Abhishek Dutt Tripathi
Kianoush Khosravi-Darani
Suresh Kumar Srivastava *Editors*

Novel Food Grade Enzymes

Applications in Food Processing and Preservation Industries

Novel Food Grade Enzymes

Abhishek Dutt Tripathi •
Kianoush Khosravi-Darani •
Suresh Kumar Srivastava
Editors

Novel Food Grade Enzymes

Applications in Food Processing and Preservation Industries

Editors
Abhishek Dutt Tripathi
Department of Dairy Science & Food Technology
Banaras Hindu University
Varanasi, Uttar Pradesh, India

Kianoush Khosravi-Darani
Department of Food Technology Research
National Nutrition and Food Technology Research Institute
Tehran, Iran

Suresh Kumar Srivastava
School of Biochemical Engineering
Indian Institute of Technology BHU
Varanasi, India

ISBN 978-981-19-1287-0 ISBN 978-981-19-1288-7 (eBook)
https://doi.org/10.1007/978-981-19-1288-7

© The Editor(s) (if applicable) and The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
This work is subject to copyright. All rights are solely and exclusively licensed by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Enzymes play a significant role in pharma, agriculture, and other allied industries. In the modern era, there is significant application of enzymes in food processing industries and our proposed book will give deeper insight into food applications of enzymes. This book covers all the aspects of enzyme including its classification, kinetics, microbial production, biosynthetic pathways, commodity-wise industrial applications, and its downstream processing strategies. The broad focus of this book will be on the application of various classes of enzymes in dairy, fruits and vegetable, cereals and oilseeds, meat and poultry, brewing and food packaging industries. The first chapter of this book gives a brief introduction about enzymes classifications and their kinetics. Chapters 2, 3, 5, 6, 7, and 8 provide information regarding enzyme applications in different food commodities like fruits and vegetables, dairy, beverage, and meat processing industries. These chapters also focus on different food grade enzyme biosynthesis mechanism in simpler and illustrative way. Besides that, this book will also allow the readers to understand the biosynthesis, production, optimization, and purification of certain less known enzymes such as CGTase and naringinase. Chapters 9 and 12 of this book will be giving some novel information related to application of enzymes specifically for improvement of flavor, color, organoleptic attributes, shelf-life extension, and consumer acceptability of value-added diversified food products. Chapters 10 and 11 also cover some of the very interesting aspects of enzymes application in the food packaging and development of biosensors which will be highly beneficial for those researchers and students who are interested in learning the food safety issues and indulged in product quality improvement. Enzymes play a significant role in the development of GM (Genetically Modified) food production. Chapters 13 and 14 provide detailed information related to enzymes application in GM foods. Chapter 15 highlights the various enzyme infusion strategies adopted in the food processing industries that will enable the students and entrepreneurs to understand the basic concept of enzyme activity and stability after infusion in food. Future prospects of enzymes and their nanotechnological applications have been discussed in Chapters 16 and 17 of this book. The illustrated case studies related to certain novel enzymes will allow the academicians and researchers to broaden their knowledge and will provide an opportunity to carry out extensive research work pertaining to the applications of such enzymes. The authors contributing to this book have already done extensive

research work in the proposed area and the contributed chapters will also be highlighting some of the salient research work already published in the reputed journals by internationally recognized authors in the proposed area. The flowchart described in certain chapters will provide all the necessary information related to the production, optimization, and characterization of food grade enzymes in simpler manner. This book will not only cover the basic enzyme kinetics part but also provide information related to the efficient enzyme recovery and purification process. It also gives broad information on the factors affecting enzyme activity and stability as well as involvement of enzyme in the development of functional food combating chronic diseases and disorders.

As per our survey, only few books are available on the role of enzymes in food processing although this is a prominent area of study and needs to be covered in detail. The earlier books primarily focused on the general enzyme properties and its application. However, no deep insight knowledge pertaining to the commodity-wise application of food grade enzymes has been previously illustrated. We have tried to cover all the aspects of food grade enzymes including its general properties, kinetics, wide application in all the food processing and packaging industries. Although numerous books on medical and therapy-based application of enzymes are available in the market, no books on wider perspective of food grade enzymes applications are available till now. The simpler language, clearly illustrated concepts, and broadly discussed case studies related to various food grade enzymes will enable the readers to strengthen their concept and knowledge. This book will be beneficial for the faculty members, undergraduate, postgraduate, and PhD students of Microbiology, Food Technology, Dairy Technology, Horticulture, Biotechnology, Biochemistry, and other allied discipline of Life Science. This book will also be beneficial to those authors who are indulged in the research activities related to the above-mentioned subject areas such as food science, enzyme and fermentation technology, bioprocess technology, and biochemical engineering. The involvement of foreign authors and editors will provide deeper insight about the subject knowledge and will improve the quality of book content making it readable and acceptable at global level. I hope the readers will enjoy reading this book and will develop interest in the area of enzyme technology.

Varanasi, Uttar Pradesh, India — Abhishek Dutt Tripathi
Tehran, Iran — Kianoush Khosravi-Darani
Varanasi, Uttar Pradesh, India — Suresh Kumar Srivastava

Acknowledgments

I am grateful to my co-editors, Prof. Suresh Kumar Srivastava, School of Biochemical Engineering Indian Institute of Technology (IIT), Banaras Hindu University, India, and Prof. Kianoush Khosravi-Darani, National Nutrition and Food Technology Research Institute of Iran for providing the immense support in compilation of this book. I am thankful to all my authors from India, Iran, and Nigeria who contributed different chapters for this book as per their expertise. I am grateful to Springer Nature group for selecting our book proposal and permitting us to complete this task. I am thankful to external reviewers who approved the book proposal after thorough evaluation. I pay my sincere gratitude to our Director and Esteemed Dean for their constant encouragement. I am grateful to my Head of the Department Prof. Dinesh Chandra Rai, Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, India, for his immense motivation and providing necessary facilities required for compilation of this book. I would like to pay my sincere thanks to my parents Shri Vishnu Dutt Tripathi and Mrs. Urmila Tripathi for their encouragement and support. I would like to pay my sincere thanks to my beloved wife Mrs. Priyanaka Upadhyay and my kids Master Ojas and Baby Prajul for their kind support and cooperation. Lastly, I am thankful to almighty who blessed me with the completion of this task.

Contents

1 **Food Enzymes: General Properties and Kinetics** 1
S. M. Khade, S. K. Srivastava, L. H. Kamble, and J. Srivastava

2 **Plants- and Animal-Derived Enzymes and Their Potential Application in Food Processing and Preservation** 17
Mahmoud Aminlari

3 **Role of Enzymes in Fruit and Vegetable Processing Industries: Effect on Quality, Processing Method, and Application** 65
Memthoi Devi Heirangkhongjam, Kanika Agarwal, Aparna Agarwal, and Nidhi Jaiswal

4 **Production of α, β, and γ-Cyclodextrin Gluconotransferase (CGTase) and Their Applications in Food Industry** 107
Rizul Gautam and Shailendra Kumar Arya

5 **Enzyme in Milk and Milk Products: Role and Application** 139
Aparna Agarwal, Naman Kaur, Nidhi Jaiswal, Memthoi Devi Heirangkhongjam, and Kanika Agarwal

6 **Enzymes in Brewing and Wine Industries** 165
S. Pati and D. P. Samantaray

7 **Enzymes in Meat, Fish, and Poultry Products Processing and Preservation-I** 183
Khadijeh Abhari and Hedayat Hosseini

8 **Enzymes in Meat, Fish, and Poultry Product Processing and Preservation-II** 193
Sandesh Suresh Karkal, Anushma Venmarath, Suresh Puthenveetil Velappan, and Tanaji G. Kudre

9 **Enzymes in Functional Food Development** 217
Iran Alemzadeh, Asma Sadat Vaziri, Kianoush Khosravi-Darani, and Pierre Monsan

10 Enzymes as Active Packaging System 253
Amir Heydari and Negin Mahmoodi-Babolan

11 Enzymes as a Tool in Food Analysis and Foodborne Pathogen Detection 265
Preethi Sudhakara, Jerrine Joseph, S Priyadharshini, Jemmy Chirsty, Alex Anand, Davamani Christober, and Aruni Wilson Santhosh Kumar

12 Enzymes in Flavor Development and Food Preservation 317
Fataneh Hashempour-Baltork and Parastou Farshi

13 Enzymes from Genetically Modified Organisms and Their Current Applications in Food Development and Food Chain . . . 357
Senthilkumar Muthusamy, Shilpa Ajit, Asha V. Nath, J. Anupama Sekar, and T. S. Ramyaa Lakshmi

14 Current Applications of Enzymes in GM (Genetically Modified) Food Development and Food Chain 383
Nafiseh Sadat Naghavi, Fatemeh Mahmoodsaleh, and Masoumeh Moslemi

15 Enzyme Immobilization and Its Application Strategies in Food Products 411
Nafiseh Sadat Naghavi, Nazanin Sanei, and Martin Koller

16 Prospects and Challenges in Food-Grade Enzymes Industrial Production 439
Musliu Olushola Sunmonu and Mayowa Saheed Sanusi

17 Nanotechnology and Food Grade Enzymes 455
Zahra Beig Mohammadi, Khadijeh Khoshtinat, Sanaz Ghasemi, and Zahra Ahmadi

About the Editors

Abhishek Dutt Tripathi is an assistant professor in the Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, India. He has published more than 75 research papers and articles in journals of national and international repute. His field of specialization is food chemistry, enzyme technology, fermentation technology, and food microbiology. He has also published two books, two manuals, and many book chapters associated with food and bioprocess technology. He has received several prestigious national and international awards and is also serving as editor and reviewer with many international and national journals.

Kianoush Khosravi-Darani (co-editor) is a professor at the National Nutrition and Food Technology Research Institute of Iran. She has more than 20 years of teaching and research experience. She has published more than 125 research papers in journals of national and international repute. She is editor-in-chief of the journal *Applied Food Biotechnology* indexed in web of science. She has received a number of prestigious awards in the field of food biotechnology. She has done extensive research work in the area of food science and technology, enzyme and bioprocess technology, and biopolymers. She has published number of books in reputed publication.

Suresh Kumar Srivastava is an institute professor at the School of Biochemical Engineering, Indian Institute of Technology (BHU), India. He has more than 30 years of teaching and research experience. He has published more than 70 research papers in journals of national and international repute. He has received number of prestigious awards from different government organizations and held prestigious positions in board of studies at different Indian Universities and also served as member of different administrative committees related to R&D. His area of specialization in enzyme engineering, enzyme technology, food technology, and biochemical engineering.

Check for updates

1 Food Enzymes: General Properties and Kinetics

S. M. Khade, S. K. Srivastava, L. H. Kamble, and J. Srivastava

Abstract

Enzymes are the biocatalysts having a catalytic power that speeds up a chemical reaction without changing the equilibrium of the reaction. Almost all enzymes are protein in nature and the biocatalytic power lies in the integrity of their structural conformation. The enzymes are highly specific with their substrate molecules that convert into the product by decreasing the activation energy, without getting changed itself. However, the biocatalytic power of the enzymes also depends on several physico-chemical parameters such as temperature, pH, salt concentrations, etc. of the reaction. The enzyme catalysis can be quantitatively revealed by the enzyme kinetics mechanism which measures the reaction rates and the affinity of enzymes towards the substrates and inhibitors. The enzymes can be isolated from the various biological factories such as plant, animal, or microbial cells depending upon the type of enzyme. However, in order to produce at large scale, the microbial sources are the best choice which can effectively reduce the production and purification cost of enzymes. The enzymes are widely used in various sectors such as agriculture, environmental, leather tanning, paper and pulp, chemical and pharmaceutical, detergent, food and beverages, etc. In this chapter, we are mainly focussing on the role of enzymes in the food industry.

S. M. Khade (✉) · J. Srivastava
School of Engineering, Ajeenkya D Y Patil University, Pune, Maharashtra, India

S. K. Srivastava
School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University), Varanasi, Uttar Pradesh, India

L. H. Kamble
School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded, Maharashtra, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_1

Keywords

Enzyme · Biocatalyst · Substrate · Physico-chemical parameters · Enzyme kinetics · Inhibitors

1.1 Introduction

An enzyme is a biomolecule which acts as a biocatalyst that increases the rate of a chemical reaction without altering the equilibrium of the reaction. The enzyme transforms a substrate into a product, without getting changed itself. Enzymes are produced by the living cells. The term ‘enzyme’ was first named by Kuhne in 1878 for the catalytically active substances earlier called **ferments**. However, a German biochemist Eduard Buchner in 1897 was the first to discover the enzyme zymase from the yeast which is involved in the fermentation of sugar into alcohol. In 1907, he was awarded the Nobel Prize for the biochemical investigations of non-cellular alcoholic fermentation (Kohler, 1971). Most of the metabolic reactions are catalysed by the enzymes in the living cell. All the enzymes are not present at all the time in the cell, but are produced whenever the cell needed from the blueprint is present in DNA. There are nearly 75,000 enzymes that are functional in the human body which are divided into three types: metabolic enzymes (responsible for anabolic and catabolic processes), digestive enzymes (mainly extracellular responsible for the digestion of food), and food enzymes from raw foods (responsible for initiation of food digestion) (Barnes-Svarney & Svarney, 2014).

