformis* with increased specific activity at low temperatures were granted as a patent to Novozymes in 2004 (Borchert et al., 2004).

47.4.2 β-Galactosidases

β-Galactosidases are valuable food enzymes that hydrolyze lactose into galactose and glucose, which helps in the production of lactose-free food products. It also helps in the degradation of lactose degradation in milk, cheese whey bioremediation, and sweetener production. Cold active enzymes form *Pseudomonas fragi*, which is used in cheese manufacturing and dairy products (Alquati et al., 2002). Cold active lactases active in refrigerated milk help in the hydrolysis of lactose, which reduces the production cost. β-Galactosidases was isolated from psychrophilic marine bacterium and characterized, with hydrolysis at 80% of lactose in raw milk at 20°C at a pH of 6.5 (Pulicherla et al., 2013). A patent was granted for cold active lactase active at ˂8°C (Schmidt and Stougaard, 2012). Tagatose, a novel sweetener, is produced by the help of β-galactosidases in which lactose is broken down into galactose and glucose, and then galactose is enzymatically transformed into tagatose. Cold active β-galactosidase was isolated from Antarctic marine bacterium *Pseudoalteromonas haloplanktis*, which hydrolyzes lactose from whey permeate that helps in tagatose production (Van de Voorde et al., 2014).

47.4.3 Pectinases

Pectinases degrade pectin, which plays an important role in the food industry such as fruit juice processing (clarification, vinification, reducing viscosity, and extraction of oils) (Adapa et al., 2014). Only a few pectinases are characterized as cold active pectinases. However, several pectinases show activity at low temperatures. For example, Novoshape (Novozymes) a pectinmethylesterase produced from *Aspergillus oryzae* and Pectinase 62L (Biocatalysts Ltd.), consists of polygalacturonase and pectin lyase. These enzymes are active at a wide range of temperatures from 10 to 60°C. A mixture of polygalacturonase, pectin esterase, and pectin lyase (Lallemand) active at 5–20°C is used in the clarification of juice, musts, and wine.

47.4.4 Xylanases

Xylanases help in the conversion of insoluble hemicellulose into soluble sugars before baking to get fluffy and voluminous loaves of bread with soft and elastic properties. It will be beneficial if the process is done at a low temperature. There are several advantages to using cold active xylanases over thermophillic and mesophillic xylanases (Collins et al., 2005). There are many reports on cold active xylanases from different organisms (Dornez et al., 2011; Wang et al., 2010; Zheng et al., 2014). Three psychrophilic bacteria producing cold active xylanases were isolated, which increased the bread volume up to 28% when compared with mesophillic xylanases (Dornez et al., 2011).

47.4.5 Proteases

In the food industry, the property of having high catalytic activity at a low temperature allows the transformation of heat labile products. It can be used in processes such as fermentation of fish or soy sauce with no spoilage or alterations in flavor and nutritional value. Cold active proteases along with lipases can be used as rennet substitutes to accelerate the ripening of slow-ripening cheeses. Additionally, cold active proteases can find utility in the softening and taste development of frozen or refrigerated meat products. Apart from this, the thermal lability of such proteases can result in rapid inactivation by mild heat treatment (Morita, 1975). This feature will prove beneficial in preserving quality in the food industry (Joshi and Satyanarayana, 2013). Protein polymerization and gelling in fish as well as improvement in food texture and flavor modification can also be carried out with cold active proteases (Cavicchioli and Siddiqui, 2004).

47.4.6 Lipases

Lipases have become an integral part of the modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years. Several cold active lipases have been reported to be used in food industries to improve processes. The selection of these enzymes is based on their biochemical parameters (Table 47.2). Microbial lipases are best utilized for food processing. A few, especially psychrotrophic bacteria of *Pseudomonas* sp. and a few molds of *Rhizopus* sp. and *Mucor* sp., cause havoc with milk and dairy products as well as soft fruit (Stead, 1986; Coenen et al., 1997). Cold

TABLE 47.2 Biochemical Properties of Cold Active Lipases and Other Enzymes Related to Their Application

Microorganism	Enzymes	Properties	Application	Reference
C. antarctica	Lipase	Stability up to 50°C for 2 h and Long chain alcohol	Synthesis of lipophilic antioxidants	Buisman et al., 1998
Acinetobacter sp. Strain no. 6		T_{opt} 20°C; ½ life: 30 min at 50°C	Laundry detergents additive and catalyst for organic syntheses at low temperatures	Suzuki et al., 2001
Psychrobacter okhotskensis sp. nov		T_{opt} 25°C; growth at 0%–10% NaCl solution	Synthesis of fatty acid ester, Reduces nitrate to nitrite	Yumoto et al., 2003
Aspergillus nidulans WG312		k_{cat} or V_{max} (temp) 29,640/min (40°C) 8829/min (0°C); K_m Substrate 0.28 mM (*p*-[Nitrophenyl phosphate]	Hydrolysis of esters of short- and middle-chain fatty acids	Mayordomo et al., 2000
Pseudomonas fragi		T_{opt} 29°C; good stability in the organic solvent	Cheese manufacture and dairy product	Alquati et al., 2002
C. antarctica		Stability up to 60°C	Synthesis of short chain flavor thio ester in solvent free medium	Cavaille-Lefebvre and Combes, 1997
Sclerotinia borealls	Polygalacturonase	K_m or V_{max} (temp.) 700 U/mg (5°C)	Cheese ripening, fruit juice, and wine industry	Lonheinne et al., 2001
Arthrobacter sp.		13,500/min and 15 min stability at 40°C	Food, health products	Takasawa et al., 1997
Cryptococus adellae	Xylanase	888/min (5°C)	Dough fermentation, wine and juice industry	Petrescu et al., 2000

active lipase is used in the production of fatty acids and the interestrification of fats (Jaeger and Eggert, 2002). An example of the application of a cold-adapted enzyme in nonaqueous biotransformation is the use of a lipase from *Pseudomonas* strain P38 for synthesis in n-heptane of the flavoring compound, butyl caprylate (Tan et al., 1996). *Candida antarctica* lipase is used in the synthesis of short chain flavor thio ester in a solvent-free medium (Cavaille-Lefebvre and Combes, 1997). Immobilized lipases from *C. antarctica* (CAL B), *Candida cylindracea* AY30, *Humicola lanuginosa*, *Pseudomonas* sp., and *Geotrichum candidum* were used for the esterification of functionalized phenols for the synthesis of lipophilic antioxidants to be used in

sunflower oil (Buisman et al., 1998). A whole-cell biocatalyst of mutated *C. antarctica* lipase B (mCAL B) by a yeast molecular display system and its practical properties were studied (Kato et al., 2008). When mCAL B was displayed on the yeast cell surface, it showed a preference for short chain fatty acids, an advantage for producing flavors.

47.5 FUTURE OUTLOOK

Cold habitats are a potential source for a diverse group of microorganisms adapted to low temperatures. There is a huge potential for exploring and identifying cold active enzymes from psychrophilic microorganisms to be used in the food industry. Additionally, a metagenomics approach can also help to identify novel biocatalysts that can replace mesophilic enzymes, which helps in huge energy savings. Further expressing the genes responsible for the cold active enzymes are expressed in expression systems for cost-effective production. These enzymes can be modified according to the needs of industries. A few attempts have been made to manipulate the catalytic properties of these enzymes. Cold active enzymes in the field of food processing is an emerging research field and is expected to attain remarkable success in the coming years.

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CHAPTER

48

Enzymes Used in the Food Industry: Friends or Foes?

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48.1 INTRODUCTION

Enzymes play an important role in the food industry in both traditional and novel products. Since ancient times, enzymes found in nature have been used in the production of food products such as cheese, beer, wine, and vinegar (Kirk et al., 2002). Enzymes, the natural catalysts for chemical reactions, are produced by all living cells. Their role in food processing has also been recognized for many centuries. Even before the knowledge about enzymes, they have been used in a number of processes, such as tenderization of meat using papaya leaves, soy sauce preparation, curd or cheese making, baking, brewing, etc. Enzymes provide a powerful, varied set of specialized tools for food and beverage preparation. Besides their catalytic ability, enzymes can enhance reactions by >1010 times. They can function exceptionally well to control process time, enrich flavor, improve texture, increase shelf life, and decrease the use of chemical food additives. One advantage of enzymes is their high activity, which makes them the best cost-effective choice of ingredients in addition to their reputation. Enzymes decompose complex molecules into smaller units such as carbohydrates into sugar. They are natural substances involved in all biochemical reactions. Because of the enzyme specifications, each substratum has corresponding enzymes. Enzymes are produced by all living cells and act as catalysts for specific chemical reactions. Enzymes are present in all foods at some time, and have played an important role in food processing practices for thousands of years (Dewdney, 1973). Adding exogenous enzymes to enhance reaction processes or to create new products has been going on since the early 20th century; gradually, it became a significant part of the food industry. From animals to plants to microbial sources, enzymes may be extracted from any living organism. Of the hundred or so enzymes being used in industries, more than half are of microbial origin. In the food industry, microbial enzymes have been extensively used to increase the diversity, variety, and quality of food. Microorganisms as enzyme sources are always preferred over other sources as large amounts of enzymes can be produced from them in a controlled manner that is also faster and cheaper. The chances of

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https://doi.org/10.1016/B978-0-12-813280-7.00048-7

other potentially harmful content (phenolics in plants or endogenous enzyme inhibitors and proteases in animals) are also minimized. Some of the important microbial enzymes used in the food processing industry are lipases, amylases, proteases, rennet, pectinases, invertases, cellulases, and glucose oxidase. Apart from these, many other enzymes such as raffinase, pullulanase, catalase, and lactase that have specific roles in the food industry are also being produced from microbial sources. Fungi *Aspergillus niger, Aspergillus oryzae, Saccharomyces cervisae*, and *Bacillus subtilis* bacteria are some examples of potential microbial sources that have been harnessed for production of many commercially important enzymes. This chapter deals with the types of microbial enzymes used in food processing and the food industry as well as their physicochemical and biological properties and industrial applications. The chapter also covers recent developments in this area.

48.2 MAJOR MICROBIAL ENZYMES AND THEIR APPLICATIONS IN THE FOOD INDUSTRY

The use of microorganisms in food preparation such as bread, curd, cheese, and alcoholic beverages has been done since ancient times. Some yeast, mold, and bacterial species have been highly explored and were found to be highly useful in the fermentation and mass-scale production of substances. Species of *Aspergilus* (*A. niger*), *Saccharomyces* (*S. cerevisiae*), *Mucor, Serratia*, *Bacillus* (*B. subtilis, B. amyloliquefaciens, B. licheniformis*), *Lactobacillus* (*L. casei, L. acidophilus*, *L. delbruekii*), *Corynebacterium* (*C. glutamicum)*, and *Rhizopus* (*R. oryzae*) are some significant examples of microbial species that are being extensively used commercially. Production of organic acids (citric acid, lactic acid, ascorbic acid, gluconic acid), enzymes (rennet, α-amylase, glucoamylase, glucose isomerase), amino acids (glutamate, methionine, glycine, lysine, arpartate, phenyl alanine), dairy products (yogurt, cheese, fermented foods such as idli, pickled vegetables), alcoholic beverages (beer, wine), and specialized microbial products (single cell proteins—SCP, flavor enhancers) is being done primarily with the use of microorganisms. The largest application of enzymes is in the food industry, and almost 50% of all enzymes produced for industrial purposes are used in food processing. Microbial enzymes predominate in this, comprising more than $2 billion in the world market. In the food industry, enzymes have been used to yield and to enhance the quality and variety of food. Ancient traditional knowledge of brewing, cheese making, meat tenderization with papaya leaves, and condiment preparation (e.g., soy sauce and fish sauce) depended on proteolysis, although the methods were established prior to our knowledge of enzymes.

48.2.1 Alpha-Amylases

Alpha-amylases belong to the family of *endo*-amylases and act on starch to yield glucose and maltose. The first starch-degrading enzyme was discovered in 1811, then named α-amylases by Kühn in 1925. Later, many other forms including β-amylases have been isolated and described. It belongs to two classes, namely, *endo*-amylases and *exo*-amylases, on the basis of the sessile glycosidic bond. Among the various extracellular enzymes, α-amylase is one of the most important industrial enzymes; it has been extensively used commercially. It has successfully replaced the chemical hydrolysis of starch in the starch-processing industries (Pandey et al., 2000; Reddy et al., 2003).

48.2.1.1 Sources of α-Amylase

Although α-amylases is widely distributed in various bacteria, fungi, plants, and animals as well as human beings, commercial production has been limited to only a few selected strains of fungi and bacteria. In animals, the pancreas and salivary glands are the main sources of α-amylase. In plants, it is generally present in the green parts, but grains and starchy parts have its maximum concentrations. β-Amylase is found only in plants and yields maltose as its major product. Among microorganisms, α-amylase is produced by several fungi and bacteria. The most widely used bacterial species is the mesophilic *Bacillus* spp., namely, *B. amyloliquefaciens* and *B. licheniformis,* which are extensively used for commercial production of the enzyme. *B. subtilis, B. cereus, B. stearothermophilus,* and *B. licheniformis* are also much explored as good producers of thermostable α-amylase (Coronado et al., 2000; Konsoula and Liakopoulou-Kyriakides, 2007). Halophilic amylases from halophilic bacteria such as *Chromohalobacter* sp., *Halobacillus* sp., *Haloarcula hispanica*, *Halomonas meridiana* (Kathiresan and Manivannan, 2006), and *Bacillus dipsosauri* have been reported with promising advantages in many harsh industrial processes where concentrated salt solutions are used. Fungal sources of α-Amylase are mostly the *Aspergillus* species and few species of *Penicillium*, such as *P. brunneum*, *P. fellutanum* (Erdal and Taskin, 2010).

48.2.1.2 Applications of α-Amylases in the Food Industry

Many industrial, environmental, and food biotechnology processes employ this enzyme at some stage or another. Besides being used as a major food source, starch is very much harvested and processed into a variety of products such as starch hydrolysates, glucose syrup, fructose, malto-dextrin derivatives, etc. Some important industrial applications of α-amylase are:

48.2.1.2.1 GLUCOSE AND FRUCTOSE PRODUCTION FROM STARCH

One of the major commercial uses of α-amylase is the production of glucose. The chemical conversion of starch into glucose syrup was developed in the early 19th century. For many decades, the diluted acid hydrolysis method was used for commercial conversion of starch into glucose syrup. However, by the late 1990s this was replaced by using a mixture of enzymes (Crabb and Shetty, 1999). The enzymatic process for starch conversion into high glucose syrup starts with liquefaction into short chain dextrins by the action of α-amylase from *B. amyloliquefaciens, B. stearothermophilus*, or *B. licheniformis*. Then saccharification to form a high concentration glucose syrup (>95%) is done by treating starch hydrolysate with fungal *exo*-glucoamylases for which *A. niger* is the primary source (Haq et al., 2010). Another significant industrial use of starch processing is the conversion of high glucose syrup into high fructose syrup. This process is carried out using the enzyme glucose isomerase. Fructose syrup is an important sweetener and additive used extensively in a wide variety of processed foods and beverages ranging from soft and fruit drinks to yogurts and breads (Parker et al., 2010).

48.2.1.2.2 BAKERY INDUSTRIES

In the baking industry, α-amylase plays a major role in the enhancement of quantity, aroma, and taste of the product. Being the main constituent of bread, starch causes hardness and makes it unpleasant to eat with age as the starch crystallizes. The addition of amylase and lipase enzymes in bread making reduces this crystallization and extends the shelf life (Jegannathan and Nielsen, 2013). The enzyme is frequently used during the preparation of

muffins, soft rolls, buns, and breads wherever additional characteristics are desired, such as dough conditioning or improved crust color. α-Amylase also affects antistaling in baking bread and helps to improve the softness (Gupta et al., 2008). All undesirable changes such as a decrease in moisture content and a loss of crispiness after removal from the oven are called staling. The mechanism of bread staling remains a major area of research and extensive study because of the huge economic loss to baking industries. Different supplementations are added during bread baking to reduce staling. The additives may be chemicals, enzymes, or their combinations. The substances that may be added include hydrocolloids such as sodium alginate, carrageenan, and xanthan; emulsifiers such as lecithin and sugar esters; and oxidants such as potassium bromate, ascorbic acid, etc. (Gray and Bemiller, 2003; Guarda et al., 2004; Spendler and Jorgensen, 1997). Enzymes have also been studied as bread improvers and find more acceptance from consumers as being a natural substance. Hemicellulase, lipase, protease, xylanase, and glucose oxidase are some of the examples of bread improvers. However, starch hydrolyzing enzymes, α-amylase, and branching and debranching enzymes were found to be of more use as antistaling agents (Cole, 1982). However, the use of α-amylase still remains out of favor because a small overdose results in sticky bread.

48.2.1.2.3 ALCOHOL INDUSTRY

Raw starch is extensively converted by hydrolysis and fermentation into ethanol, other distilled spirits, and biofuels. Starches such as grains and potatoes are frequently used as the substrate of ethyl alcohol (Juge et al., 2006). However, the conventional method of hydrolysis of starch to liquefy requires the use of strong chemicals (caustic soda, lime, and sulfuric acid) and heating procedures. The energy requirements for temperature control and measures of pH control add to the cost of such procedures (Gray et al., 2006; Robertson et al., 2006). Therefore, many processes have been described to maintain temperature and carry out hydrolysis, then the fermentation of starch, in one step (Reddy et al., 2009; Robertson et al., 2006). Yeast is the most widely utilized organism for industrial bioethanol production (Reddy et al., 2009; Rudolf et al., 2009) due to high productivity, better tolerance, and being in the generally regarded as safe (GRAS) category. A researcher has recently reviewed the possibility and development of starch-degrading amylases by different strains of yeast, recombinant *S. cerevisiae*, and its comparison with the exogenous addition of α-amylase. The production of raw starch-degrading amylases by recombinant *S. cerevisiae* provides opportunities for the direct hydrolysis and fermentation of raw starch to ethanol without cooking or exogenous enzyme addition. Such a consolidated bioprocess (CBP) for raw starch fermentation will substantially reduce costs associated with energy usage. *B. licheniformis*, engineered strains of *E. coli*, and *B. subtilis* are some of the good sources of thermostable α-amylases producing bacterial strains used for the hydrolysis of starch suspensions (Souza, 2010). The combination of α-amylases with gluco-amylase has been described to be the better option for improving raw starch fermentation to ethanol. The most effective mixture found was α-amylase from *Streptococcus bovis* and glucoamylase from *Rhizopus oryzae.*

48.2.1.2.4 FEED INDUSTRY

A major concern for the industrial production of animal feed is that it is not fully degraded and digested by livestock, which causes underutilization of feed. The protein and minerals are also not fully utilized. The undigested feed excreted by animals leads to environmental

problems as well. To improve this, enzymes are mixed in the animal feed during mass-scale production. α-Amylase, xylanase, phytase, and protease are mixed for the purpose (Jegannathan and Nielsen, 2013). The use of α-amylase in the feed industry is being done to improve the body weight gain and feed conversion ratio. The enzyme hydrolyzes the starch polymers into fructose and glucose, which increase the digestibility of carbohydrates (Sidkey et al., 2011; Silva et al., 2006).

48.2.2 Protease

Protease belongs to a group of proteolytic enzymes that is to hydrolyze peptide bonds of proteins. These enzymes are widely distributed in nearly all plants, animals, and microorganisms. The partial digestion by proteases produces peptide sequences that may have biological properties and important functional food ingredients (Udenigwe and Aluko, 2012). Proteases constitute one of the most important groups of industrial enzymes, capturing almost 60% of the total enzyme market (Mala et al., 1998). The vast variety of proteases, with their specificity of action and application, have been exploited widely in the detergent, food, pharmaceutical, and leather tanning industries (Gupta and Khare, 2007; Kalpana et al., 2008). This has been considered as ecofriendly because the appropriate producers of these enzymes for commercial exploitation are nontoxic and nonpathogenic.