General Properties:

1. Almost all enzymes are protein in nature except a small group of catalytic RNA molecules. The catalytic activity of the enzyme relies on the structural integrity of native protein conformation.
2. Enzymes are very specific to their substrates.
3. Enzymes possess huge catalytic power which increases the rate of a chemical reaction by reducing the activation energy without altering the equilibrium position of the reaction.
4. The biocatalytic power of the enzyme depends on the optimum physio-chemical parameters of the reaction such as pH, temperature, and salt concentration.
5. Enzymes are highly regulated in nature.

Generally, the enzymes are classified as

(a) *Simple enzymes*: The enzymes which are entirely made up of the proteins constituting the amino acids only, e.g. trypsin, pepsin, etc. (Bisswanger, 2017).
(b) *Conjugated enzymes*: The enzymes which contain the non-protein part called **cofactor** along with the protein part, called **apoenzyme**. In these enzymes, the apoenzyme part is generally biologically inactive, if the cofactor is removed. The complete biologically active conjugated enzyme (apoenzyme and cofactor)

is called **holoenzyme**. The cofactor may be inorganic or organic in nature that linked either covalently or non-covalently to the apoenzyme. The inorganic cofactors are simple metal ions, whereas the organic cofactors are complex organic groups called **coenzymes**. Also, the organic cofactors which are firmly attached to the apoenzyme are called **prosthetic group**. The conjugated enzymes are alkaline phosphatase, horseradish peroxidase, etc. (McComb et al., 2013).

1.2 Nomenclature and Classification

Most of the enzymes are named with the suffix –ase; however, some old names are exceptions, e.g. trypsin, ptyalin, pepsin, chymotrypsin, renin, etc. Also, some of the old names indicate the source rather than the action, e.g. papain from Papaya and bromelain from Pineapple (Bromeliaceae family). Many enzymes are named based on substrate and the chemical reaction they catalyse with the suffix –ase, e.g. pyruvate kinase that helps in the synthesis of pyruvate from the phosphoenol-pyruvate, whereas pyruvate dehydrogenase enzyme which converts pyruvate to acetyl-CoA. To avoid the confusion arose due to the common names, an International Commission on enzymes was established for the systematic nomenclature of enzymes.

The enzyme commission (EC) has set a regulation for the nomenclature of enzyme. According to EC, each enzyme is classified and named based on the type of chemical reaction, and for each enzyme, number with four parts is given, e.g. EC 1.7.3.3 (Uricase); EC 3.5.1.1 (Asparaginase). The first three numbers denote major class, subclass, and sub-subclass, respectively, and the last number is a serial number in the sub-subclass, which indicates the order in which each enzyme is added to the list (Boyce & Tipton, 2001) (Table 1.1).

All the enzymes are classified into six classes based on the type of chemical reaction they catalyse. The first integer in the EC number denotes the class of enzymes.

Table 1.1 Common name and EC numbers of some enzyme

Common name of an enzyme	EC number	Reference
Hexokinase	EC 2.7.1.1	Dai et al. (1995)
Lactate dehydrogenase	EC 1.1.1.27	Vanderlinde (1985)
α-Amylase	EC 3.2.1.1	Janeček (2009)
Pectinase	EC 3.2.1.15	El Enshasy et al. (2018)
Lipase	EC 3.1.1.3	Kouker and Jaeger (1987)
Invertases	EC 3.2.1.26	Vu and Le (2008)
Xylanase	EC 3.2.1.8	Huhtanen and Khalili (1992)
Alcohol dehydrogenase	EC 1.1.1.1	Heinstra et al. (1989)
Maltase	EC 3.2.1.20	Nawaz et al. (2015)

1.2.1 EC 1 Oxidoreductase

The enzymes which catalyse the oxidation-reduction reaction are classified under this class. The enzymes are involved in the transfer of electrons from one molecule (oxidant) to another molecule (reductant) (Table 1.2).

$$X_{oxi} + Y_{red} \rightarrow X_{red} + Y_{oxi}$$

1.2.2 EC 2 Transferase

The enzymes which catalyse the transfer of groups from one molecule to another are classified under transferase class. The groups involved in the transfer are phosphoryl ($–PO_3^{2-}$), methyl ($–CH_3$), carboxyl (–COOH), amino ($–NH_2$), acyl (–RC=O), and carbonyl (–C=O). The common names for these enzymes start with prefix *trans* with exceptions of kinases, phosphorylases, etc. (Table 1.3).

$$X - A + Y \rightarrow X + Y - A$$

Table 1.2 Some examples of enzymes under oxidoreductase class

Oxidoreductase	Type of reaction	Reference
Peroxidases	Utilize H_2O_2 as an electron acceptor	Gramss and Rudeschko (1998)
Dehydrogenases	Oxidize a substrate by reducing the electron acceptor other than oxygen, e.g. NAD^+	Pumirat et al. (2014)
Oxygenase	Directly incorporate oxygen into the substrate	Huang et al. (2008)
Oxidases	Use molecular oxygen as an electron or hydrogen acceptor	Kiess et al. (1998)

Table 1.3 Some examples of enzymes under transferase class

Transferase	Type of reaction	Reference
Transaminases	Transfer amino group from amino acids to keto acids	Smit et al. (2009)
Kinases	Transfer of phosphate group from ATP to substrate	Martín et al. (2010)
Methyltransferases	Transfer of methyl group from methyl donor to the substrate	Shixian et al. (2006)
Acyltransferases	Transfer of acyl group to the substrate	Kuhajda et al. (2011)

1.2.3 EC 3 Hydrolases

These are the enzymes which catalyse the cleavage of a covalent bond by adding water (Table 1.4).

$$X - Y + H_2O \rightarrow X - H + Y - OH$$

1.2.4 EC 4 Lyases

These are the enzymes which cleave C–C, C–O, C–N, C–S, and other bonds without hydrolysis or oxidation, but by means of elimination, which results into the formation of double bond or a new ring or contrarily adding groups to double bonds (Table 1.5).

$$X = Y + SP \rightarrow X - S + Y - P$$

1.2.5 EC 5 Isomerases

The isomeric structures are created by means of intramolecular arrangements that convert one isomer to another (Table 1.6).

$$X\,Y \rightarrow Y\,X$$

Table 1.4 Some examples of enzymes under hydrolases class

Hydrolases	Type of reaction	Reference
Esterases	Cleave esters into an acid and an alcohol	Faulds et al. (2004)
Phosphatases	Remove phosphate group from a substrate	Morisseau et al. (2012)
Peptidases	Cleave amide bonds present in the proteins	Perrier et al. (2005)

Table 1.5 Some examples of enzymes under lyases class

Lyases	Type of reaction	Reference
Decarboxylases	Removal of CO_2 by elimination reactions	van der Werf et al. (1994)
Dehydratases	Formation of double or triple bond by elimination of water	Shibata et al. (2010)
Ferrochelatases	Addition of divalent metal cations to the tetrapyrrole structure	Gazel and Yildiz (2016)

Table 1.6 Some examples of enzymes under isomerases class

Isomerases	Type of reaction	Reference
Racemases	Interconversion of L and D stereoisomers	Staudigl et al. (2014)
Tautomerases	Interconversion of keto- and enol-groups	Orita et al. (2001)
Mutases	Intramolecular transfer of functional groups	Rhimi et al. (2008)

Table 1.7 Some examples of enzymes under ligases class

Ligases	Type of reaction	Energy source	Reference
Carboxylases	Catalyse the formation of C–C bond by using CO_2	ATP	Portevin et al. (2005)
T4 DNA ligases	Catalyse the formation of phosphodiester bond	ATP	Wilson et al. (1997)
E. coli DNA ligases	Catalyse the formation of phosphodiester bond	NAD	Nandakumar et al. (2007)

1.2.6 EC 6 Ligases

These are the enzymes which join the two molecules by forming a chemical bond like C–C, C–N, C–O, and C–S. The energy required for these is gained by the cleavage of ATP or NAD. Ligases are also called synthetases, because of synthesis of new molecule (Table 1.7).

$$X + Y + ATP\ (NAD) \rightarrow X - Y + ADP\ (NAM)$$

1.3 Role of Enzyme

The collision of an atom, ion, or molecule of one substance with an atom, ion, or molecule of other results into a chemical reaction between the two substances. After this collision, the intermediate product is produced, having higher chemical energy than the chemical energies of both the reactants together. The transition state in the reaction is achieved with the help of some of the energy entering the reaction apart from the chemical energy of the reactants. The transition state is the stage with highest free energy. The difference in free energy between the transition state and the reactants is known as the Gibbs free energy of activation or only the activation energy.

The enzyme plays an important role in lowering the activation energy of a reaction which results in increase in the rate of reaction in both the directions. However, the enzyme cannot alter the relative energies of the initial and final states. In addition, the catalyst does not alter the position of equilibrium of a chemical

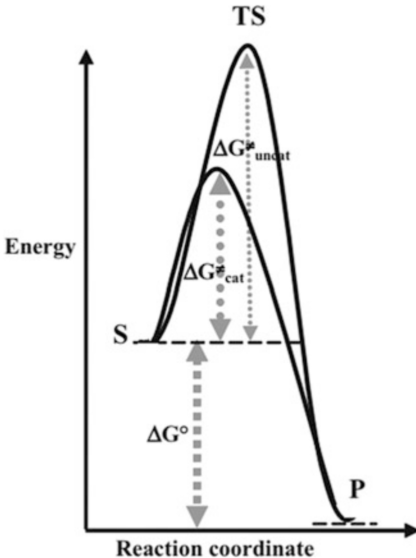


Fig. 1.1 Free energy profile of uncatalysed vs. catalysed reactions. The standard free energy of reaction (ΔG^0) and free energy of activation for uncatalysed ($\Delta G^{\#}_{\text{uncat}}$) and catalysed ($\Delta G^{\#}_{\text{cat}}$) reactions are shown here (Punekar, 2018)

reaction, but only increases the rate of a reaction by lowering the activation energy (Fig. 1.1).

The biocatalysis of a chemical reaction begins with the binding of enzyme and the substrate, the substrate to be catalysed. The substrate specifically binds at the specialized region in the enzyme called active site with multiple weak non-covalent interactions such as hydrophobic, hydrogen bond, ionic interaction, or reversible covalent bonds. Upon binding of the enzyme and substrate, a large amount of free energy is released, called binding energy, which is used to lower the activation energy. The general catalysed reaction between enzyme and substrate may be represented as

$$\mathrm{E} + \mathrm{S} \leftrightharpoons \mathrm{E} - \mathrm{S} \rightarrow \mathrm{E} + \mathrm{P}$$

where E is enzyme, S is substrate, the complex E–S is the enzyme-substrate complex, and P is the product formed.

The binding of substrate at the active site of the enzyme has been proposed with two models.

1.3.1 Lock-and-Key Model

This model assumes that the binding of substrate at the active site of enzyme is highly compact, which may be due to the high degree of similarity between the shape of the substrate and the geometry of the binding site, i.e. active site on the enzyme. This binding of substrate and enzyme is just like a lock and a specific key (Fig. 1.2).

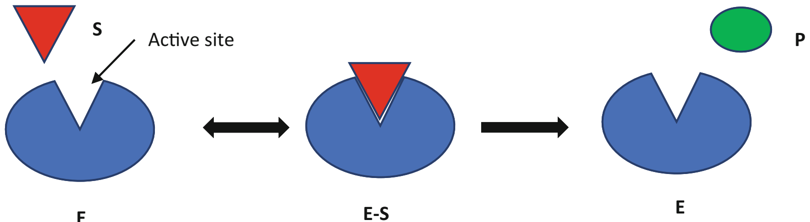


Fig. 1.2 Lock-and-key model of enzyme-catalysed reaction

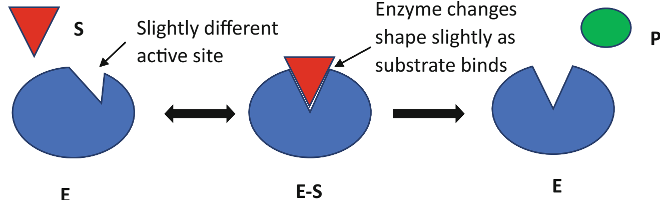


Fig. 1.3 Induced-fit model of enzyme-catalysed reactions

1.3.2 Induced-Fit Model

This model describes the behaviour of protein with some three-dimensional flexibility. Based on this model, the conformational change occurs in the enzyme upon binding of the substrate which leads to complementary fit as soon as substrate is bound. However, prior to substrate binding, the active site has slightly different three-dimensional shape. After binding of the substrate, the enzyme-substrate complex with transition state is formed which subsequently converts into product and free enzyme (Fig. 1.3).