48.2.2.1 Sources of Proteases

Proteases from all sources, that is, bacteria, fungi, virus, plants, animals, and humans, have been identified because of their important physiological roles. On the basis of site of action on protein substrates, they are broadly classified as *endo*-peptidases or *exo*-peptidases. Papain, bromelain, keratinases, and ficin are well-known proteases of plant origin (Mala et al., 1998). All these can be categorized into five major classes: cysteine, serine, aspartic, threonine, and metallo-proteases (Zamyatnin Jr., 2015). Papain, extracted from the latex of *Carica papaya* fruits, is the plant protease that has a long history of use. Bromelain, a cysteine protease, is extracted and purified from the fruits of pineapples. Other potential sources of plant erine-proteases are latex of *Wrightiatinctoria, Ipomoea carnea, Fistulosa, Euphorbia milii*, etc. (Guevara et al., 2001; Sanna and Sayed, 2001).

48.2.2.2 Proteases From Microbes

Microorganisms represent a brilliant source of enzymes because of the ease of genetic manipulation and extensive diversity. On an industrial scale, they are always preferred because of various advantages, such as lower manufacturing costs, large-scale production in industrial fermentors, extensive physical and chemical characteristics, rapid culture development, and lack of effect from seasonal variations. Microbial proteases are responsible for approximately 40% of the total worldwide enzyme sales. Most commercial neutral and alkaline proteases are produced by members of the genus *Bacillus*. Neutral proteases of bacteria are active at a narrow pH range (pH 5–8) and show relatively low thermotolerance. But due to their intermediate rate of reaction, these proteases cause less bitterness in hydrolyzed food proteins compared with the animal proteinases; therefore, they are used frequently in the food industry. *Pseudomonas* is a gram-negative bacterium that produces alkaline proteolytic enzymes. A variety of diverse proteases have been isolated from multiple strains of

Pseudomonas aeruginosa. Fungi produce a wider variety of proteolytic enzymes than bacteria, for example, *A. oryzae* produces neutral, acid, and alkaline proteases.

48.2.2.3 Applications of Proteases in Food Industry

Proteases are of great importance in the food industry because of their biochemical characteristics such as temperature, pH, metal requirement, activity, and stability. The cost of production, ease of development, markets, and the economy of applications are added advantages. The proteases have been regularly used for purposes such as cheese making, baking, making of soy hydrolysates, and meat tenderization.

48.2.2.3.1 DAIRY INDUSTRY

The important application of proteases in the dairy industry is in the manufacturing of cheese. The milk-coagulating enzymes are divided into four main categories: animal rennets, microbial milk coagulants, vegetable rennet, and genetically engineered chymosin. Both animal and microbial milk coagulating proteases belong to a class of acid aspartate proteases. In cheese making, the basic role of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-casein and macro-peptides. Chymosin is preferred because of its high specificity for casein, a major reason for its excellent performance in cheese making.

48.2.2.3.2 BAKING INDUSTRY

Proteinase from *A. oryzae* has been permitted in baking since 1952 (Lyons, 1982). When proteases are mixed in flour dough, they reduce mixing time and generate a bread loaf with better texture and crust color. Bacterial proteinases are derived primarily from *B. subtilis*. These powdered enzymes are very stable and remain 95% active for up to 1 year. Proteases have been used to degrade and decrease gluten content in bakery products (Heredia-Sandoval et al., 2016). Sourdough bread, a staple food of Central and Eastern Europe, is prepared by a long fermentation process that mainly uses lactic acid bacteria such as *Lactobacillus alimentarius, Lactobacillus brevis*, etc.

48.2.2.3.3 SOY SAUCE PRODUCTION

A salty seasoning agent, soy sauce is one of the most widely used Asian fermented products. It is prepared by microbial fermentation of soybeans and wheat in salt water. On an industrial scale, proteolytic and amylase enzymes are used in the digestion of deflated soybeans in salt brine. This significantly reduces the time for brine fermentation. Large amounts of protease and amylase enzymes are produced by the mold *A. oryzae* after a 48-h culture during soybean koji fermentation (Chancharoonpong et al., 2012). Further immobilized cells of *Pediococcus halophilus* bacterium, *Zygosacchromyces rouxii*, and *Candida versatilis* are used in bioreactors for lactic acid formation during soy sauce production (Luh, 1995).

48.2.2.3.4 BREWING INDUSTRY

In beer production, sugar fermentation is converted into alcohol. During mashing, enzymes from malt, which is germinated barley, act on the starch of different grains to form sugars. Jegannathan and Nielsen (2013) have described an alternative malting process that uses industrially produced amylase and protease enzymes for fermentation of starch. Because malting requires grains and heat for drying, bypassing this process will save energy as well

as agricultural land. *B. subtilis* proteases are used to solubilize proteins from barley adjuncts for production of wort. Haze formation due to proteinanceous substances in beer is also hydrolyzed by microbial proteases.

48.2.2.3.5 MEAT TENDERIZATION

The organoleptic quality of meat is of paramount importance for its marketability. Numerous publications have stated the importance of tender meat and consumers' willingness to pay more for such meat. Exogenous proteases such as collagenase from *Clostridium histolyticum*, aspartic protease from *A. oryzae*, thermophile protease from *Bacillus* strain, and caldolysin from *Thermus* strain are being used commercially to improve the tenderness of meat (Bekhit et al., 2014).

48.2.2.3.6 INDUSTRIAL PRODUCTION OF ASPARTAME

Aspartame, also known as Nutrasweet or Equal, is an artificial noncarbohydrate, zero-calorie sweetener that is the methyl ester of dipeptide L-aspartic acid and L-phenylalanine. Aspartame is an important ingredient in >5000 consumer goods and beverages worldwide. Therefore, it has an industrial production of 3000–6000 metric tons every year. Because the L-forms only synthesize sweet aspartame, the maintenance of the stereospecificity always adds to the cost of production by chemical methods. Thus, enzymatic synthesis of aspartame is carried out by proteases, which catalyze the reverse reaction to maintain stereospecificity of the two amino acids.

48.2.3 Lipase

Lipases are the biocatalysts that carry out esterification, interesterification, hydrolysis, alcoholysis, acidolysis and aminolysis as well as break down fats and oils. Due to their wide applicability, lipases become one of the most important industrial enzymes. Several sources of lipases having different catalytic properties have been identified.

48.2.3.1 Sources of Lipases

In eukaryotes, lipases are involved in various stages of lipid metabolism including absorption, lipoprotein metabolism, fat digestion, and reconstitution. If lipase is produced in sufficient quantity, it can use fat stores to be burnt as fuel. In animals, lipases are found in a wide diversity of sources such as blood, gastric juices, adipose tissues, intestinal juices, and pancreatic secretion.

In the plant kingdom, lipases appear to have wide distribution, where lipases are present in energy reserve tissues. Lipases of plants origin were isolated frequently from barley, corn, and cotton (Hasan et al., 2006). The seeds belonging to the families Euphorbiaceae, Ranunculaceae, and Papaveraceae have a high lipase content. Microbial lipases may originate from fungi, molds, or bacteria and most of them are formed extracellular, therefore they are easy to recover or isolate. Yeast such as *Candida* and *Torulopsis* and filamentous fungi such as *Rhizopus, Geotrichum,* and *Humicola* are some of the sources of extracellular lipases of microbial origin. Some of the lipolytic bacterial species are *B. subtilis, P. fragi, B. megaterium, S. aureus, Burkholderiacepacia, P. aeruginosa* (Sachan and Singh, 2015), and *P. pseudo alcaligenes*. Some other common lipase-producing fungal species are *Helvinal anuginosa, Rhizopus delemar,*

Eurotrium herbanorium, *A. niger*, *M. circinelloi*, and *Penicillium citrinum*. Microbial lipases are commercially important because of their low production cost, greater stability, and greater widespread availability than plant and animal lipases. Therefore, they are most useful for industries. Maximum production of microbial lipases on an industrial scale is being done by submerged culture as well as solid state fermentation.

48.2.3.2 Applications of Lipases in the Food Industry

Lipases form an integral part of the industries that include cosmetics, leather, textile, paper, pharmaceuticals, agrochemical, detergents, tea, and bioremediation processes along with almost every field of the food processing industry (Hasan et al., 2006; Treichel et al., 2009). The first recombinant and commercial lipase was isolated from the fungus *Thermomyces lanuginosus*, expressed in *A. oryzae*. Since then, many other recombinant lipases have been made for commercial purposes.

48.2.3.2.1 DAIRY INDUSTRY

In dairy industries, lipases are extensively used for the hydrolysis of milk fat. They are also used to enhance the flavor of cheeses in acceleration of cheese ripening, in the manufacturing of cheese products, and the lipolysis of cream. When acting on milk fat, lipases generate free fatty acids that lead to the production of many dairy products, mainly cheese (Aravindan et al., 2007). All blue veined cheese relies on *Penicillium roqueforti* and varieties of it for flavor enhancement. The major bacterial flora in a rennet cheese is the lactic streptococci.

48.2.3.2.2 BAKERY FOOD

In the baking industry, there is an increasing interest in lipolytic enzymes. They can be used to replace traditional emulsifiers as the enzymes degrade wheat lipids to produce emulsifying lipids in situ. It is basically used to improve the flavor of bakery products by releasing short-chain fatty acids through esterification along with flavor enhancement.

48.2.3.2.3 BEVERAGE PROCESSING

Barley is the most important grain in use for making beer all over the world. The total lipid content in barley ranges from 3% to 5% of a grain's dry matter. Lipase is used to hydrolyze the lipid of barley and also improve the aroma in alcoholic beverages such as sake. The Japanese company Tanabe Seiyaku has been using a patented lipase isolated from *R. delemar* or a *Candida* species during fermentation in the preparation of apple wine to improve the aroma and increased alcohol content.

48.2.3.2.4 MEAT OR FISH PROCESSING AND FOOD DRESSING

In the processing of meat and fish products, lipases are used for fat removal and flavor development. Lipases are also found in the fat of meat, fish, eggs, milk, and cereals. Micrococcaceae and Lactobacilli lipase are being used for flavor development and ripening of dry sausages. Some Micrococcus species were found to be lipolytic to pork fat while various volatile and nonvolatile fatty acids were identified in ripened sausages (Caserio and Gervasini, 1969). Lipases are being used extensively in mayonnaise, dressing, and whipping to improve the quality and texture.

48.2.3.2.5 LIPASES IN TEA PROCESSING

The quality of black tea is dependent mainly on the dehydration, enzymatic fermentation, and mechanical breaking. While processing black tea, the enzymatic breakdown of membrane lipids initiates the formation of volatile products with characteristic flavor properties (Verma et al., 2012). The exogenous addition of lipase secreted by *Rhizomucor miehei* has resulted in an increase in flavor volatiles and the aroma of tea (Ramarethinam et al., 2002).

48.2.3.2.6 LIPASES IN FAT AND OIL PROCESSING

Modification of fats and oils is one of the prime areas in the food processing industry. Chemical modification of fats and oils is not only nonspecific but also energy consuming whereas lipase-mediated modifications are highly specific and can be carried out under mild conditions. Microbial lipases, which can be used for converting cheap oils into nutritionally rich oils, low calorie tri-acylgylcerols, PUFA, and oleic acid-rich oils, have been extensively reviewed (Gupta et al., 2003). Unilever Ltd. has a patent for a mixed hydrolysis and synthesis reaction to produce a cocoa butter substitute using an immobilized lipase (Jaeger and Reetz, 1998). The removal of phospholipids in vegetable oils is also a recently developed environmentally friendly process.

48.2.4 Rennet

Rennet is a complex of enzymes containing chymosin, pepsin, and lipase. The enzyme is synthesized in a weaning ruminants' stomach to digest mother's milk. Industrially, rennet is the main enzyme in cheese making, where it is used to separate milk into solid curds for cheese making and liquid whey. All the manufacturing of cheese with a variety of flavors and textures is dependent on the rennet enzyme. In milk, casein accounts for 80% of total milk proteins and exists as a large organized soluble structure, termed the micelle. There are three major caseins (α, β and κ-casein) distributed all over the micelle. The percent of each type of casein varies according to the micelle size, but approximately it is α—55%, β—30%, and κ—15%. The smaller the micelle size, the higher the κ-casein content in the micelle. The action of rennet is on κ-casein, in which it splits the peptide bond between phenylalanine (Phe^{105}) and methionine (Met^{106}) to form glycomacropeptide with *N*-acetyl neuraminic acid (Morr, 1975). Rennet action is, however, inhibited if the milk is heated. The reduced availability of the peptide bond in the κ-casein for rennet may be the reason.

48.2.4.1 Sources of Rennet

The source of the animal rennet enzyme is the fourth stomach of young ruminants. It may contain 50%–95% chymosin, depending on the age of the animal (Addis et al., 2008). Plant origin proteolytic enzymes are also milk coagulants but the high proteolytic action decreases the cheese yield and increases the bitter taste in cheese (Lo Piero et al., 2002). Plant origin rennet may be used in the production of kosher and halal cheeses, but nearly all kosher cheeses are produced with microbial rennet. For commercial purposes, rennet is generally obtained from the mold *R. miehei*. Spain and Portugal have a great variety of cheeses that use Cynara sp. as a plant coagulant (Tejada et al., 2008). Initially, the mammalian rennet was exclusively used in the preparation of all types of cheese. But the demand for cheese production and the

need for a nonanimal derivative have led to substitute sources, such as microbial rennets. Presently, almost one-third of the cheese produced worldwide is through microbial rennets. Marketed since the 1970s, microbial rennets from various microorganisms have proved suitable for the production of different kinds of cheeses. These rennets are more proteolytic in nature in comparison to those isolated from animal sources and may result in the production of some bitter peptides during the cheese ripening (Fox and McSweeney, 1997). Therefore, efforts have been made to clone the gene for calf chymosin, and to express it in selected bacteria, yeasts, and molds. Microbial rennets have advantages over animal rennets such as bulk production, unlimited availability, less cost, and no risk of disease transmission, as may be in case of animal rennets. Many microorganisms have been identified as good sources of rennet. Microorganisms such as *R. pusillus*, *R. miehei*, *A. oryzae*, and *Irpex lactis* are widely used for the production of rennet in cheese making (Bailey and Siika-Aho, 1988; Escobar and Barnett, 1993; Neelakantan et al., 1999). Also the calf rennet or chymosin has been expressed in genetically modified microorganisms (GMOs) such as yeast or fungi (Dutta and Banerjee, 2006). The rennets by GMOs have been commercially produced since the 1980s in India; only the microbial rennet is being used as there is a ban on calf rennet (Pai, 2003).

48.2.4.2 Applications of Rennet in the Food Industry

Cheese is an extensively consumed fermented dairy product with a growing consumer demand. More than a hundred varieties of cheese are produced in the world. It is an excellent dietary source of protein, vitamins, and minerals such as calcium. The variety of cheese depends on the type of milk, the animal's diet, the butter fat content, bacteria and mold, and the processing and aging conditions of cheese (Fox et al., 1996; Miller et al., 2007). The use of the rennet enzyme in cheese production is one of the major applications of enzymes in food processing. Rennin acts on the milk protein in two stages, by enzymatic and by nonenzymatic action that results in coagulation of milk. Recently, Kethireddipalli and Hill (2015) have critically reviewed in detail the role of heat processed milk on rennet coagulation during cheese making as well as the addition of whey proteins to cheese milk. Mild heating conditions with some other process adaptations such as ultrafiltration and pH adjustments will lead to the production of cheeses with the desired organoleptic qualities and a higher protein content.

48.2.5 Catalase

Catalases (EC 1.11.1.6), one of the most studied enzymes, is also named hydroperoxidases. It is a tetrametric enzyme of four identified subunits of 60 kDa each. Catalase is one of the major enzymes which are important in revenue generation. One catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen per second.

48.2.5.1 Source of Catalase

Catalase is a common enzyme found in nearly all living organisms (Chelikani et al., 2004). Catalase enzymes are usually obtained from the bovine liver and microorganisms ((Tukel and Alptekin, 2004; Costa et al., 2001). Plants such as *M. sylvestris* L. leaves with high antioxidant properties, including radical-scavenging activity, are good sources of catalase (Barros et al., 2010). The enzyme has been studied, purified, and characterized from many plants such as black gram (*Vignamungo*) seeds (Kandukuri et al., 2012), cotton, sunflower (Eising et al., 1990), and pumpkin.

48.2.5.2 Industrial Applications of Catalase

Catalase has great industrial importance for its applications in the removal of hydrogen peroxide used as an oxidizing, bleaching, or sterilizing agent (Arıca et al., 1999; Ertaş et al., 2000). The enzyme can be used in a limited amount in cheese production. It is used in the food industry and also in egg processing, along with other enzymes. Catalase together with glucose oxidase is used in some food preservation, where superoxide dismutase, an antioxidant used in foods, produces H_2O_2, which is removed by catalase. Glucose oxidase and catalase are often used together in selected foods for preservation.

48.2.6 Lactases

Lactose, the sugar found in milk and whey, and its corresponding hydrolase, lactase, or β-galactosidase, have been widely researched in the past decade (Mehaia and Cheryan, 1987). This is because of the enzyme immobilization technique, which has given new and exciting prospects for the utilization of this sugar. Due to an insufficient intestinal enzyme, some individuals and/or populations show lactose intolerance and difficulty in consuming milk and dairy products. Therefore, a low-lactose or lactose-free food aid program is essential for lactose-intolerant people to prevent severe tissue dehydration and even diarrhea. Another advantage of lactase-treated milk is the improved sweetness of the milk, thus reducing the addition of sugars in the production of flavored milk drinks. Producers of ice cream, yogurt, and frozen desserts use lactase to increase scoop and creaminess, sweetness, and digestibility. Cheese manufactured from hydrolyzed milk ripens more rapidly than the cheese produced from normal milk.

48.2.6.1 Industrial Applications of Lactases

Lactose crystallizes easily, which restricts its use in the dairy industry. The use of lactase to overcome this problem cannot be exploited completely due to its associated high costs. Also, the discharge of large quantities of cheese whey pollutes the environment. However, the discharged whey could be utilized as a substitute source of lactose for the production of lactic acid by fermentation. The whey permeate, which is a by-product in the manufacture of whey protein concentrates, by ultrafiltration could be fermented efficiently by *L. bulgaricus* (Mehaia and Cheryan, 1987). Lactose can be isolated from several sources such as plants, animal organs, bacteria, yeasts (intracellular enzyme), or molds. Some of these sources are utilized for the preparation of the enzyme. Lactase production from *A. niger*, *A. oryzae*, and *Kluyveromyces lactis* is considered safe because it has been subjected to numerous safety tests and has a history of safe use. The most explored *E. coli* lactase is not used in food processing due to its cost and toxicity issues. The properties of the enzyme depend on its source. Temperature and pH depends on the type of source and the method of preparation. Immobilization of the enzymes, the procedure of immobilization, and the type of carrier can also affect these optima values. Universally, fungal lactase has a pH optima in the acidic range 2.5–4.5, and yeast and bacterial lactases in the neutral region 6–7 and 6.5–7.5, respectively. The difference in the pH optima of lactases marks them as appropriate for specific applications. For example, yeast and bacterial lactases are suitable for milk (pH 6.6) and sweet whey (pH 6.1) hydrolysis whereas fungal lactases are used for acid whey hydrolysis. Lactases from the *Bacillus* species are superior with respect to thermostability, pH operation range, product inhibition, and sensitivity against

high-substrate concentration. Its high activity for skim milk and less inhibition by galactose has made it suitable for use as a production organism for lactase (Gekas and Lopez-Levia, 1985). The enzymatic hydrolysis of lactose can be attained either by free enzymes (in the batch fermentation process) or by immobilized enzymes. Though various hydrolysis methods have been studied, only a few of them have been successfully scaled up and very few have been applied at an industrial level. Large-scale methods that use the free enzyme process have been established for the processing of UHT-milk and the treating of whey, using *K. lactis* lactase (Maxilact, Lactozyme). Beta-galactosidase is a very significant enzyme used in the dairy industry. It is involved in the hydrolysis of lactose into glucose and galactose with improved solubility and digestibility of milk and dairy products. Food thus obtained with a low lactose content is perfect for lactose-intolerant consumers (Mahoney, 1997; Pivarnik et al., 1995). It is also a good choice for consumers who have less tolerance to dairy products.