1.4 Enzyme Kinetics

Enzyme kinetics is the quantitative study of chemical reactions catalysed by the enzymes. The kinetic study helps to determine the reaction rates and the affinity of enzymes for the substrates and the inhibitors. Let us consider a chemical reaction $X + Y \rightarrow P$, where X and Y are the reactants and P is the product obtained, the rate of reaction can be determined by either the rate of disappearance of one of reactants or the rate of formation of product. The rate of disappearance of X is $-\Delta[X]/\Delta t$, where Δ represents change, [X] is the concentration of A in mol/L, and t denotes time. Similarly, the disappearance of another reactant Y is $-\Delta[Y]/\Delta t$ and the rate of formation of product P is $\Delta[P]/\Delta t$. So, from these, the rate of reaction can be measured which is related by the stochiometric equation for the reaction.

$$\text{Rate} = -\frac{\Delta[\text{X}]}{\Delta t} = -\frac{\Delta[\text{Y}]}{\Delta t} = \frac{\Delta[\text{P}]}{\Delta t}$$

1.4.1 Kinetics of Enzyme-Catalysed Reactions

Leonor Michaelis and Maud Menten in 1913 proposed a model for the kinetics study of enzyme-catalysed reaction of hydrolysis of disaccharide sugar and sucrose by invertase enzyme into monosaccharide sugars, glucose, and fructose units. Michaelis-Menten assessed the relationship between the reaction velocity and the substrate concentration. The results of this disaccharide hydrolysis into the two monosaccharides by the invertase are represented with the following reaction:

$$\text{E} + \text{S} \underset{k_{-1}}{\overset{k_1}{\leftrightharpoons}} \text{E} - \text{S} \overset{k_2}{\rightarrow} \text{E} + \text{P}$$

Here, k_1 represents the rate constant for the formation of enzyme-substrate complex, ES after the binding of substrate S with the enzyme E; the rate constant k_{-1} denotes the reverse reaction of dissociation of enzyme-substrate complex to the free enzyme and the substrate; whereas the rate constant k_2 represents the conversion of ES complex to product P and the free enzyme.

As per Michaelis-Menten, the rate or velocity of enzyme-catalysed reaction depends upon the substrate concentrations [S], when measured at different substrate concentrations with keeping all the physio-chemical parameters constant throughout the course of experiment. At the low concentration of substrate, the initial velocity (V_0) increases at a linear rate with the increase in the concentration of substrate to the point beyond which the velocity does not increase further called maximum velocity (V_{max}) with increase in the substrate concentration (Fig. 1.4).

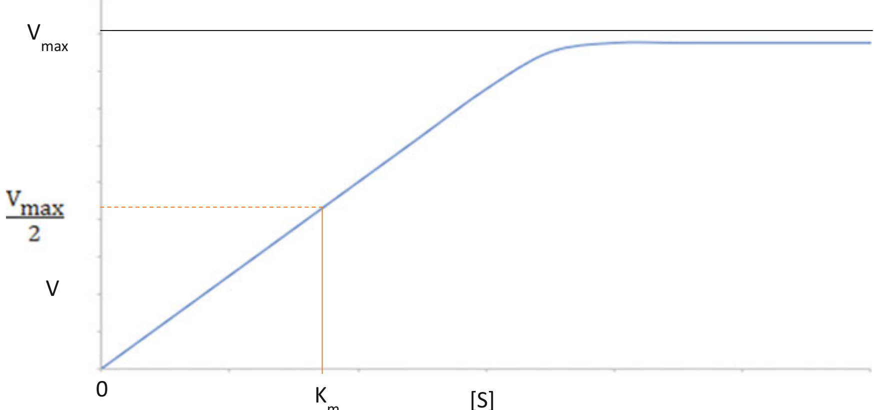


Fig. 1.4 Kinetic study of substrate-saturation curve

1.4.1.1 Lineweaver–Burk Plot

In Lineweaver–Burk plot or doubsle reciprocal plot, this graph was plotted in between reciprocal of velocity against the reciprocal of substrate concentration as in the following equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \tag{1.1}$$

In this plot, x and y intercepts represent $\frac{-1}{K_m}$ and $\frac{1}{V_{max}}$, respectively (Fig. 1.5).

1.4.1.2 Eadiae-Hofstee Plot

The errors in kinetic parameters in Double reciprocal plot were minimized by Eadiae-Hofstee plot which was plotted in between the rate of reaction (v) and v/[S] and represented by the following equation:

$$v = -K_m \cdot \frac{v}{[S]} + V_{max} \tag{1.2}$$

where V_{max} is the y-intercept and $\frac{V_{max}}{K_m}$ is the intercept on x-axis (Fig. 1.6).

1.4.1.3 Hanes-Woolf Plot

The kinetic parameters were determined by the advanced plot called Hanes-Woolf plot. The graph was plotted between the substrate concentrations vs. the rate of reaction 'v'. The following equation was used for the plot:

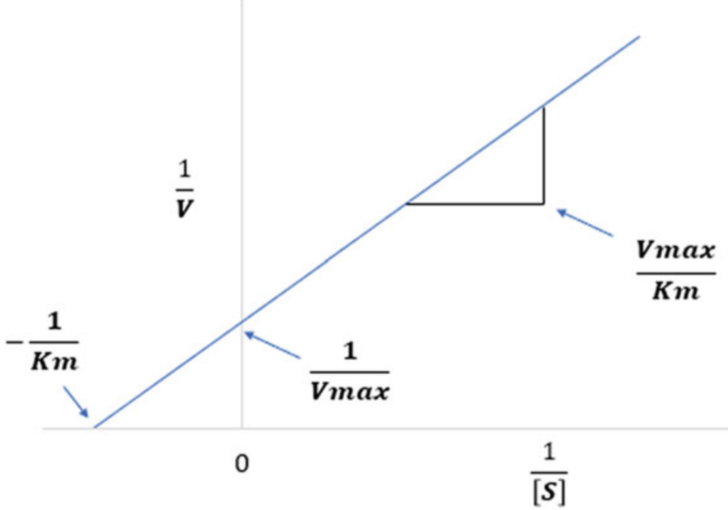


Fig. 1.5 Lineweaver–Burk plot to determine kinetic parameters

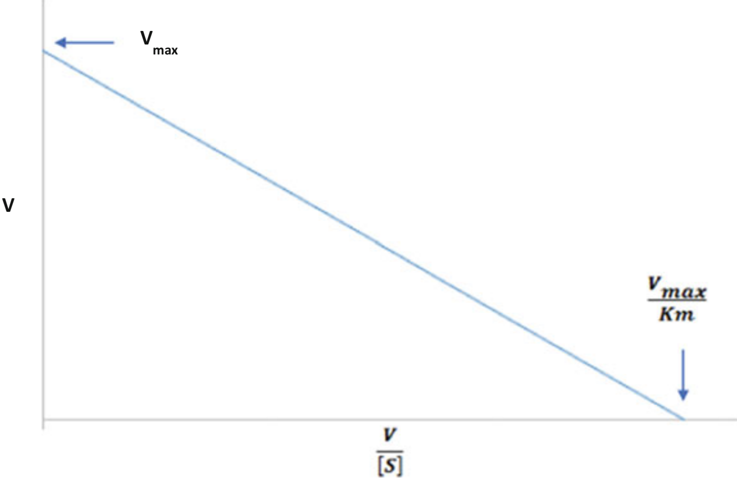


Fig. 1.6 Eadiae-Hofstee plot to determine kinetic parameters

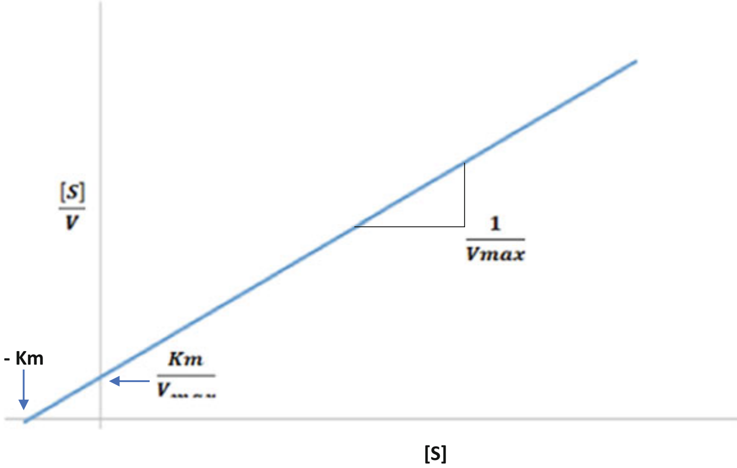


Fig. 1.7 Determination of kinetic parameters by Hanes-Woolf plot

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \tag{1.3}$$

The straight line graph will yield $\frac{1}{V_{max}}$ as the slope, whereas $\frac{K_m}{V_{max}}$ as the *y*-intercept and $-K_m$ as the *x*-intercept (Fig. 1.7).

1.5 Applications of Enzymes in Food Industries

The enzymes play a vital role either in the food processing or additives in the food industries. The major applications of food enzymes are in the baking industries, dairy industries, starch processing, juice industries, brewing industries, etc.

1.5.1 Baking Industry

To produce a valuable high-quality bakery product, the baking industry utilizes yeasts and enzymes since long ago. The starch containing foods can be processed by the addition of amylolytic enzymes like amylases to improve the softness, freshness, and shelf life qualities of food. The other enzymes, such as xylanases, oxidoreductases, lipases, and proteases, are also used to improve the dough stability, gluten strength, stability of the gas cells in the dough, and by reducing the protein content in the flour, respectively (Li et al., 2012).

1.5.2 Dairy Industry

The enzymes employed for the processing of dairy products become essential in the improvement of the quality and physiologically health benefits. The enzymes, chymosin (coagulation), lipases (low-down fat level), and lysozymes (to avoid late blowing), are used in the production of cheese (Kilcawley et al., 1998; Marseglia et al., 2013). In addition, β-galactosidases and lactases enzymes are predominantly employed in the hydrolysis of lactose sugar in milk into glucose and galactose to avoid lactose intolerance.

1.5.3 Starch Industry

The α-amylase is the prominent enzyme used in the baking industry to act on the starch present in the dough of the bread into the dextrins, which enhances the fermentation process. The released extra sugar in the dough improves the taste and textural properties of the bread (Souza, 2010).

1.5.4 Juice Industry

The enzymes, amylases and glucoamylases, are mainly used in the clarification of cloudy juices. Pectinases are used in the hydrolysis of structural heteropolysaccharide of fruit cell wall pectin, leading to increase in the juice production. Naringinases are mainly employed to reduce the bitterness of the citrus juices (Tapre & Jain, 2014).

1.5.5 Brewing Industry

The α-amylase hydrolyses the starch into maltose and glucose units with reduced viscosity. β-Glucanases hydrolyse glucans to reduce the viscosity and smooth wort separation. Proteases are used to process the malt which allows favourable content for yeast growth. Xylanases are used to enhance the 5-C pentose utilization in malt, barley, and wheat with extraction and beer filtration (Li et al., 2012).

References

Barnes-Svarney, P., & Svarney, T. E. (2014). *The handy biology answer book*. Visible Ink Press.

Bisswanger, H. (2017). *Enzyme kinetics: Principles and methods*. John Wiley & Sons.

Boyce, S., & Tipton, K. F. (2001). Enzyme classification and nomenclature. *eLS*. https://doi.org/10.1038/npg.els.0000710

Dai, N., Schaffer, A. A., Petreikov, M., & Granot, D. (1995). Arabidopsis thaliana hexokinase cDNA isolated by complementation of yeast cells. *Plant Physiology, 108*(2), 879.

El Enshasy, H. A., Elsayed, E. A., Suhaimi, N., Abd Malek, R., & Esawy, M. (2018). Bioprocess optimization for pectinase production using Aspergillus niger in a submerged cultivation system. *BMC Biotechnology, 18*(1), 71.

Faulds, C. B., Mandalari, G., LoCurto, R., Bisignano, G., & Waldron, K. W. (2004). Arabinoxylan and mono-and dimeric ferulic acid release from brewer's grain and wheat bran by feruloyl esterases and glycosyl hydrolases from humicolainsolens. *Applied Microbiology and Biotechnology, 64*(5), 644–650.

Gazel, N., & Yildiz, H. B. (2016). Enzyme-based biosensors in food industry via surface modifications. In *Surface treatments for biological, chemical, and physical applications* (pp. 227–252). Wiley.

Gramss, G., & Rudeschko, O. (1998). Activities of oxidoreductase enzymes in tissue extracts and sterile root exudates of three crop plants, and some properties of the peroxidase component. *New Phytologist, 138*(3), 401–409.