48.2.7 Cellulases

Cellulases enzymes break the glucosidic bonds of cellulose microfibrils and release oligosaccharides, cellobiose, and glucose (Dillon et al., 2004). Apart from the use of these hydrolytic enzymes in the food, drug, cosmetics, detergent, and textile industries, they are also used in wood pulp and paper industry, in waste management, and in the medical and pharmaceutical fields (Bhat and Bhat, 1997). In the food industry, cellulases are used in the extraction of constituents from green tea, soy protein, essential oils, aromatic products, and sweet potato starch. Together with hemicellulases and pectinases, these are used in the extraction and clarification of fruit juices. After the crushing of fruit, the enzymes are used to enhance liquefaction through the degradation of the solid phase. These enzymes are also used in the making of orange vinegar and in the extraction and clarification of citrus fruit juices. Cellulases complement pectinases in the juice and wine industries as extraction, clarification, and filtration services, with an increase in yield and flavor (Pretel, 1997). Cellulase is manufactured by a variety of fungal populations, such as *Trichoderma, Chaetomium, Penicillium, Fusarium, Aspergillu,s* and *Phoma*; aerobic bacteria, such as *Acidothermus, Bacillus, Celvibrio, Pseudonoma, Streptomyces, Staphylococcus,* and *Xanthomonas*; and anaerobic bacteria, such as *Bacteroides, Butyrivibrio, Caldocellum, Acetovibrio, Clostridium, Erwinia, Eubacterium, Ruminococcus, Pseudonocardia,* and *Thermo anaerobacter* (Soares et al., 2012). Filamentous fungi, *Aspergillus* stands out as key producers of cellulolytic enzymes. *A. niger*, a fermenting microorganism, produces cellulolytic enzymes, organic acids, and other products with great value by solid-state fermentation processes (Castro and Pereira Jr., 2010; Chandra et al., 2007; Couto and Sanroman, 2006).

48.2.8 Glucose Oxidase

Glucose oxidase (GOx) is an enzyme that catalyzes the oxidation of beta-D-glucose with the formation of D-gluconolactone. This enzyme consists of the prosthetic group flavin adenine dinucleotide (FAD), which allows the protein to catalyze oxidation-reduction reactions. Guimarães et al. (2006) accomplished a screening of filamentous fungi that could majorly yield glucose-oxidase. The result of the study showed high levels of GOx in *Rhizopus stolonifer* and *Aspergillus versicolor*. The literature states that the genus *Aspergillus* is a chief producer. This enzyme is used in the food industry for the elimination of harmful oxygen. Packaging materials

and storage environments are primarily responsible for the quality of products containing probiotic microorganisms because the metabolism of the microbial group is basically anaerobic or microaerophilic (Mattila-Sandholm et al., 2002). Therefore, the oxygen level during storage should be minimal to circumvent toxicity, the death of the organism, and the consequent loss of the product's functionality. Glucose oxidase may be a great strength to biotechnology to improve the stability of probiotic bacteria in yogurt without chemical additives.

48.2.9 Glucose Isomerase

Glucose isomerase (GI) (D-xylose ketolisomerase) catalyzes the reversible isomerase from D-glucose and D-xylose into D-fructose and D-xylulose, respectively. The enzyme is very significant in the food industry because of its uses in the manufacturing of fructose-rich corn syrup. Saprophytic bacteria has nutritional requirement for interconversion of xylose into xylulose by GI, which has a major application in the bioconversion of hemicellulose into ethanol. The hunt for GI thermostable enzymes has been the main goal of protein engineering (Hartley et al., 2000). Biotechnology plays a significant role in isolating mutants of promising prospects for the commercialization of the GI enzyme.

48.2.10 Invertase

Invertase is S-bD-fructo-furanosidase isolated from *S. cerevisiae* and other microorganisms. Hydrolysis from sucrose to fructose and glucose is catalyzed by this enzyme. The production of inverted sugar is one of invertase's numerous applications. Due to its sweetening effects, which are more than sucrose, it has great industrial significance and has good prospects for its use in biotechnology. Invertase is more active at temperatures between 40 and 60°C and pH ranging from 3 to 5. Microorganisms such as filamentous fungi are good producers of invertase with potential use in various industries. Soares et al. (2012) cultivated the filamentous fungus *Rhizopus* sp. in a wheat bran medium and obtained invertase. Another potential invertase-producing fungus is *Aspergillus casiellus*. It was inoculated in a soybean meal medium and after 72 h its crude extract was isolated. As most invertases used in the industry are produced by yeasts, the search for high yielding fungi is a requisite.

48.3 FRIENDS OR FOES

Very few enzymes show hazards, due to their catalytic activity, to those handling them in normal conditions but the major areas of risk are their source and chemical nature. These are allergenicity, activity-related toxicity, residual microbiological activity, and chemical toxicity.

All enzymes, being proteins, are possible allergens and have particularly strong effects if inhaled as a dust. Once an individual has developed an immune response as a result of inhalation or skin contact with the enzyme, reexposure produces increasingly severe responses that can become dangerous or even fatal. Because of this, dry enzyme preparations have been replaced to a large extent by liquid preparations. Enzyme producers and users recognize that allergenicity will always be a potential problem and provide safety information concerning

enzyme preparations. Liquid preparations are inherently safer but it is important that any spilt enzyme is not allowed to dry as dust formation can then occur. The formation of aerosols (e.g., by poor operating procedures in centrifugation) must be avoided as these are at least as harmful as powders. Activity-related toxicity is very rare but it must be remembered that proteases are possibly dangerous, particularly in concentrated forms, if inhaled. No enzyme has been found to be toxic, mutagenic, or carcinogenic by itself as might be expected from its proteinaceous structure. Enzyme preparations cannot be regarded as completely safe as such dangerous materials may be present as contaminants, derived from the enzyme source, or produced during its processing or storage. The organisms used in the production of enzymes may themselves be sources of hazardous materials and have been the chief focus of attention by the regulatory authorities.

48.4 FUTURE PROSPECTS

Being natural products, enzymes are always preferred by the consumers. The industrial demand for replacing chemical additives with enzymes and other such substances is increasing, especially as regards to preservatives and additives in food products. The use of enzymes has been successfully done for the production of gluten-free food, fiber-rich bread (Damen et al., 2012), glucose syrup, and natural sweetener. Microorganisms are being explored for the production of industrial enzymes because of the ease for bulk production and the reduction in cost. Various strategies are being employed for the purpose ranging from isolation of novel enzymes from microorganisms obtained from unique environment to genetically modifying organisms, rational protein design and protein engineering for recombinant proteins directed evolution to modify enzyme properties and high throughput screening to select proteins (Miguel et al., 2013).

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CHAPTER

49

Future Prospectives for Enzyme Technologies in the Food Industry

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49.1 INTRODUCTION

Enzymes are "green" biological catalysts that have altered the way we process our food. Enzymes are extensively used in various food and feed industries, widely covering baking, juice and beverages, brewing, meat, dairy, dietary supplements, vegetable processing, fats, and oils (Robinson, 2015; Schäfer, 2007). The desired traits such as thermostability, the ability to act at a wide pH and pressure range, nonmetal ion dependency, fast reaction rate, wide substrate utilization, and the ability to perform under the susceptibility of typical inhibitory molecules have been developed by using single or integrated approaches such as screening, rDNA technology, protein engineering, etc. (Fig. 49.1). The immobilization of enzymes has also enabled them to be employed more economically so they can be reused with minor loss, or without any loss in the activity (Fernandes, 2010). With the advancement in genetic engineering techniques, different genes encoding enzymes of interest could be transferred to and expressed in the required suitable host microbes for large-scale production. Today, the implementation of gene technology is of paramount significance for the production of the majority of industrial enzymes via novel enzyme discovery as well as optimization of the existing proteins. The usage of enzyme subsidies provides many benefits in food industrial applications, including improved product consistence and quality, reduced dependence on raw materials for processing, acting as a replacement for chemical food additives, and the prevention of potential harmful by-products in food (Schäfer, 2007). Novice enzyme technologies are also opening up new avenues for enzyme utilization to enhance the aromatic, structural, textural, and umami properties of food products. The use of genetic engineering augments the enzyme's extraction and purification as well as its large-scale production with cheaper costs and less energy utilization from plants and microbes to be used in food manufacturing and processing. With the advent of bioinformatics, in silico designing of novel enzymes and

https://doi.org/10.1016/B978-0-12-813280-7.00049-9

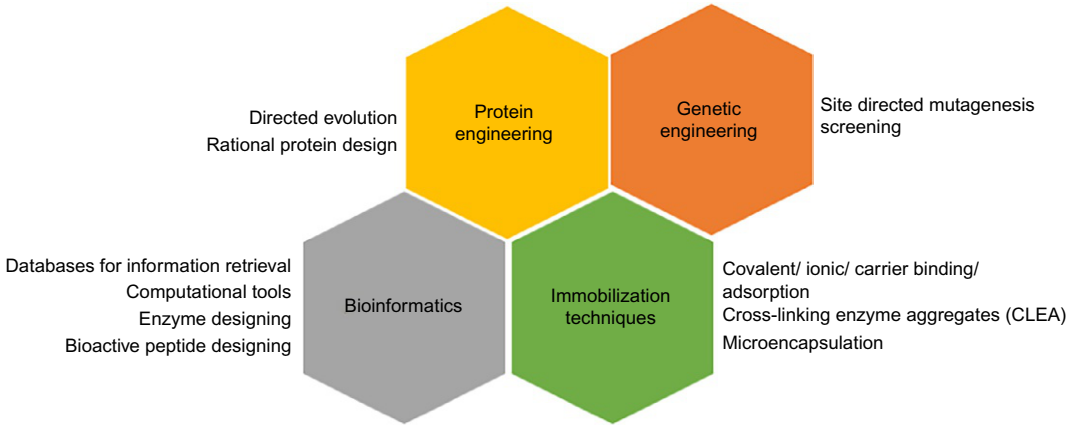

FIG. 49.1 Consolidated view of the techniques employed in enzyme technology and further dimensions.

enhancing the catalytic efficiency of existing enzymes also have a profound impact (Hilvert, 2013; Minkiewicz et al., 2016; Penning and Jez, 2001), ranging from food industries to other sectors for contribution, as in pharmacological and biomedical interventions (Alderson et al., 2012; Gurung et al., 2013).

49.2 BIOTECHNOLOGICAL TOOLS FOR IMPROVING ENZYMES

49.2.1 Recombinant DNA Technology

The utilization of recombinant DNA technology or genetic engineering has complemented the improvement in the microbial strains as well as the enzymes associated with food processing and fermentation techniques. Since the advent of this tool in the 1970s, it has completely revolutionized the food industry. Novel as well as known enzymes can be specifically tailored using this technology to produce enzymes with the desired specificity and sensitivity along with reducing the production cost of the enzymes. The considerable success in the development of improved microbial strains has led to many applications in the food industry, such as engineering microbial host strains to enhance enzyme yields by deleting native genes encoding for extracellular proteases. Certain fungal strains have been manipulated to reduce or eliminate their potential for releasing metabolites that are toxic to the final product formation (Olempska-Beer et al., 2006). Genetically engineered strains can be effectively implemented for various batch and continuous operations. This reduces the requirement for the enzyme extraction and purification and can be easily cultured using cheaper raw materials. The genetically improved strains in the production of high fructose syrup from starch is also made suitable for several variable parameters, such as compatibility with addition of high concentrated substrates in the intermediate steps of a multistep biotransformation, metal ions removal/addition step, processing temperatures, and pH adjustments. Therefore, this technique has increased productivity and reduced the cost connected with upstream product processing (Liu and Xu, 2008; Panesar, 2010). Other enzymes (such as α-amylases and pullulanases) used in starch hydrolysis are actually produced by utilizing

improved strains. Genetically modified (GM) microbial cultures, along with the production of enzymes, are also utilized for the production of monosodium glutamate (MSG), polyunsaturated fatty acids, and amino acids. However, the use of genetically improved strains is also restricted in some food technologies, owing to incongruity with production and purification procedures, lack of being certified as safe for consumption in the United States and the European Union, and the rising public consciousness regarding the nonconsumption of GMO or GMO-processed products (Agarwal and Sahu, 2014; Olempska-Beer et al., 2006; Spök, 2006).

The first commercial recombinant enzyme approved by the US Food and Drug Administration for used in food processing was bovine chymosin, involved in cheese manufacturing (Flamm, 1991). Another example of enzymes manipulated using genetic engineering (extensively reviewed in Olempska-Beer et al., 2006) and used in food processing includes alpha amylases engineered for increased heat stability for use in the production of high-fructose corn syrups. The Phospholipase A1 gene from *Fusarium venenatum* was expressed in GM *Aspergillus oryzae* to over-produce the phospholipase A1 enzyme. It is used in the dairy industry for cheese manufacture to improve process efficiencies and yields.

49.2.2 Screening

Screening methods aim for the isolation of enzymes from an extremophile source so that enzymes operating under a harsh environment for industrial applications should remain stable and show high activity, specificity, and turnover numbers (Asgeirsson and Kristjansson, 2002; Gomes and Steiner, 2004; Synowiecki et al., 2006). Among extremophiles, various thermophiles sourced from higher temperatures such as a hydrothermal vent are most commonly utilized for the food industries. These include alpha amylase, amylopullulanases, fructosyltransferases, glucoamylases, glucose (xylose) isomerases, glucosidases, inulinases, levansucrases, pullulanases, β-galactosidases, and xylanases in industries relying on starch hydrolysis. Examples of these referenced and various other enzymes sourced from extremophiles are extensively reviewed by Fernandes (2010) and Gomes and Steiner (2004). The isolation of bacteria from marine environments has also received great attention for sourcing other genes of interest, such as heat-stable invertase/inulinase from *Thermotoga neapolitana DSM 4359*, inulinase from *Cryptococcus aureus* (Dipasquale et al., 2009; Sheng et al., 2007), amylolytic enzymes, glucosidases and proteases from several genera (Mohapatra et al., 2003), esterase from *Vibrio fischeri* (Ranjitha et al., 2009), and glycosyl hydrolases (Giordano et al., 2006; Tramice et al., 2007). A few food industries such as dairy and meat need enzymes to be operated at a lower temperature, so screening for cryophiles has also identified potential microbes that are used in different applications such as milk processing with lactase for lactose-free milk (Nakagawa et al., 2006); cheese maturing and milk coagulation with proteases (Wang et al., 2008); clarification of fruit juices with pectinases to produce clear juice (Nakagawa et al., 2004); or production of oligosaccharides (Mao et al., 2010). However, screening approaches have the caveat that the microbes screened and identified are difficult to cultivate in laboratory conditions, so current genetic engineering methods are exploited to express the genes obtained from extremophiles in a mesophilic microbe. This is easy to culture in a lab, and protein extraction and purification can be done with ease.

49.2.3 Protein Engineering

With the vast amount of technology accessible through the latest automated PCR techniques for gene amplification and mutant insertion using a primer subset, a generation of random and site-specific mutagenesis has provided steadiness to different catalytic reactions with improvements in enzyme stability and tolerance to harsh temperatures, pH, and/or organic solvents (Chiang, 2004). A plethora of enzymes with different functional domains/motifs can be easily generated using recombinant tools. X-ray crystallography and NMR (Yee et al., 2002) can enable an enzyme's substrate binding/recognition sites, residues involved in an active site, and the whole landscape of the tertiary structure of an enzyme (Schneider et al., 2009). This information facilitates further improving its catalytic rate and modifying amino acids for enhanced substrate specificity. Two different approaches used in protein engineering are rational protein design (Tiwari et al., 2012) and directed evolution (Jackel et al., 2008); these might be used exclusively or in conjunction with each other. The rational design approach requires a prior knowledge of the structure and function of a protein to introduce the desired traits using site-directed mutagenesis (Singhania et al., 2010). Homology modeling, protein threading, and multiple sequence alignment methods are utilized to predict the overall structure and function of the enzyme (Poluri and Gulati, 2016). In directed evolution, prior information about the protein is not mandatory and random mutagenesis is performed via chemical mediation or by using error-prone PCR methods. Multiple rounds of mutation and selection are applied, mimicking natural evolution to produce variants with desired modifications (Jackel et al., 2008). The major impetus in applying the rational design approach is that very little effort is required to screen for the improved properties as a limited number of protein variants is generated (Singhania et al., 2010). For example, the mutant glucose isomerase generated utilizing this approach from *Actinoplanes missouriensis* displays similar enzymatic activity but with improved stability at different pH as well as enhanced thermal stability as compared to its parental enzyme (Quax et al., 1991). When the double mutant isomerase (G138P, G247D) was designed by the Zhu et al. (1999) group, a 2.5-fold increase in half-life and a 45% increase in the specific activity was displayed as compared to its wild type. This approach was also used by Lin and coworkers in creating amylase mutants from *Bacillus* sp. *strain TS-23* for enhancing the thermal stability of the enzyme (Lin et al., 2008). Xylanase obtained from *Trichoderma* sp. is also utilized on a large scale in the feed industry. By designed mutagenesis, its thermal stability has been enhanced about 2000 times at 70°C while the half-life has also increased from approximately 60 s to 4 min via stabilization of the alpha-helix region and the N-terminus (Fenel et al., 2004). On the other hand, directed evolution uses combinatorial methods to generate changes in a more random fashion and produce multivariants that need high-throughput methods to be validated for its function. Various biocatalysts such as proteinases, cellulases, α-amylases, lipases, and glucoamylases have been designed using the directed evolution approach (Singhania et al., 2010).

49.3 ENZYME IMMOBILIZATION

Enzyme immobilization has innumerable advantages, such as bringing thermal, operational, and storage stabilization of enzymes that need to be operated at adverse and variable environmental conditions; acting as a regulator for the extension of the reaction; and simplifying the

downstream application of product purification and separation from the biocatalyst. Enzyme immobilization precludes enzyme denaturation from autolysis or organic solvents and hence, can be loaded at higher amounts. The operation in bioreactors with immobilized enzymes can be automated and run as a continuous or a batch operation on a drain-and-fill basis, depending on user requirements. This is provided that enzyme immobilization has sufficiently encapsulated the enzyme without the loss of its activity. Immobilized biocatalysts can be easily recovered and reused, leading to improved productivity per enzyme unit as well as minimized substrate inhibition (Mateo et al., 2007; Sheldon, 2007; Swaisgood, 2003). Several applications of immobilized enzyme reactors routinely used in food industrial applications are (1) biocatalysts filled with immobilized glucose isomerase for the production of high-fructose corn syrup; (2) aminoacylase for the production of amino acids; (3) lactase for whey hydrolysates and for the production of tagatose; (4) invertase for the production of inverted sugar syrup; (5) lipases for the interesterification of edible oils, for the production of trans-free oils, cocoa butter equivalents, and for modification of triacylglycerols; (6) β-fructofuranosidase for the production of fructooligosaccharides; (7) isomaltulose synthase for the production of isomaltulose (Fernandes, 2010; Nakakuki, 2003; Swaisgood, 2003; Walsh, 2007).

Numerous alternate modes of immobilization are entrapment/microencapsulation, binding to a solid carrier via ionic or covalent interactions, cross-linking of enzyme aggregates, and adsorption methods (Brady and Jordaan, 2009). Adsorption (electrostatic, biospecific, hydrophobic) of enzymes to a matrix or support is more recommended than chemical immobilization. The matrix can be easily stripped from the enzyme, but it can be reloaded and reused and the steps are less intricate as compared to the covalent mode of immobilization (Swaisgood, 2003). However, the caveat of this approach is the desorption of the enzyme with time, leading to a decrease in the function activity. Various matrices with less-abrasive properties broadly used in immobilization techniques are agarose, Sephadex, Sepharose, nylon, glass, silica, celite, polystyrene, polyacrylamide, polyvinyl alcohol, polyethylene, and glycol derivatives (Cao, 2006; Datta et al., 2013). Glutaraldehyde cross-linking with the enzyme is an example of covalent immobilization as well as CLEA (cross-linked enzyme aggregates). However, the addition of a spacer has improved the enzyme activity by ensuring the enzyme reaction with the substrate by providing appropriate distance (LI and WALSH, 2000; NAM and Walsh, 2005). The entrapment method employs a polymer network of an organic polymer or a sol-gel (alginate, gelatin, k-carragenan, gellan), which can serve as a microcapsule or hollow fiber (Kailasapathy and Lam, 2005). Alternatively, enzymes are also encapsulated within liposomes according to the reaction specifications (Rodríguez-Nogales and López, 2006). Various modes and applications of the immobilized enzymes in the food industry are also broadly reviewed in Fernandes (2010).