Heinstra, P. W., Geer, B. W., Seykens, D., & Langevin, M. (1989). The metabolism of ethanol-derived acetaldehyde by alcohol dehydrogenase (EC 1.1. 1.1) and aldehyde dehydrogenase (EC 1.2. 1.3) in Drosophila melanogaster larvae. *Biochemical Journal, 259*(3), 791–797.

Huang, S. K., Chiu, A. W. H., Pu, Y. S., Huang, Y. K., Chung, C. J., Tsai, H. J., et al. (2008). Arsenic methylation capability, heme oxygenase-1 and NADPH quinone oxidoreductase-1 genetic polymorphisms and the stage and grade of urothelial carcinomas. *Urologia Internationalis, 80*(4), 405–412.

Huhtanen, P., & Khalili, H. (1992). The effect of sucrose supplements on particle-associated carboxymethylcellulase (EC 3.2. 1.4) and xylanase (EC 3.2. 1.8) activities in cattle given grass-silage-based diet. *British Journal of Nutrition, 67*(2), 245–255.

Janeček, S. (2009). Amylolytic enzymes-focus on the alpha-amylases from archaea and plants. *Nova Biotechnologica et Chimica, 9*(1), 5–25.

Kiess, M., Hecht, H. J., & Kalisz, H. M. (1998). Glucose oxidase from Penicillium amagasakiense: Primary structure and comparison with other glucose-methanol-choline (GMC) oxidoreductases. *European Journal of Biochemistry, 252*(1), 90–99.

Kilcawley, K. N., Wilkinson, M. G., & Fox, P. F. (1998). Enzyme-modified cheese. *International Dairy Journal, 8*(1), 1–10.

Kohler, R. (1971). The background to Eduard Buchner's discovery of cell-free fermentation. *Journal of the History of Biology, 4*, 35–61.

Kouker, G., & Jaeger, K. E. (1987). Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology, 53*(1), 211–213.

Kuhajda, F. P., et al. (2011). Pharmacological glycerol-3-phosphate acyltransferase inhibition decreases food intake and adiposity and increases insulin sensitivity in diet-induced obesity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 301*(1), R116–R130.

Li, S., Yang, X., Yang, S., Zhu, M., & Wang, X. (2012). Technology prospecting on enzymes: Application, marketing and engineering. *Computational and Structural Biotechnology Journal, 2*(3), e201209017.

Marseglia, A., Castellazzi, A. M., Valsecchi, C., Licari, A., Piva, G., Rossi, F., Fiorentini, L., & Marseglia, G. L. (2013). Outcome of oral provocation test in egg-sensitive children receiving semi-fat hard cheese Grana Padano PDO (protected designation of origin) containing, or not, lysozyme. *European Journal of Nutrition, 52*(3), 877–883.

Martín, M. A., Ramos, S., Granado-Serrano, A. B., Rodríguez-Ramiro, I., Trujillo, M., Bravo, L., & Goya, L. (2010). Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation via extracellular regulated kinases and phosphatidylinositol-3-kinase/protein kinase B pathways in HepG2 cells. *Molecular Nutrition & Food Research, 54*(7), 956–966.

McComb, R. B., Bowers Jr, G. N., & Posen, S. (2013). *Alkaline phosphatase*. Springer Science & Business Media.

Morisseau, C., Schebb, N. H., Dong, H., Ulu, A., Aronov, P. A., & Hammock, B. D. (2012). Role of soluble epoxide hydrolase phosphatase activity in the metabolism of lysophosphatidic acids. *Biochemical and Biophysical Research Communications, 419*(4), 796–800.

Nandakumar, J., Nair, P. A., & Shuman, S. (2007). Last stop on the road to repair: Structure of E. coli DNA ligase bound to nicked DNA-adenylate. *Molecular Cell, 26*(2), 257–271.

Nawaz, M. A., Karim, A., Aman, A., Marchetti, R., Qader, S. A. U., & Molinaro, A. (2015). Continuous degradation of maltose: Improvement in stability and catalytic properties of maltase (α-glucosidase) through immobilization using agar-agar gel as a support. *Bioprocess and Biosystems Engineering, 38*(4), 631–638.

Orita, M., Yamamoto, S., Katayama, N., Aoki, M., Takayama, K., Yamagiwa, Y., & Takeuchi, M. (2001). Coumarin and chromen-4-one analogues as tautomerase inhibitors of macrophage migration inhibitory factor: Discovery and X-ray crystallography. *Journal of Medicinal Chemistry, 44*(4), 540–547.

Perrier, J., Durand, A., Giardina, T., & Puigserver, A. (2005). Catabolism of intracellular N-terminal acetylated proteins: Involvement of acylpeptide hydrolase and acylase. *Biochimie, 87*(8), 673–685.

Portevin, D., de Sousa-D'Auria, C., Montrozier, H., Houssin, C., Stella, A., Lanéelle, M. A., et al. (2005). The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth identification of the carboxylation product and determination of the ACYL-CoA carboxylase components. *Journal of Biological Chemistry, 280*(10), 8862–8874.

Pumirat, P., Vanaporn, M., Pinweha, P., Tandhavanant, S., Korbsrisate, S., & Chantratita, N. (2014). The role of short-chain dehydrogenase/oxidoreductase, induced by salt stress, on host interaction of B. pseudomallei. *BMC Microbiology, 14*(1), 1.

Punekar, N. S. (2018). *Enzymes*. Springer.

Rhimi, M., Haser, R., & Aghajari, N. (2008). Bacterial sucrose isomerases: Properties and structural studies. *Biologia, 63*(6), 1020.

Shibata, N., Tamagaki, H., Hieda, N., Akita, K., Komori, H., Shomura, Y., & Toraya, T. (2010). Crystal structures of ethanolamine ammonia-lyase complexed with coenzyme B12 analogs and substrates. *Journal of Biological Chemistry, 285*(34), 26484–26493.

Shixian, Q., VanCrey, B., Shi, J., Kakuda, Y., & Jiang, Y. (2006). Green tea extract thermogenesis-induced weight loss by epigallocatechin gallate inhibition of catechol-O-methyltransferase. *Journal of Medicinal Food, 9*(4), 451–458.

Smit, B. A., Engels, W. J., & Smit, G. (2009). Branched chain aldehydes: Production and breakdown pathways and relevance for flavour in foods. *Applied Microbiology and Biotechnology, 81*(6), 987–999.

Souza, P. M. D. (2010). Application of microbial α-amylase in industry—A review. *Brazilian Journal of Microbiology, 41*(4), 850–861.

Staudigl, P., Haltrich, D., & Peterbauer, C. K. (2014). L-Arabinose isomerase and D-xylose isomerase from Lactobacillus reuteri: Characterization, coexpression in the food grade host Lactobacillus plantarum, and application in the conversion of D-galactose and D-glucose. *Journal of Agricultural and Food Chemistry, 62*(7), 1617–1624.

Tapre, A. R., & Jain, R. K. (2014). Pectinases: Enzymes for fruit processing industry. *International Food Research Journal, 21*(2), 447–453.

van der Werf, M. J., van den Tweel, W. J., Kamphuis, J., Hartmans, S., & de Bont, J. A. (1994). The potential of lyases for the industrial production of optically active compounds. *Trends in Biotechnology, 12*(3), 95–103.

Vanderlinde, R. E. (1985). Measurement of total lactate dehydrogenase activity. *Annals of Clinical and Laboratory Science, 15*(1), 13–31.

Vu, T. K. H., & Le, V. V. M. (2008). Biochemical studies on the immobilization of the enzyme invertase (EC. 3.2. 1.26) in alginate gel and its kinetics. *ASEAN Food Journal, 15*(1), 73–78.

Wilson, T. E., Grawunder, U., & Lieber, M. R. (1997). Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature, 388*(6641), 495–498.

2 Plants- and Animal-Derived Enzymes and Their Potential Application in Food Processing and Preservation

Mahmoud Aminlari

Abstract

Enzymes are biological catalysts which increase the rate of biochemical reactions in living cells. It is important that enzymologist understands the specific action of particular enzyme in a plant or animal tissue and applies these properties in vitro and in a food product. Most enzymes can be used as processing aids and as protection agents against microbial and deteriorative processes. Although the advent of recombinant DNA technology and advances made in food applications by microorganisms are more practical and economic, some of these enzymes are sufficiently abundant in their natural sources to make them amenable to large-scale production (for example, egg-white lysozyme and plant proteases). In this chapter, several plant and animal enzymes, their occurrence, and potential applications in food industry will be presented. Emphasis will be made on enzyme working on carbohydrates, proteins, and lipids. A section is devoted to miscellaneous enzymes used in food industry, such as phenylalanine ammonia lyase of wheat seedling which metabolizes Phe, thereby rendering foods suitable for PKU patients. In the final section of this chapter, examples of chemical modification of enzymes to improve their properties will be discussed and examples of the studies on modification of chicken egg-white lysozyme to enhance its functional and antimicrobial activities, performed in the laboratory of this author, will be presented.

M. Aminlari (✉)
Department of Food Science and Technology, School of Agriculture, Shiraz University, Shiraz, Iran

Department of Biochemistry, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
e-mail: aminlari@shirazu.ac.ir

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_2

Keywords

Plant enzymes · Animal enzymes · Food application · Antimicrobial · Bioactive peptides · Chemical modification

2.1 Introduction

Enzymes perform many biochemical functions in living cells. They participate in multitude of biochemical reactions which are vital for cell well-being, growth, and production of various biochemical products. As such, enzymes are indispensible in any living system. Some enzymes are unique to particular cell type, while others are present in many different types of cells. It is the responsibility of the food enzymologist to determine and adapt the functionality of a particular enzyme in a plant or animal tissue to a functional property in food systems.

Enzymes from plants and animals have been utilized in the food industry for millennia. The maturation of sugar to liquor by yeast is an earliest illustration of a biotechnological cycle. Enzymes present in various raw materials have been used to produce traditional foods such as cheese, yoghurt, and bread and fermented beverages such as wine, beer, and vinegar for many thousands years (Poulsen & Buchholz, 2003). As early as 1783, it was shown that pepsin gastric juice could digest meat in vitro. The first industrial application of enzymes involved using crude protease mixture from animal pancreas as laundry detergents in the beginning of twentieth century.

In view of abundance of starting raw material the greater part of the first intracellular enzymes came from yeast and skeletal muscle. Starting late 1940s, significant improvements in the separation techniques such as chromatography applicable to proteins resulted in preparation of large quantities of industrially important enzymes (Aehle, 2007). With the advent of recombinant DNA technology, it became possible to transfer and express the genes encoding the enzymes of interest to selected host microbes for production at industrial scale. Today, molecular biology and genetic engineering play major roles in both the discovery of novel enzymes and the optimization of existing enzymes and are the basis for the production of the majority of industrial enzymes.

The grade or degree of purity of enzyme preparation depends on its application. For many industrial applications, partially purified enzyme preparations can be used, provided they do not contain interfering contaminating activities.

They are less costly and, therefore, preferred to highly purified products. They are commonly produced in large scale worldwide and are mostly obtained from animal or plant sources with few purification steps. Examples include proteases and enzymes working on carbohydrates. Enzyme destined for analytical purposes must be pure and do not contain unknown by-products that can interfere with enzymatic analyses, for example, glucose oxidase and restriction endonucleases. Finally, clinically important enzyme which must be highly purified is usually sold in crystalline form and must be devoid of any contaminating activity. A very famous example of

this group of enzymes is asparaginase which is used for treatment of childhood leukemia (Chahardahcherik et al., 2020). The last two types of enzyme are produced from microbes, plants or animals. These enzymes are produced in mg-g quantities worldwide using sophisticated purification procedures.

Food applications of enzymes represent wide and highly diverse fields including baking, dairy, fruit juice, vegetable processing, and meat. Several factors must be taken into consideration when one attempts to employ an enzyme in foods; reaction specificity, requirement for mild conditions, possibility of formation of an unacceptable by-product by a chemical process, and relative cost-benefit.

Uses of exogenous enzymes are used in the production of many commodities (glucose, high-fructose corn syrup, invert sugar, and other sweeteners), protein hydrolysates, and modified lipids; modification of components inside a food matrix (such as brew adjustment, cheese making, meat tenderization, citrus juice debittering, and crumb relaxing), process improvement (such as cheese maturing, juice extraction, juice clarification, fruit and oil seed extraction, drink filtration, quicker batter blending, baked product raising, and adjustment), process control (for example, online biosensors), and food analysis. The enzymes are utilized to acquire various advantages, like more proficient cycles, prompting decreased utilization of raw materials, improved or consistent quality, substitution of synthetic food-additives and evasion of potential destructive side effects in the food, and enhancements of nutritional attributes.

There are some regulations in food industry which allow adding certain chemical agents to foods to achieve particular goals, such as oxidizing and reducing agents (cysteine, bisulfites, and potassium bromide) in baking industry and preservatives such as benzoate and sorbates in different foods. However, in recent years, consumer demand for “natural” foods has driven development of products without additives. In order to meet this demand, much attention and interest have been directed toward identification and application of naturally made compounds. In many cases, the beneficial effect of enzymes to replace chemical agents can be demonstrated.

This chapter aims to present an overview of recent developments in identification of novel plant and animal enzymes with potential application in the food industry. The aim is to increase awareness of and stimulate interest in developing novel enzyme technologies to meet the new and changing needs of the food industry. The activities of many endogenous enzymes continue postharvest and postmortem, and in most cases, contribute to the food quality deterioration, such as excessive ripening of fruits by pectic enzymes, hydrolytic rancidity of milk lipids due to lipase activation, and extra meat tenderization by calpain system. However, the aim of this chapter is to exclusively address the application of exogenously added enzymes of plant and animal origin for their beneficial effects. Because of space limitation, this chapter focuses on enzymes working on major food constituents (i.e., carbohydrates, proteins, and lipids). A few enzymes with specific action in foods will be briefly described under the heading “miscellaneous enzymes”. A section is devoted to production of bioactive peptides through use of plant and animal proteases. In the final section of this chapter, examples of chemical modification of enzymes to improve their properties will be discussed and some of the studies done on lysozyme

in the laboratory of this author will be presented. Table 2.1 summarizes the use and suggested use of enzymes in food industry.

2.2 Enzyme Working on Carbohydrates

Most of the enzymes working on food carbohydrates are hydrolase, i.e., use water as one of the substrates. These enzymes are commonly referred to as glycosyl hydrolases or glycosidases.

They catalyze the hydrolysis of glycosidic bonds in complex sugars. They are amazingly normal enzymes with roles in nature including degrading of complex biomass like starch (amylase), cellulose (cellulase), hemicellulose, and as antibacterial safeguard methodologies (e.g., lysozyme), in pathogenesis components (e.g., viral neuraminidases), and in ordinary cell work (e.g., managing mannosidases associated with N-connected glycoprotein biosynthesis). Along with glycosyltransferases, glycosidases' are the major synergist apparatus for the synthesis and breakage of glycosidic bonds (Bourne & Henrissat, 2001). Glycosidases hydrlyze α-1,4 or α-1,6 glycosidic bonds. Glycosidases can be classified either as "retaing" or "invering" types, in light of the destiny of the anomeric configuration (α or β) of the hydrolyzed glycosidic bond. One more broad differentiation among glycosidases is whether they are "endo" or "exo" splitting. Exo-acting works for the most part on the non-reducing terminal of the substrate, while endo-acting types rondomly assault internal glycosidic bonds of the substrate. Naming glycosidases as "α" and "β" (as in amylases and glucosidases) perceives the anomeric configuration of the freed reducing group as being axial and equatorial, respectively (Whitaker, 1994). Specialty applications for various glycosidases continue to emerge. Glycosidases account for about half of enzyme used as processing aids in the food industry, primarily for the production of low molecular weight sweetener and bulking or thickening agents (dextrins) from starch (such as amylases), and for carbohydrate modification in baking and fruit products (such as cellulases and pectic enzymes) (Parkin, 2017). Most of these enzymes are commercially available. A summary of the types and classification of glycosidases of most importance in foods is provided in Table 2.2.

2.2.1 Amylases

2.2.1.1 α-Amylases

The amylases are utilized to hydrolyze starch into lower molecular weight dextrins and consequently "thin" starch suspensions. α-Amylase is an endo- splitting glycosidase that hydrolyses α-1,4 glycoside bond. The initial product is dextrin and the final main products are maltose or maltotriose. The α-1,4 bonds close to α-1,6 branches are impervious to hydrolysis. Broad hydrolysis of amylopectins with α-amylases produces "α-limit dextrins," as cleavage stops at the α-1,6 bonds. Figure 2.1 shows the impacts of various glycosidases on starch molecule (Wong & Robertson, 2007;

Table 2.1 Some uses and suggested uses of enzymes in food industries (from Whitaker, 1994)

Enzyme	Food	Purpose or action
Amylases	Baked goods	Increase in maltose content for food fermentation
	Brewing	Conversion of starch to maltose for fermentation, removal of starch turbidity
	Cereals	Conversion of starch to dextrins and maltose, increase water absorption
	Confectionary	Recovery of sugar from candy scraps
	Fruit juices	Removal of starch to increase sparkling properties
	Jellies	Removal of starch to increase sparkling properties
	Pectin	Aid in preparation of pectin from apple pomace
	Syrup	Conversion of starch to low molecular weight dextrins (corn syrup)
	Vegetables	Hydrolysis of starch as in tenderization of peas
Cellulase	Brewing	Hydrolysis of complex carbohydrates cell walls
	Coffee	Hydrolysis of cellulose during drying of beans
	Fruits	Removal of graininess of pears, peeling of apricot, tomatoes
Invertase	Artificial honey	Conversion of sucrose to glucose and fructose
	Candy	Manufacture of chocolate-coated soft-cream candies
Lactase	Ice cream	Prevention of crystallization which results in grainy, sandy texture
	Milk	Stabilization of milk proteins in frozen milk by removal of lactose. Hydrolysis of lactose, permitting use by adults deficient in lactase in intestinal and infants with congenital lactase deficiency
Tannase	Brewing	Removal of polyphenolic compounds
Pentosanase	Milling	Recovery of starch from wheat flour
Naranginase	Citrus	Debittering citrus juice by hydrolysis of the glucoside naringin
Pectic enzymes	Chocolate, cocoa	Hydrolytic activity during fermentation of cocoa
	Coffee	Hydrolysis of gelatinous coating during fermentation of beans
	Fruits	Softening
	Fruit juice	Improving yield of press juice, prevention of cloudiness, improving concentration process
	Olive	Extracting oil
	Wines	Clarification
Proteases	Baked goods	Softening action in dough, cut mixing time, increase extensibility of dough, improvement in grain, texture, loaf volume, liberate β-amylase
	Brewing	Body, flavor, and nutrient development during fermentation, aid in liberation and clarification, chill-proofing
	Cereals	Modification of proteins to increase drying rate, improve product handling characteristics, manufacture of misu and tofu
	Cheese	Casein coagulation, characteristic flavor during aging
	Chocolate, cocoa	Action on beans during fermentation

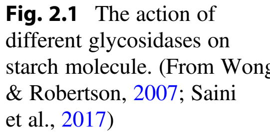
(continued)

Table 2.1 (continued)

Enzyme	Food	Purpose or action
	Eggs	Improve drying properties
	Meat, fish	Tenderization, recovery of proteins from bones and trash fish, liberation of oils
	Milk	In preparation of soy-milk
	Protein hydrolysate	Condiments such as soy sauce, specific diets, dehydrated soups, gravy powder, processed meats, production of bioactive peptides, aids in digestion of proteinous foods
	Wines	Clarification
Lipase	Cheese	Aging, ripening, and general favor characteristics
	Oils	Conversion of lipids to glycerol, fatty acids, and monoglycerides
	Milk	Production of milk with slightly cured flavor for use in milk chocolates

Table 2.2 Catalytic properties of some glycolytic enzymes important in food technology (from Whitaker, 1994; Parkin, 2017)

Enzyme	Bond selectivity	Product selectivity	Products
α-Amylase (endo)	α-1→4 glucose	Retaining α→α	Initial major product dextins, final product maltose, maltotriose
β-Amylase (exo)	α-1→4 glucose	Inverting α→β	β-Maltose
Pullulanase (endo)	α-1→6 glucose	Retaining α→α	Acts on pullulan to give maltotriose and on starch to give linear dextrins
Glucoamylase	α-1→4 (α-1→6) glucose	Inverting α→β	β-Glucose
Cyclomaltodextrin transferase	α-1→4 glucose	Retaining α→α	Acts on cyclodextrins and linear dextrins to give maltose and maltotriose
Cellulase	β-1→4 glucose		1,4-β-dextrins, mixed 1,3–1,4-β-dextrins
Invertase	β-1→2 fructose	Retaining β→β	Glucose and fructose
β-Galactosidase	β-1→4 galactose	Retaining β→β	Galactose and glucose
β-Gluctosidase	β-1→4, β-1 aglycan, glucose	Retaining β→β	Aglycan and glucose
Polygalactourinase	α-1→4	Inverting α→β	Galactouronic acid
Xylanase	α-1→4 xylose	Retaining β→β	
Lysozyme	α-1→4 NAM-NAG	Retaining α→α	



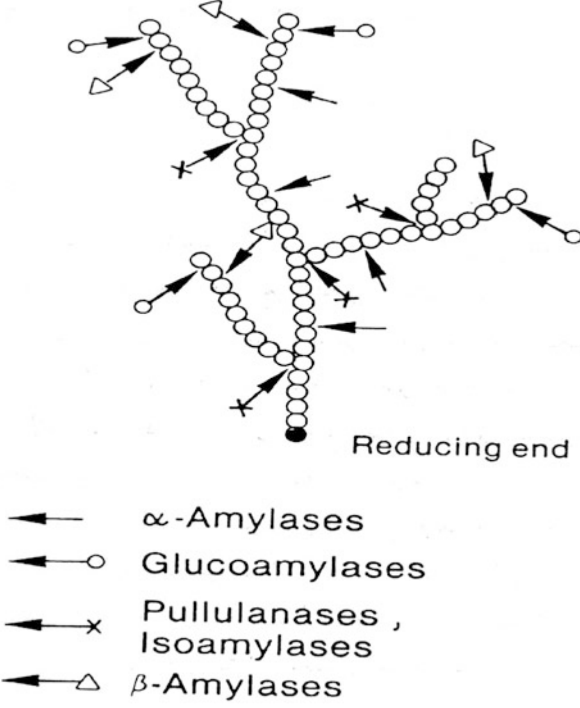


Fig. 2.1 The action of different glycosidases on starch molecule. (From Wong & Robertson, 2007; Saini et al., 2017)

Saini et al., 2017). α-Amylase is an important enzyme in carbohydrate metabolism of microorganisms, plants, and animals. The active site requires a substrate of no less than three glucose units long. The normal final results of α-amylase activity are α-limit dextrins and maltooligosaccharides of up to 12 glucose units. The random action of this enzyme on starch results in rapid decrease in the viscosity of starch solution (Fig. 2.2). The average molecular mass of α-amylase from different sources is 50–70 kDa. α-Amylases requires Ca^{2+} for activity. Ca^{2+} is firmly bound and serves to widen the pH optimum of the catalyst to between pH 6 and 10 as displayed in Fig. 2.2. α-Amylase from different sources are stable at temperatures in the range of 30–130 °C (Pandey et al., 2000). There are many commercial sources of α-amylases available, the majority of which are microbial, despite the fact that malt (grain or wheat) amylases are also accessible. The α-amylase routinely is purified from barley using different purification steps, including salt precipitation, centrifugation, ion exchange, and affinity column chromatographic methods such as CM Sepharose CL-6B column and cyclohexaamylose (CHA)-Sepharose affinity column. The enzyme can be also expressed in yeast and then purified from yeast extract as described above (MacGregor & Morgan, 1992).

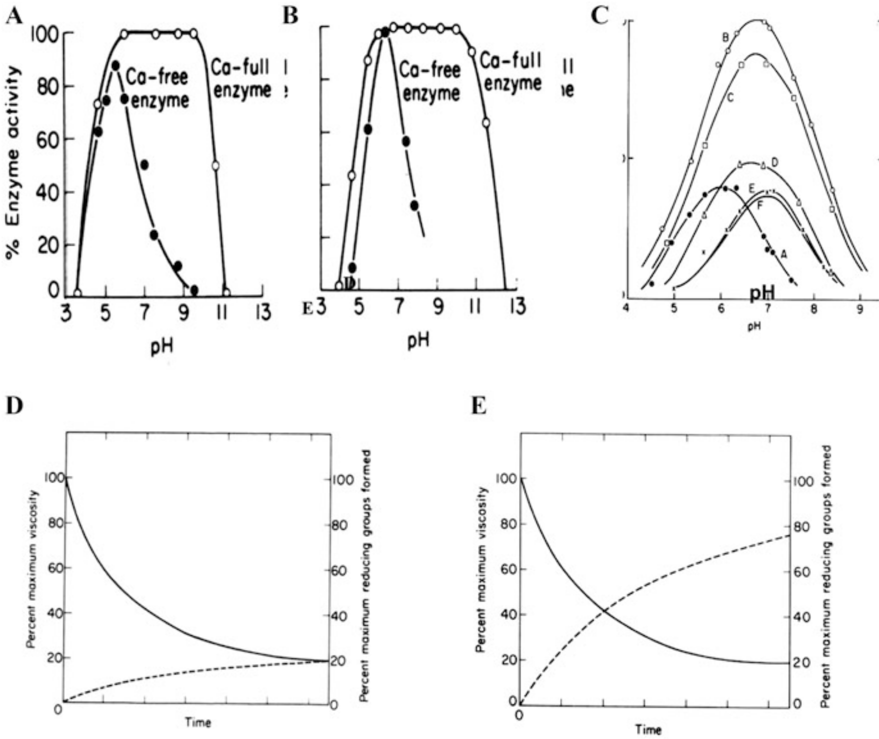


Fig. 2.2 Effect of pH, calcium ion, and salts on α-amylases: (**a**) pancreatic, (**b**) *Bacillus subtilis* α-amylases, (**c**) effect of different salts on α-amylase activity (A: No salt, B: 0.04 M NaCl, C: 0.04 M bromide, D: 0.04 M iodide, E: 1.0 M nitrate, F: 1. 0 M chlorate), effect of β-amylase (**d**) and α-amylase (**e**) on viscosity and reducing sugar content during starch hydrolysis. (From Wong & Robertson, 2007; Saini et al., 2017)

Food Application of α-Amylases

Starch Hydrolysis

Ordinary applications include preparation of starch syrup, dextrose, liquor, brewing, and bakery. α-Amylase is utilized economically in the liquefaction of starch to create dextrins which are further saccharified by glucoamylase to yield glucose feed stock for corn syrup, fuel ethanol, or alcoholic beverages. Heat stable α-amylase in the presence of calcium ion and a 105 degree centigrade is used to produce a 30-40% starch slurry.