There are some intrinsic disadvantages identified with enzyme immobilization techniques that vary from various strategies to various sorts of catalysts that are exemplified. Immobilization procedures can lead to the loss of enzyme activity because of chemical association or steric hindering of the dynamic site; mass transfer limitations; a chance of enzyme leakage amid bioreactor operation because of deterioration of the casted material in which the enzyme is encapsulated or cross-linked, or due to mechanical or chemical stress during the reaction. However in economic perspectives, the embedding of the expensive enzymes make the overall process less cost-intensive (Fernandes, 2010).

49.4 ENZYME INFORMATICS

49.4.1 Use of Bioinformatics Tool in Enzyme Engineering

Enzymes are prerequisites for performing metabolic reactions that are critical for the survival of microorganisms to living beings (Bairoch, 2000). The sheer knowledge and understanding of systems biology and metabolism rely on the detailed understanding of enzyme function and the activity–structure relationship (Li et al., 2010). Use of the bioinformatics tool and the advantage of its being in the public domain have helped in integrating and understanding vast amount of sequencing and structural data coming out of NGS (next generation sequencing), mass spectrometry, X-ray crystallography and NMR spectroscopy (Nuclear magnetic resonance) for enzyme informatics. The bioinformatics allow us to perform sophisticated computations analysis of the information present in enzyme databases in minimal time either using online or off the network (Alderson et al., 2012).

49.4.2 Resources for Retrieval of Enzyme Information

NCBI RefSeq and UniPort is a consortium of a large number of peptide and protein sequences; PDB contains three-dimensional (3D) structural information of thousands of proteins (Jin et al., 2015; The UniProt Consortium, 2008). Pfam is a protein family database sourced from EMBL-EBI (Xu and Dunbrack, 2012); CATH provides protein structure, evolution, function and conserved site information (Sillitoe et al., 2015). Many of the useful enzyme databases are interconnected so that information can be easily retrieved. ExplorEnz is a database solely dedicated to enzymes while also providing introductory resources for other databases (McDonald et al., 2009). BRENDA contains a plethora of enzyme information pertaining to biological sources, substrate specificity, kinetics, and chemistry (Chang et al., 2015). The networking maps present in KEGG (Kyoto Encyclopedia of Genes and Genomes) provide useful information on the role of enzymes in metabolic pathways (Kanehisa et al., 2017, 2016). Other relevant protein-related databases are MEROPS, the peptidase database at the Sanger Center (Rawlings and Barrett, 2000), CAZy, the carbohydrate-active enzymes database (Lombard et al., 2014), and ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins (Lenfant et al., 2013). EzCatDB (http://ezcatdb.cbrc.jp/EzCatDB/) interprets the enzyme catalytic mechanism and contains 878 enzyme reactions data (Nagano et al., 2015). The Catalytic Site Atlas (CSA) (http://www.ebi.ac.uk/thornton-srv/databases/CSA) catalogues the enzyme's active sites and catalytic residues identified in the 3D structure of the enzymes. The 3D template provided in the CSA is used to examine a protein structure for spatial signatures of enzymatic activity (Furnham et al., 2014). The Structure–Function Linkage Database (SFLD) provides a hierarchical classification of the evolutionarily related and divergently evolved enzymes. They also classify "unknown" sequences and annotate them while also mapping the shared functions to the conserved active site residues (Akiva et al., 2014; Brown and Babbitt, 2014). MACiE is another dataset evolved from CSA and provides in-depth information about the mechanism and the reactive center of the enzyme reactions, rate determining steps, and reversibility evidence (Holliday et al., 2007, 2012). The Human Metabolome Database (HMDB) is an exemplary database containing information supporting experiments involving a metabolomics approach (Wishart et al., 2013, 2009).

FooDB is also a comprehensive resource on biology, chemistry, and constituents present in food, including enzymes. Enzymes present in food can be assessed based on the mass spectrometry or nuclear magnetic resonance data (Scalbert et al., 2011).

49.4.3 InterPro for Automated Enzyme Function Prediction

For the automated annotation process, a manually curated dataset is a prerequisite. But manual curation of enzyme information in databases is inevitably a time-consuming task (Baumgartner et al., 2007). Specific tools based on the machine learning algorithm rely on the attributes (alias features), which are obtained via evolutionarily conserved patterns and sequence signatures that are already annotated with its complete information. A signature is specified with the annotations for its vivid structures, mutant information, in vitro or in vivo functionality check, and literature accessible for the designated enzyme or protein (Alderson et al., 2012). InterPro is a database that contains a boutique of signatures sourced from other databases such as: CATH-Gene3D, CDD, MobiDB, HAMAP, PANTHER, Pfam, PIRSF, PRINTS, ProDom, PROSITE, SFLD, SMART, SUPERFAMILY, and TIGRFAMs (Finn et al., 2017) as well as online software. InterProScan to match signatures to the sequences is also presented in their archives (Jones et al., 2014). Gene Ontology for its functional curation also relies on IntroPro. The InterPro2GO method assigns Gene Ontology terms that find a consistent match to InterPro signatures (Burge et al., 2012). The limitation of automated prediction is its dependency on manually curated datasets and the lack of validation unless an experimental approach is conducted for cross-checking (Furnham et al., 2009). EnzML also relies on InterPro signatures to predict multiple EC numbers on around 300,000 Swiss-Prot proteins, using a K-nearest neighbors multilabel algorithm (De Ferrari et al., 2012).

49.4.4 Computational Enzyme Designing

With the advent of in silico tools, enzymes can be redesigned to improve the structure–activity relationship. Important properties can also be deduced via comparison with the functionally annotated proteins and enzymes (Gro and Plaxco, 1997; Tao and Cornish, 2002). Enzymes can be engineered on the basis of activity, specificity, and stability. Two main applications of the enzyme designing approach involve de novo designing, where novel proteins can be structured and their catalysis can be interpreted using bioinformatics tools (Huang et al., 2016); and redesigning (Penning and Jez, 2001), where the natural protein is improvised for its catalytic power as well as the functional dynamics in the environment under which it operates. The *ROSETTA* and *ORBIT* are the most widely used software suites for de novo enzyme designing. Others recently surfacing include *WISDOM, EVODESIGN,* and *POCKETOPTIMIZER* (extensively reviewed in Damborsky and Brezovsky, 2014). Designed enzymes can catalyze nonbiological reactions, including multistep retro-aldol transformation, Diels-Alder cycloaddition, and proton transfer (Hilvert, 2013; Kries et al., 2013). These designed enzymes do not yet meet the efficiencies of natural enzymes, but can be improvised using the directed evolution approach. Other molecular modeling-based simulations are also performed to improve the kinetics of the existing enzymes, such as the software tool *CAVER* to analyze tunnel dynamics and buried active sites; *PRETHERMUT* to evaluate the free energy of unfolding; and *PAREPRO* to calculate the site evolutionary entropy (Chovancova et al., 2012; Damborsky and Brezovsky, 2014; Tian et al., 2013).

49.4.5 Application of Bioinformatics in Detecting Bioactive Peptides

Easy availability and utilization of inexpensive protein raw materials derived from by-products of dairy, meat, oilseed, fish processing meals, and seed storage proteins can be used to produce bioactive peptides that have the capability to ameliorate several health conditions pertaining to hypertension, hyperlipidemia, inflammation, diabetes, cancer, microbial infections, and immune disorders. Bioactive peptides are released during processing and consumption of food proteins by gastric digestion, endogenous and exogenous proteolysis, and by the action of the microbial enzyme in fermentation (Agyei and Danquah, 2012; Beermann and Hartung, 2013; Udenigwe and Aluko, 2012). Bioactive peptides can form a peptide-protein interaction to deviate abnormal protein functioning and have their applications in the maintenance of physiological homeostasis and enhancement of hepatic cholesterol metabolism (Howard and Udenigwe, 2013).

The bioinformatics approach for detecting the bioactive peptides encrypted in food proteins can make the process less daunting as compared to the conventional discovery method. BIOPEP (http://www.uwm.edu.pl/biochemia) and PepBank (http://pepbank.mgh.harvard.edu) are the reservoirs of bioactive peptide information and are used to identify new bioactive peptides of pharmacological activity from food proteins (Ahn et al., 2014; He et al., 2013). In silico proteolysis tools such as PoPS, BIOPEP, and ExPASy PeptideCutter can be used to determine the putative or de novo bioactive peptides from the protein sequences (Udenigwe, 2014). Several groups have used this approach to study the distribution of disease-related enzyme-inhibiting peptides within the primary structure of typical food proteins (Gu et al., 2011; Lacroix and Li-Chan, 2012). The presence of Cys- and Trp-containing peptides for antioxidant and therapeutic interventions was also identified after in silico hydrolysis of dioscorin (yam storage protein) with pepsin. In cellular systems, the peptides displayed better antioxidative and antiglycation properties compared to physiological peptides, glutathione, and carnosine (Han et al., 2014, 2013). The open-access PeptideRanker server (http://bioware.ucd.ie/wcompass/biowareweb) is a tool based on the N-to-1 neural network. It has the capability to rank large peptide sets and assign scores of 0 to 1 according to structural patterns that indicate the likelihood of showing bioactivity, with scores 0 and 1 being the least likely and most likely, respectively (Mooney et al., 2012). This integrated bioinformatics approach yielded ribulose-1,5-bisphosphate carboxylase, a plant photosynthesis enzyme, as a sustainable precursor for the formation of bioactive peptides (Udenigwe et al., 2013).

49.5 EXTENSIVE APPLICATIONS OF ENZYME TECHNOLOGIES TO PRODUCE, ENHANCE, OR MODIFY THE FLAVOR OF FOOD PRODUCTS

49.5.1 Usage of Vanilla and By-products for High-Value Flavor Molecules

Vanilla (*Vanilla planifolia* Andr.) is a very popular spice used in various food products. Is has a very pleasant fragrance and taste due to the generation of a fragrant fruit: the vanilla pod. The vanilla pod in its green state does not have the aroma that characterizes it.