This yields a mixture of linear and branched dextrins (maltodextrins) with extent of hydrolysis ranging from 8 to 15 DE (DE: dextrose equivalence). The product of this reaction is used for the production of 15–40 DE maltodextrins (corn syrups, used as thickening, bulking, and viscosity building), sweetener production, and a 95–98% glucose syrup (95 DE) in which other enzymes such as glucoamylase (which is often used as an immobilized enzyme column), with or without pullulanase, might also be

used. The glucose syrup can then be treated with an immobilized glucose isomerase column to generate a high-fructose corn syrup of 42% fructose (52% glucose) (Wong & Robertson, 2007; Van der Maarel et al., 2002).

In future, improving starch handling and change will zero in on broadening optimum pH (to pH 4–5), heat stability and decreasing Ca^{2+} prerequisite of α-amylase, thereby improving starch processing and transformation, and improving the capacity to process crude starch by β-amylases. For all enzymes included, improving heat stability is of significance as this will increase efficiency in handling just as advancing a single-step process. Likewise, improvement of determinants of selectivity is also important.

Baking and Baked Goods Industries

Over the course of bread making, proteins have consistently assumed a significant role. Indeed, even the old Egyptians utilized proteins present endogenously in the flour, in spite of the fact that they might not have known about the efffect.

The first application of enzymes in baked goods was supplementation of α-amylase by addition of malt to correct the concentration of endogenous α-amylase in the flour. Today, a whole range of enzymes is available for end users of flour. These make it possible to correct suboptimal concentrations of endogenous flour enzymes. Virtually all of the glycosidases presented in Table 2.2 have been added for some benefit in baking applications. The most widely used enzyme in baking industry in terms of amount and value is α-amylase. Initially, amylases were believed to function primarily by mobilizing fermentable carbohydrate for yeast.

The essential impact of amylase supplementation is getting satisfactory gassing power by degradation of damaged starch granules in the mixture, which helps maltose production by endogenous β-amylase. It is presently perceived that amylases added straightforwardly to the dough will decrease batter viscosity and further develop portion volume, bread softness(antistaling), and color. The majority of these impacts can be considered to be due to incomplete hydrolysis of starch during baking as the starch gelatinizes. Decrease in viscosity advances volume and texture by permitting the reactions engaged with mixture molding and baking to happen quicker (mass transfer effect) (Christophersen et al., 1998).

The most broadly utilized α-amylase in baking industry is the alleged α-amylase from fangus or Taka-amylase from *Aspergillus oryzae*. The most well-known other options, malt amylases from grain or wheat malt, have higher protease contamination and higher thermostability, and this makes them more inclined to negative incidental effects when used too much. The second important goal for use of amylases in baking is antistaling, i.e., improving the fresh keeping of baked goods.

Staling is a highly complex phenomenon, but it is generally accepted that retrogradation of amylopectin is the main contributor to bread staling (Morgan et al., 1997). This happens during the first hour of cooling subsequent to baking, the initial crumb structure is set by amylose gelation, which makes an organization in which the gelatinized starch granules are implanted. Recrystallization of amylopectin side chains leads to rigidification of the starch granules and to a general

reinforcing of the crumb structure, considered as an increment in crumb firmness. An inter- and intragranular amylose recrystallization is additionally associated with the staling process. Estimates of worth of lost baked goods due to staling in the United States in 1990 was about US $1 billion. All the more as of late, the maltogenic types of α-amylases as and β-amylase have been perceived as being far better antistaling agents, since they will generally produce smaller maltooligosaccharides (DP 7–9) and larger dextrins (which are plasticizers) (Hebeda et al., 1991).

Brewery and Fermentations

One of the primary uses of enzymes in brewing industry is preparing the materials for fermentation and whether the enzyme is endogenous in kernel or been added from outer sources, a profound knowledge and examination of those enzymes is required for better production and better overall quality. Fermenting is one of the most established food processes done by man. Brewing is characterized as the course of beer production where the sugars in starch are converted to ethanol through the activity of yeast. These days, brewing is one of the lead food industries in many countrie. Use of enzymes is one of the fundamental mainstays of brewing industry (Gomaa, 2018).

The four most normal enzymes utilized in brewing are β-glucanase, protease, α-amylase, and β-amylase. The shared factor in the beer production is the conversion of starch in the grains to ethanol. In the first stage, enzymes hydrolyze starch into fermentable sugars. In a subsequent interaction stage, these sugars are changed over to ethanol and carbon dioxide by yeast. The customary sources of enzymes utilized for the change of cereals into beer lager is malted grain, and malted barely is a vital factor in brewing. Commercial enzymes can be utilized for additional quality ascribes, for example, clarification, color, texture or flavor, and it is obligatory to utilize the enzymes from outer source since the amylases endogenous to malted grain are insufficient to use all of the fermentable starch because of low, absence of heat stability, as well as presence of endogenous inhibitors in the grains (Bamforth, 2009).

Thus, α- and β-amylases are added to maximize the availability of fermentable carbohydrate. Auxiliary enzymes such as glucoamylase, pullulanase, glucanases, xylanases, and cell wall hydrolyzing enzymes are added to complete the degradation of starch to α- and β-limit dextrins that the thermally labile malt amylases cannot achieve, to render limit dextrins fermentable and to hydrolyze glucans (similar to cellulose, but with β-1,3 and β-1,4 linkages) and xylans. The remaining limit dextrins provide body to the final product. Exogenous enzymes are added during (or right after) the “mashing” step, which is conducted at moderate temperatures (45–65 °C), and they are destroyed during the subsequent boiling stage. A range of exogenous enzymes, such as glucanases, acetolactate decarboxylase, and prolyl endopeptidase, are available for enhancement of the existing brewing process (Bamforth, 2009).

2.2.1.2 β-Amylases

β-Amylase is an exoglucanase that catalyzes the hydrolysis of 1,4-α-D-glucosidic bonds in polysaccharides to separate progressively maltose units from the nonreducing end of α-1,4-glucans like starch and glycogen. Sweet potato contains a bountiful β amylase which represents 5% of the total soluble proteins in the tuber; while only little α-amylase is available. The activity of this endogenous catalyst brings about production of maltose which renders a pleasant sweet flavor to potato (Thacker et al., 1992).

Properties and Applications

There are several similarities and differences between β-amylase and α-amylase. Both enzymes cleave α-1,4 glucosidic bonds in amylose and amylopectin. β-Amylase cleaves successively maltose units from the nonreducing end. Both have an average molecular weight of about 50 kDa. While α-amylase is widely distributed in nature, β-amylase is only found in plants.

Seeds of higher plants and sweet potatoes are the essential source of β-amylase. β-Amylase is liable for the pleasantness of aged natural product since it hydrolyses starch into maltose during ripening of fruits. pH 4.0–5.5 is optimum for the hydrolytic action of β-amylase. The product in α-amylase activity is an oligosaccharide with α configuration, and in β-amylase, it is β-maltose.

α-Amylase is an endo-splitting glycosidae, while β-amylase is exo-splitting. α-Amylase does bypass the branch point (i.e., α-1,6 glucosidic bonds) and β-amylase cannot bypass. Both enzymes cause reduction in viscosity as demonstrated in Fig. 2.2d, e, α-amylase acts more rapidly than β-amylase (Das & Kayastha, 2019).

The amino acid sequences of β-amylases from different plants, including sweet potato, soybean, barley, wheat, maize, rye, cowpea, alfalfa, and several species of bacteria, have been deduced from their respective gene sequences. The sequences between plant and bacterial β-amylases show only 30% homology (Thalmann et al., 2019).

β-Amylase has been prepared in a crystalline form from a variety of plant sources including germinated barley, sweet potato, and soybean. Purification mostly involves multisteps fractionation by acetone, ethanol, or ammonium sulfate precipitation followed by cation/anion exchange chromatography and gel filtration and the affinity chromatography using cyclodextrin Sepharose 6B column.

β-Amylase has been used for various research and industrial applications. β-Amylase is employed by the starch industry in the production of high maltose syrups. Compared with glucose syrups, maltose syrups have a higher viscosity and lower hygroscopicity, less tendency to crystallize, and more resistance to browning. These characteristics make them particularly suitable for use in the confectionery and baking industry (Aiyer, 2005).

2.2.2 Pectic Enzymic

2.2.2.1 Introduction

Pectins or pectic substances are collective names for a group of closely associated polysaccharides present in plant cell walls, where they contribute to complex physiological processes like cell growth and cell differentiation and so determine the integrity and rigidity of plant tissue. They also play an important role in the defense mechanisms against plant pathogens and wounding. Commercial pectins are polymers of α-D-galactopyranosyluronic acids with various contents of methyl ester groups and are obtained from citrus peel and apple pomace. Pectins have a unique ability to form spreadable gels in the presence of sugar and acid or in the presence of calcium ions and are used primarily in these types of applications. By definition, preparations in which more than half of the carboxyl groups are in the methyl ester form are classified as high-methoxyl (HM) pectins, the remainder of the carboxyl groups will be present as a mixture of free carboxyl group and their sodium salt. Preparations in which less than half of the carboxyl groups are esterified with methanol are called low-methoxyl (LM) pectins. The percentage of carboxyl groups is the degree of esterification (DE) or degree of methylation (DM) (Benen et al., 2003; Schols & Voragen, 2003; Yapo, 2011).

The pectin degrading enzymes or pectic enzymes are classified into three general groups, namely polygalacturonase, pectate and pecyin lyases, and pectin methyl esterase. The specific responses brought about by these three pectinase activities are displayed in Fig. 2.3. The depolymerizing enzymes are hydrolases and lyases. The methylesteraseases hydrolyze the methylesterase at O6 of a galacturonicacid residues, though acetylesterases hydrolyze the acetylester at O2 or potentially O3 of a galacturonic acids. These enzymes are commonly found in plants and microorganisms (particularly in fungi like *A. niger*) and have been purified and widely studied (Benen et al., 2003).

Pectic enzymes are normally present in many vegetables and fruits (endogenous enzymes), yet they are also added as processing aids as exogenous enzymes. In higher plants, especially pectin methyl esterase and polygalacturonase, both endo- and exo-asplitting are present. Pectin acetylesterase and pectate lyase have been found in citrus products of plants. The endogenous enzymes are expected to assume significant parts in plant improvement and during maturing. The cutting edge genomic approach will empower us to more readily comprehend their functions in plants in various isoforms and their role in plant improvements. Taking into account that pectic enzymes alone record for around one fourth of the world's food enzymes production, one can securely conclude that the technological innovations and applications designed to reduce economical costs and increase the productivity of these enzymes will be of great value (Alkorta et al., 1998; Whitaker, 1990).

2.2.2.2 Polygalacturonases

Polygalacturonases hydrolyze the α-1,4-D-galacturonosidic linkage (Fig. 2.3). Whereas endo-polygalacturonases hydrolyze the polymer substrate randomly, the exo-polygalacturonases are confined to cleave off galacturonic acid monomers or

Fig. 2.3 Site of action and reaction mechanism of pectin-degrading enzyme. (From Whitaker, 1994)

digalacturonides from the nonreducing end. The enzymes, as found for the pectate lyases, use both polygalacturonic acid and low to moderate degree of esterification of galactouronic acids as their substrate (Benen et al., 2003). Polygalacturonases are widely distributed in higher plants and are believed to contribute to fruit softening of pears, peaches, and avocado.

These enzymes assume significant parts in, food technology, for example, vegetable and fruit juice extraction, such as, apples, grape wine, strawberries, grapes, raspberries, and so on, degumming of plant fiber, waste water treatment, oil extraction, coffee and tea aging, paper and pulp industry, and animal feed. To clarify fruit juices, a combination of amylases and pectinases is utilized. The time of filtration is decreased up to half. Tomato is a rich source of polygalactouronase. Brilliant practices are utilized in the tomato processing to heat inactivate the enzymes momentarily ("hot break" process) to prepare thick juice liked by the purchaser and the high-consistency concentrated juice (tomato pase) utilized for sauces, soups, catsup, and comparable items. At the point when tomatoes are utilized for color and flavor, and consistency is obtained by different agents like starch, thiner, "cold break", juice is the beginning material for paste production. A holding time is applied

between smashing and heat treatment to guarantee breakdown of the pectins by joined pectin methylesterasease/polygalacturonase activity (Benen et al., 2003). To the food enzymologist, the most intriguing endogenous polygalacturonases are those present in the parts of the plant that are utilized as or in food. The pernicious impact of endogenous polygalacturonases, i.e., diminishing in consistency of the homogenate during aging, has become progressively clear. To prevent the activity of these enzymes, tomatoes are homogenized after heat pretreatment to inactive the enzymes and yield the purported hot break paste (Javed et al., 2018).

2.2.2.3 Pectin Methylesteraseases

Pectin Methylesteraseases have been industrially utilized in the commercial sector for wine and fruit juice industry since 1930. Apart from the presence of many genes encoding putative pectin methylesteraseases, these enzymes have also been detected in various plants by their activity, which confirms their wide distribution (Gupta et al., 2015; Dixit et al., 2013). Pectin methyl esterases are of major importance for the preparation of pectins for specific applications. These enzymes have been identified in higher plants and vegetables. Within each species, multiple forms of pectin methylesterases can be present. These enzymes, along with polygalacturonases, are used in clarification of juices in fruit juice industry. The break down of pectins additionally decreases the viscosity of juices and facilitates their filtration and concentration. One more utilization of pectin methylesterases in natural product juice industry includes liquefaction of natural product mashes. In such an application, the pectinases are joined with cellulases and hemicellulases for the total interruption of the cell walls. In this multitude of enzymatic applications, degradation of pectin facilitate the process and increase juice yield. Likewise, the favorable aroma of juices is increased and amount of waste material decreases. Pectin methylesterasease from *Datura stramonium* in blend with polygalacturonase expanded clearity of orange, apple, pomegranate, and pineapple juices by 2.9, 2.6, 2.3, and 3.6 fold, respectively (Dixit et al., 2013).

2.2.2.4 Pectate Lyases

Pectate lyases cleave the α-1,4-D-galacturonosidic linkage by β-elimination, resulting in the formation of a Δ4,5-unsaturated newly formed nonreducing end as is shown in Fig. 2.3b. Pectin lyases perform the same reaction. However, the latter enzymes require the galacturonic acid residues adjacent to the scissile bond to be methylesteraseified. Pectate lyases have been identified in plants (Uluisik & Seymour, 2020). The first clear cut demonstration of plant pectate lyase activity was reported in the plant zinnia eleganse (*Zinnia elegans*) (Semenova et al., 2006; Domingo et al., 1998). Pectate lyases have not found many applications in food industry because of their harsh reaction conditions (Semenova et al., 2006).

2.2.3 Cellulases

Cellulose is the most plentiful biopolymer on the planet. It is mostly created in higher plants in which it shapes the inflexible skeleton of the plant. Cellulose is a homopolymer comprising of up to 1000 β-1,4-linked-anhydroglucopyranoside units. Single glucose polymers are packed onto one another to shape an exceptionally crystalline fibrillar material in which the singular cellulose chains are held together by hydrogen bonds. Cellulose microfibrils likewise contain some amorphous areas, the level of which depends on the source. The most crystalline cellulose is produced by algae, and the least crystalline by plants (Klemm et al., 2005).

Cellulases belongs to the group of β-glucan hydrolases that can break down cellulose. Several microorganisms, including filamentous organisms, yeast, and microbes existing in the digestive tract and colon of monogastric, can hydrolyze cellulose to oligosaccharides and in the long run to glucose. Ruminants can completely degrade cellulose in their rumen by the wide range of microorganisms present. Cellulases have generally been classified into two specific groups endoglucanases and exoglucanases, which act in the middle or at either ends of the cellulose chain, respectively (Tenkanen et al., 2003). Cellulases are produced by fungi, bacteria, protozoans, and animals. There are additionally a few reports on the presence of cellulases in plants. Up to this point, just the endoglucanases (1,4-β-D-glucan-4-glucanohydrolase) have been found in plants. This enzyme catalyzes random cuts in cellulose chains, subsequently creating more shorter cello-oligomers, which can be additionally degraded by exoglucanases. Endogenous plant endoglucanases have been recommended to assume a part in plant development, for example, in fruit maturing and leaf abscission, or in the rearranging of polysaccharides in developing cells. The producion of microbial cellulase in transgenic plants, for example, sugarcane and corn, has been reported. These advances can offer one potential utilization of plant biomass as biofuel harvests or remaining horticultural waste as a sustainable fuel source (Hefferon, 2017).

Cellulases are not among the central enzymes used in the food industries. They are, nevertheless, applied in treatment of grain-based beverages and food assortments like beer and bread. Cellulases are used in rather immense totals in animal feed (Zhang & Zhang, 2013). Cellulase has a wide scope of utilizations in industrial biotechnology and is the second most utilized modern enzyme after protease. Cellulases are utilized in the textile industry, in cleansers, pulp and paper industry, further developing edibility of animal feeds, and food industry. In numerous food applications, cellulases are not utilized alone, but are added to help the activity of other enzymes. In collaboration with enzymes, such as pectinases and hemicellulases, cellulases disrupt the underlying rigidity of the plant cell walls and upgrade the extraction efficiency of the molecules of interest. Thus, they are utilized in any process of handling plant-based materials. Cellulases are utilized in wine and production of fruit juice to facilitate the maceration, and, the extraction of color and flavor compounds, fermentable sugars and the extraction of juices. Macerating enzymes mix containing cellulases are likewise utilized in fruit juice production. Furthermore, the utilization of these enzymes offers benefits in the treatment of

waste of the fruit juice industry by further developing the extraction yield and the general process efficiency. In baking industry, cellulase is utilized to degrade gums in the dough, improving bread structure. Added enzymes are utilized progressively for improving the processes and final quality of products. Obviously, the biggest modern utilization of cellulases is outside the food and feed area but in material handling of fibers made out of cellulose (Zhang & Zhang, 2013; Autio et al., 1996). Cellulases are likewise utilized for the extraction of nutraceuticals from plants. Nutraceuticals are compounds from normal sources with extra medical advantages, other than a nutritious agent (Fernandes, 2018). Cellulases and hemicellulases can be utilized for the separation of pectin from citrus fruits to separate the cell wall, expanding the pectin yield. Cellulases can be utilized alone or in blend with other cell wall degrading enzymes in all processes in which significant mixtures like juice, oil, polysaccharides, protein, and so on are removed from the plant material (Laurikainen et al., 1998).

2.2.3.1 β-Glucosidases

β-Glucosidases are enzymes with great practical significance to natural systems. These are divided in various glycoside hydrolase families in light of their catalytic mechanism and amino acid sequences. Most investigations completed on β-glucosidases are centered around their applications rather than their endogenous capacity in the their natural environment (Singh, 2016). In a large number of leafy foods tissues, significant flavor compounds are glycosylated. β-Glucosidases catalyze the hydrolysis of alkyl and aryl β-glycosides disaccharides and short-chain oligosaccharides. β-Glucosidases have an extraordinary potential to be utilized in different biotechnological processes from freeing flavours, fragrances, and isoflavone aglycons to the biosynthesis of oligosaccharides and alkylglycosides. The utilization of glycosidases to deliver flavor compounds from glycosidic compounds was at first analyzed in wines. β-Glucosidases are omnipresent, generally dispersed in nature, and can be found in microorganisms, plants, and animals (Romero-Segura et al., 2009; Zhu et al., 2017).

One more attribute of glucosidases critical to food processing and quality is the presence of cyanogenic glycoside in many plants that can potentially produce hydrogen cyanide during preparation or by eating. The cyanogenic glucoside linamarin in cassava roots and leaves (widely used as food staple in tropical areas of Africa, Asia, and South America), lima beans, bitter almonds, and flax seeds are examples of these glycosides. There are roughly 25 cyanogenic glycosides referred to, for example, linamarin in cassava and lima beans; dhurrin in sorghum; amygdalin in almonds, peaches, and apricot pits (Hughes, 1999). The enzyme rhodanese is a ubiquitous enzyme active in all living organisms from bacteria to man.

It is a multifunctional enzyme, yet appears to have important role in cyanide detoxification. This enzyme is likewise broadly present in plants (Chaudhary & Gupta, 2012). The primary source of this enzyme was reported to be the liver of animals. Nonetheless, broad examinations on the distribution of rhodanese in our research have exhibited that different organs, specifically the gastrointestinal system, is more prominent source of rhodanese, which show that cyanide delivered from

cyanogenic glycosides is mainly detoxified in the GI prior to being absorbed (see Aminlari & Vaseghi, 2006).

2.2.3.2 Xylanases

After cellulose, xylan is the most abundant polysaccharide in nature. Xylans are a major hemicellulose component, and together with cellulose, they comprise the bulk of the cell wall material in botanical products. Xylanases are β-retaining glycosidases capable of hydrolyzing linear β-1,4-linked polymers of xylose (with various substitutive groups such as arabinose). Multiple isoforms exist and these enzymes can be endo- or exo-acting (endo-acting are more important in foods). The xylanases are ubiquitous in nature and their presence is observed diversely in a wide range of organisms (Biely, 2003). They are in plants (especially important in cereals), bacteria, and fungi, and they typically range in molecular mass from 16 to 40 kDa. Xylanolytic enzymes also occurring in plants participate in the process of cell wall extension, cell division, seed germination, and other morphological and physiological events in plants.

During germination, endogeneous xylanases catalyze hydrolysis of these polysaccharides to eliminate the actual boundary imposed by cell wall on the free dispersion of starch and protein degrading enzymes through the germinating grain. Enzymes responsible for breaking down hemicellulose polysaccharides are likewise reported to be available in wheat grain and wheat flour (Kumar et al., 2017).

Xylanase enzymes are beneficial to food handling by depolymerizing water-unextractable arabinoxylan pentosans, which have high water-holding capacity This increases the viscosity and thickness of the dough, gluten strength, and final bread volume. The mixed xylanases are especially significant in the preparation of frozen dough. Endoxylanases are one of the hemicellulases that are utilized in fruit and vegetable processing. Xylanases are likewise utilized in brewing to lower viscosity of the wort in preparing, easeing partition/filtration steps, decreased haze formation, and marginally further developed process yields (Kumar et al., 2017; Bhardwaj et al., 2019).

2.2.3.3 Glucoamylase

Glucoamylase catalyzes the hydrolysis of α-(1,4), α,β-(1,1), α-(1,6), α-(1,3), and α-(1,2) glycosidic connections between adjoining glucosyl residues, arranged by their decreasing rates of hydrolydid. It starts hydrolysing from nonreducing end of starch-based substrates to liberate glucose. Glucoamylas assume a significant function in food processing to saccharify starch in cereals and produce glucose syrups from maltodextrins after the activity of α-amylase. Glucoamylas is produced by certain eubacteria, some archaea, various yeasts, and numerous filamentous fungi. Despite the fact that there are reports of animal and plant glucoamylas, these seem, by all accounts, to be essentially various enzymes with analytic properties that crossover those of genuine glucoamylas. Thus, and notwithstanding wide utilization of this enzyme in food industry, this catalyst won't be additionally discussed in this section.

2.3 Enzymes Working on Proteins

Proteolytic enzymes, proteinases, or proteases are used interchangeably to refer to enzymes that hydrolyze peptide bonds in proteins and peptides, according to the following equation:

$$
\begin{aligned}
&-\mathrm{NH}-\mathrm{CHR}_1-\mathbf{CO}-\mathbf{NH}-\mathrm{CHR}_2-\mathrm{CO}-\ +\ \mathrm{H_2O} \\
&\rightarrow -\mathrm{NH}-\mathrm{HR}_1-\mathrm{COOH}+\mathrm{N}+\mathrm{H}_3-\mathrm{CHR}_2-\mathrm{CO}-
\end{aligned}
$$

Proteases are found in all living life forms (creatures, plants, and microorganisms). They are fundamental for separating of proteins to peptides and amino acids. In the human digestive system, the proteolytic enzymes pepsin (stomach) and several proteolytic enzymes in small intestine (trypsin, chymotrypsin, carboxypeptidases, leucine aminopeptidases, tripeptidases, and dipeptidases) convert the proteins of ingested food varieties to amino acids.

Proteases are by far the best-portrayed enzymes as far as their fundamental roles in the human digestion and early commercialization (the originally normalized calf rennet for cheddar making was promoted in 1874) is concerned (Parkin, 2017). Peptidases breakdown food proteins in situ or are added exogenously to cause protein change.