The vanilla aroma arises only after the curing process, which can vary depending on the country and growing region (Baqueiro-Peña and Guerrero-Beltrán, 2017). The by-products of the vanilla extract were not utilized earlier, but in 2010, the enzymatic treatment and reutilization of the exhausted vanilla pods were reported. Exhausted vanilla pods contain only small amounts of aroma and flavor, but after treating them with various enzymes, the aroma and flavor can be extracted and potentially be used as food additives (Patent, 2010, US 7803412 B1: Enzymatic treatment of spent vanilla beans, United States). Flavorings can also be produced from vanilla bagasse by utilizing various microorganisms capable of producing enzymes that degrade the plant cell. The microorganisms capable of performing this biotransformation were *Bacillus* sp., *Escherichia coli, Streptomyces* sp., *Thermomyces* sp., *Chaetomium* sp., *Polyporus* sp., *Pycnoporus* sp., *Trametes* sp., *Myceliophthera* sp., *Rhizomucor* sp., *Candida* sp., *Aspergillus* sp., *Trichoderma* sp., and *Mucor* sp. (Patent, 2011, US 2011/0318805 A1: Microbial transformation of spent vanilla beans, United States). Furthermore, artificially synthesized vanillin has also been produced from several industrial by-products. Barbosa et al. (2008) prepared a system of coconut waste with strains of *Phanerochaete chrysosporium* (mold). As the result of biotransformation, they obtained 44.4 μg vanillin per gram of residue in 24 h of fermentation. But, with prior pretreating of the coconut through sun drying and mechanical pressing, the vanillin yield increased to 52.5 μg vanillin per gram of residue. Considering the cost of extraction and curing of natural vanilla, the economic benefit of this process is quite promising.

49.5.2 Utilizing Enzymes for Enhancing the Umami Taste

Umami is recognized as the fifth basic taste after sweet, bitter, sour, and salty and is characterized by a savory flavor that is naturally present in many plant- and animal-based foods. Due to its savory taste, umami has the potential to reduce dietary intake of sodium, thus making the food richer in taste and reducing the fat and salt intake (Imada et al., 2014; Miyaki et al., 2016). Mushrooms are known to be rich in proteins (Kalac, 2013) while containing significant amounts of free glutamic and aspartic acid, which are found to elicit umami flavor (Zhang et al., 2013). In order to improve the taste-contributing free amino acid contents, these proteins need to be hydrolyzed. Flavourzyme contains both *endo*- and *exo*-peptidase activities that are suitable to yield greater amounts of free amino acids while also producing flavor-active amino acids and peptides from proteins (Berends et al., 2014; Merz et al., 2015). Consequently, the treatment of mushrooms with the cell-wall degrading enzyme β–glucanase and the protease Flavourzyme significantly enhanced the umami and other taste-contributing free amino acids (Poojary et al., 2017). For ages, squid have been used in savory dishes as a flavor enhancer. The head of the dried squid, obtained as a by-product of the dried squid snack industry, had also been reported to contain rich protein content and a high quantity of umami amino acid and glutamic acid (7.45 mg/100 mg). Enzymatic hydrolysis of the dried squid head was also achieved at the optimal pH and temperature to produce a flavored-functional protein hydrolysate using Flavourzyme (Sukkhown et al., 2017). Freeze drying of the hydrolysate further retains the antioxidant property as well as volatile compounds enriching the flavor. Moreover, hydrolysis of seaweed (*Gracilaria* sp.) by-products by using the bromelain enzyme also yields seafood-like flavor peptides (having arginine, lysine, and leucine amino acids) (Laohakunjit et al., 2014).

49.5.3 Enzymatic Hydrolysates of Fish-Proteins as Flavor Enhancers

Extensive studies have shown that fish protein hydrolysates have good nutritional properties and thus can be used widely in the food industry for various purposes. These include texturing, gelling, foaming, emulsification, protein supplements, flavor enhancers, surimi production, and beverage stabilizers (Kristinsson, 2007). Enzyme hydrolysis is more convenient and productive as compared to chemical hydrolysis (Witono and Kang, 2010). Enzymatically produced bibisan fish hydrolysates also have excellent water-holding capacity because of the increased concentration of polar groups (carboxyl, COOH and amide, NH2 groups) generated by enzymatic hydrolysis (Kristinsson and Rasco, 2000; Wasswa et al., 2008). The inexpensive source of protease enzymes extracted from Biduri plants (*Calotropis gigantea*) used in Indonesia is a promising biomolecule for enzymatic hydrolysis. Its application as a flavor enhancer was demonstrated and enriched in inferior fish substrates by synergizing with papain and cysteine (Witono et al., 2011). To improve the functional properties of proteins, enzymatic modification in the hydrolysis of the substrate has been extensively employed. The higher activity of the papain tends to increase the primary amine groups by magnifying the short chain proteins. The enzymatic hydrolysis of bibisan fish by using the protease enzyme from the biduri plant and papain also resulted in the formation of protein hydrolysates having a low moisture content (9%–10%), the least lipid (0.03%) content, and a high protein content (63%–75%) (Witono et al., 2014). Hydrolyzing the fish protein by the biduri protease and papain in the ratio of 70:30 in the presence of the activator sistein (0.6%) further demonstrated good antioxidative activity (about 7%). This was due to the ability to scavenge lipid radicals, decrease the level of rancidity and protein solubility, and generate bioactive peptides (1.2 g/100 g) and the flavor enhancer glutamate (0.04 g/100 g) (Mananda et al., 2016).

49.5.4 Formation of Taste-Active Amino Acids, Amino Acid Derivatives, and Peptides in Food Fermentations

Most fermented foods are valued for their rich and specific odor and taste (Hutkins, 2008). Taste-active amino acids, their derivatives, and their peptides are generated during food fermentation in bread, cheese, soy sauce, and fermented meats by proteolysis or autolysis. Peptides particularly impart the umami taste (e.g., α-glutamyl peptides) or bitter taste (e.g., hydrophobic proline containing peptides) (Zhao et al., 2016). Common examples of taste-active peptide derivatives include γ-glutamyl peptides, pyro-glutamyl peptides, and succinyl- or lactoyl amino acids. Pyro-glutamic acid can be released form the N-terminus of proteins and peptides by the action of a specific enzyme, l-pyro-glutamylpeptide hydrolase (Mucchetti et al., 2000). Succinyl amino acids such as suc-Arg and suc-Glu, obtained from arginine catabolism or from fungal succinyl transferase activity, have also been reported to impart the umami taste (Frerot and Chen, 2013). In the case of fermentation of meat and sourdough, endogenous enzymes from meat and cereals determine proteolysis (Gänzle et al., 2008; Ordóñez et al., 1999). During the production of ham and sausage, endogenous enzymes of meat such as dipeptidyl peptidases (DPP) and cathepsin B mainly contribute to the proteolysis process (Ordóñez et al., 1999; Sentandreu et al., 2003). However, as ham ages and ripens, the content of Met, Asn, and Ile amino acids or dipeptides such as Ile-Val, Leu-Gly, Ile-Asp, and Pro-Leu increases due to the high *endo*-peptidase activity and imparts bitterness (Sentandreu et al., 2003;

Sforza et al., 2006). In fermented foods, glutamate results from the proteolysis or glutaminase activity, which imparts the umami or savory taste, if the concentration of glutamate in the food products is above the taste threshold of about 1 mM. It has been reported that γ-Glutamyl transferase (GGT) obtained from *Bacillus* and *Aspergillus* convert the glutamine present in soy sauce into glutamate and thus provides the specific savory taste of soy sauce (Ito et al., 2013; Lioe et al., 2010; Minami et al., 2003a,b). In the case of cheese ripening, the bitterness of cheese was evaluated on the parameter of unbalanced levels of proteolysis and peptidolysis by enzyme proteinases from lactic acid bacteria (LAB) (Fallico et al., 2005). Contributing factors for the specific taste of the cheese are amino acids (such as glutamate), small peptides, and amino acid derivatives such as pyro-glutamyl and lactoyl amino acids (Andersen et al., 2010; Drake et al., 2007).

49.6 CONCLUSION

The incorporation of biocatalysts in the food and feed industries has fostered various approaches and enhanced the various properties of food as well as the techniques for manufacturing food products and processing. Hitherto improvements are still under progress for further designing of novel and improved enzymes. Diversified efforts in the improvement of enzymes have been confirmed to sustain its structure-function-activity in relation to aggressive and variable environmental factors (such as temperature, pH, organic solvents, metal ions, inhibitory agents) of the bioreactor conditions. The utilization of purified enzymes without culturing of microbes is also an advent in further downstream procedures; it also eliminates the risk of microbial contamination in food processing. With advancements in biotechnology tools such as genetic engineering, protein engineering, and computational tools, commendable attempts have potentially broadened the scope of enzyme utilization in various industries, including food, leather, textile, paper, detergents, biofuel, and pharmaceutical.

Perpetual improvements in the enzyme immobilization techniques have also been a key supporting factor for the efficient usage of enzymes; they also aim at making the process more economical. Compelling attempts in the implementation of screening, high-throughput methodologies, and automated procedures also demonstrate the timely and efficient characterization of the biocatalysts and a more refined large-scale production of the products. Thus, it can be anticipated that significant endeavors made toward the enzyme technologies have broadened our knowledge and is in continuum to expand further.

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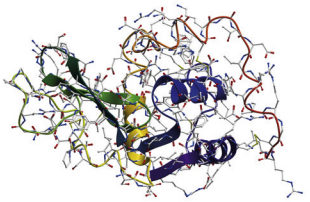
Enzymes in Food Biotechnology



Production, Applications, and Future Prospects

Edited by
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Enzymes in Food Biotechnology: Production, Applications, and Future Prospects, presents a comprehensive review of enzyme research and the potential impact of enzymes on the food sector. This valuable reference brings together novel sources and technologies regarding enzymes in food production, food processing, food preservation, food engineering, and food biotechnology useful for researchers, professionals, and students. Food applications include process of immobilization, thermal and operational stability, increased product specificity and specific activity, enzyme engineering, implementation of high-throughput techniques, screening to relatively unexplored environments, and development of more efficient enzymes.

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- Explores recent scientific research to innovate novel ideas for new foods and enzyme engineering worldwide
- Provides fundamental and advanced information on enzyme research for use in food biotechnology including microbial, plant, and animal enzymes
- Includes recent cutting-edge research on the pharmaceutical uses of enzymes in the food industry

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ISBN 978-0-12-813280-7

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An imprint of Elsevier
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