2.3.1 Specificity

The investigation of protease specificity provide information on active site structure and functionality, protein-protein association, controlling intracellular and extracellular pathways, and evolution of protease and substrate genes (Diamond, 2007). Proteolytic enzymes are specific as far as being endoproteases or exoproteases. The endoproteases, like trypsin, chymotrypsin, and chymosin, hydrolyze peptide bonds inside of the polypeptide chains. The exoproteases follow up on peptide bonds at the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) of the protein. A few proteases are said to have broad specificity, i.e., they can hydrolyze peptide bonds regardless of the side chain group adjoining the scissile peptide bond (pepsin and bacterial subtilisin from different *Bacillus* spp). Majority of proteolytic enzymes, nonetheless, are totally specific for one or few amino acid residue at the scissile peptide bond. The scissile bond of the peptide substrates is assigned as that connecting residue R1 and R2 in above equation. For instance, trypsine attacks peptides on the C-terminal side of lysine and arginine amino acid residues. The residues for chymotrypsine and elastase are aromatic amino acids, in particular phenylalanine, tyrosine, or tryptophan and small hydrophobic amino acids like alanine, respectively (Parkin, 2017; Grahn et al., 1999). The protease binding sites are chemical and spatial complements of the substrate amino acid. This specificity for the substrate is due to the favorable binding interaction of the substrate amino acid side chain with residues that form the binding site of the protease.

$$\ldots\ldots S3 - S2 - S1 - S1' - S2' - S3' \ldots\ldots$$
$$\text{-------NH} - \text{CHR} - \mathbf{CO} - \mathbf{NH} - \text{CHR} - \text{CO------}$$
$$\ldots\ldots P3 - P2 - P1 - P1' - P2' - P3' \ldots\ldots$$
$$\uparrow$$

In the protease active site, S1, S1′, . . . refer to the amino acids that provide the chemical and spatial environment that complements the substrate amino acids referred to as P1, P1′, ... Arrow shows the scissile peptide bond. These sites have been mapped and characterized for many proteases (Parkin, 2017; Vizovišek et al., 2018).

2.3.2 Classification

There are four distinct kinds of proteases, specifically, the serine proteases, the cysteine proteases, the aspartic proteases, and the metalloproteases, in light of the active site group of the enzyme engaged with the nucleophilic attack at the carbonyl carbon of the scissile peptide bond of the substrate. These are portrayed below (Whitaker, 2003).

2.3.2.1 Serine Proteases

Serine proteases share the presence of the side chain of a serine residue in the active site which function in their catalytic activity. Among the serine proteases, the most notable are trypsin, chymotrypsin and elastase, and the nine proteases engaged with blood coagulation and collagenase. The serine protease subtilisin is from *B. subtilus*. Most enzymes in this group have molecular masses of 25–35 kDa, and they are featured by a surface or internal cleft as substrate binding site. The pH ideal of these compound is around 8 (Whitaker, 2003; Hedstrom, 2002; Di Cera, 2009).

2.3.2.2 Aspartic (Acid) Proteases

Aspartic proteases are portrayed by two profoundly conserved ASP residues as the catalytic unit, with optimum pH close to 3–4. Most natural enzymes from this group are pepsin, calf chymosin (additionally called “rennin” or “rennet,” utilized in cheese making), cathepsin (engaged with after death meat tenderization), and the chymosin-substitute peptidases from *Mucor* spp. These endopeptidases are typically 34–40 kDa in mass. The main mechanism for hydrolysis includes a dyad of conserves ASP residues acting as general acid/base (Whitaker, 2003; Ulusu et al., 2016; Nair & Jayachandran, 2019).

2.3.2.3 Cysteine (Sulfhydryl) Proteases

Cysteine proteases are a large group of enzymes (more than 130 known) present in animals, plants, and microorganisms. Most of these enzymes are among the papain family, and others being chymopapain (various isoforms) and papain from tropical

tree, Carica papaya (papaya), actinidin from kiwi fruit, ficin from fig latex, bromelain from pineapple, and lysosomal cathepsins from animal tissues. Among them, papain, bromelain, and ficin address 5% of the worldwide sale of proteases (Illanes, 2008; Verma et al., 2016).

The most studied cysteine protease is papain, which will be examined later in light of its significant uses in the food industry for chill proofing of brew and meat tenderization, and for synthesis of peptides and other organic compounds. An interesting cysteine protease in muscle is calpain (numerous isoforms) that is activated by Ca^{2+} and plays a part in after death tenderization of muscle. Commonly, the enzymes of this group are 24–35 kDa in mass, are active at pH 6.0–7.5, and can endure temperatures up to 60–80 °C (due in some extent by three disulfide bonds) (Whitaker, 2003; Otto & Schirmeister, 1997).

2.3.2.4 Metalloproteases

A large group of the metalloproteases are exopeptidases. These are the carboxypeptidases A and B, glycyl-glycine dipeptidase, carnosinase (work on β-alanyl-L-histidine and related compounds), amino-acyl-histidine hydrolase, and cytosolic aminopeptidase (α-amino-acyl-peptide hydrolase). All require Zn^{2+} as cofactor. Some of these enzymes, for instance, prolidase (where proline or hydroxyproline is the carboxyl terminal residue) and iminodipeptidase (in which proline or hydroxyproline is the N-terminal residue) require Mn^{2+}. Other metalloprotases are endo-acting thermolysin (from *Bacillus thermoproteolyticus*) and the nreutal endoprotease from *Bacillus amyloliquefaciens* (Tavano et al., 2018).

2.3.3 Application of Proteases in Food Industry

Commercial proteases are available at various levels of purity, and some contain multiple proteolytic agents. Some of the important commercial applications of proteolytic enzymes are shown in Table 2.3 and are described below (Whitaker, 2003).

2.3.3.1 Production of Protein Hydrolysates

Hydrolysis of food proteins utilizing proteases, like pepsin, trypsin, chymotrypsin, papain, and thermolysin, change their functional properties, for example, nutritional, flavor/sensory, textural, and physicochemical (solubility, foaming, emulsifying, gelling) and decreased allergenicity. Broad hydrolysis by nonspecific proteases, for example, papain, causes solubilization of even inadequately soluble proteins. Such hydrolysates ordinarily contain low molecular weight peptides with two to four amino acid residues. Several functional properties of proteins, like gelation, foaming, and emulsifying properties, suffer from excessive hydrolysis. Typically, a protein is treated by an endopeptidase in a batch process for a couple of hours, after which the added enzyme is inactivated by heat treatment. This treatment brings about fast reductions in normal molecular weight of proteins, whereas exopeptidases are utilized to hydrolyze small oligopeptides to composite amino acids. Proteins

Table 2.3 Use of proteolytic enzymes in food processing

Food	Purpose of action
Baked goods	Softening action in doughs. Cut mixing time, increase extensibility of doughs. Improve texture, elasticity, and loaf volume. Liberate β-amylase.
Brewing	Body, flavor, and nutrient development during fermentation. Aid in filtration and clarification. Chill-proofing.
Cereals	Modify proteins to increase drying rate, improve product handling characteristics. Production of miso and tofu.
Cheese	Casein coagulation. Characteristic flavor development during aging.
Chocolate, cocoa	Action on beans during fermentation.
Egg, egg products	Improve drying properties
Feeds	Waste product conversion to feeds. Digestive aids, particularly for pigs.
Fish	Solubilization of fish protein concentrate. Recovery of oil and proteins from inedible parts.
Legumes	Hydrolyzed protein products. Removal of flavor. Plastein formation.
Meats	Tenderization. Recovery of protein from bones.
Milk	Coagulation in rennet puddings. Preparation of soybean milk.
Protein hydrolysates	Condiments such as soy sauce and tamar sauce. Bouillon. Dehydrated soups. Gravy powders. Processed meats. Special diets. Antinutrient factor removal. Specific protein inhibitors of proteolytic enzymes and amylases. Phytate. Gossypol. Nucleic acid.
Wines	Clarification
In vivo processing	Conversion of zymogens to enzymes. Fibrinogen to fibrin. Collagen biosynthesis. Proinsulin to insulin. Macromolecular assembly.

(ordinarily meat, milk, fish, wheat, vegetable, vegetable, and yeast sources) might be exposed to a pretreatment that renders them to some degree of denaturation as this improves peptidase access and hydrolytic activity. Protein hydrolysates can improve functional properties, for example, emulsifying capacity, foam capacity and stability, antioxidative effect, water absorption capacity, and nutritional properties (Chalamaiah et al., 2012; Thiansilakul et al., 2007; Sinha et al., 2007; Clemente, 2000). Protein hydrolysates constitute an alternative to intact proteins and elemental formulas in the development of special formulations designed to provide nutritional support to patients with different needs. The production of extensive protein hydrolysates by sequential action of endopeptidases and exoproteases coupled with the development of post-hydrolysis procedures is considered the most effective way to obtain protein hydrolysates with defined characteristics (Rocha et al., 2009). Protein levels in production of protein hydrolysates are often 8–10%, provided there are no limitations on solubility, and the amount of enzyme added is generally ~2% on a protein basis, depending on purity. Protein:enzyme levels are sufficiently high so that the enzyme reacts at nearly Vmax with limited autodigestion of the enzyme, although product inhibition by accumulating peptides may attenuate reactivity. The hydrolysates can be characterized by the degree of hydrolysis (DH), sequence of produced peptides, functional properties such as solubility, emulsifying properties,

etc. DH can be easily evaluated with *o*-phthaldialdehyde (OPA) method which determines the number of free amino groups (Mirzaei et al., 2016). Typically, a DH value of 3–6% corresponding to peptides of 2–5 kDa is desired for physicofunctional properties, while DH of 8% or more means 1–2 kDa peptides, demonstrating optimal solubility for use in sports and clinical nutrition products. More exhaustive DH (as high as 50–70%) yields small peptides and amino acids of <1 kDa which are useful for infant and hypoallergenic foods and savory flavoring ingredient preparations (soups, gravies, sauces). One problem associated with the greater DH is the possibility of production of bitter peptides (small and hydrophobic), thereby necessitating to control this potential flavor defect (Chalamaiah et al., 2012).

Proteases can likewise be utilized to detach remaining muscle protein from bones of fish and land animals as protein hydrolysates, and this normally includes heating at 55–65 °C for 3–4 h. The final product blend from enzyme hydrolysis of proteins might require posttreatment refining as well as separation to get deravatives appropriate for the planned application (Chalamaiah et al., 2012; Mizani et al., 2005).

2.3.3.2 Application of Proteolytic Enzymes in Dairy Industry

Milk is an entirely perishable food item and therefore there has been a strong motivation to change it over to more stable items, which is facilitated by specific properties of milk. Traditionally, milk has been safeguarded by fermentation, normally with addition of salt (cheddar, fermented milks, butter). More current safeguarding strategies incorporate drying, pasteurization/sterilization, and freezing. A few qualities of milk additionally render it entirely amiable to modification by enzymes. The reality that milk is a fluid facilitate enzyme addition. Cheese manufacturing is presumably the oldest, as yet the biggest, utilization of exogenous enzyme in food processing.

Since the principal components of milk are proteins (3:5%), lipids (3:6%), and lactose (4:8), the principle enzymes used in dairy technology are proteinases and peptidases, lipases, and β-galactosidase (lactase). However, several oxidoreductases have significant applications. In this section, we will focus on the proteases. Other enzymes will be dealt with in other sections. Proteolytic enzymes are the most widely used enzymes in dairy technology. Applications include cheese manufacturing, modification of functional properties, production of protein hydrolyzates, and for nutritional and other applications (Fox, 1993; Shah et al., 2014).

Cheese Manufacturing

The main gastric enzyme of neonatal ruminants is chymosin or rennet. Chymosin has low broad proteolytic action, but high milk-coagulating action. Calf chymosin (rennet) and chymosin substitutes (see beneath) are added to milk to cause the underlying milk-clotting reaction prompting cheese formation. The rennet coagulation of milk is a two-stage process. The first (essential) stage includes specific hydrolysis of the PHE105-MET106 bond of κ-casein and creation of "para-casein" and TCA (trichloroacetic acid)-soluble peptides (glycomacropeptides), while the

next stage includes the Ca-induced gelation of para-casein at a temperature of 30–35 °C. Proteolysis is essentially complete before the beginning of coagulation. The remaining from k-casein, which is referred to as para-k-casein, stays in the micelle, yet the hydrophilic peptide, called the caseino(glyco)macropeptide, diffuses into the whey and thus the micelle-settling properties of k-casein are lost. Starter cultur are added to milk at 40–45 °C to cause a pH reduction to 5.8–6.5, whereupon chymosin is added to start clotting. Because of ensuing steps of cheese manufactur, some enzyme activity stays in the curd and contributes to cheese aging and flavor improvement during aging. Proteases in the starter cultures additionally add to support proteolysis and flavor improvement during aging.

The gene for calf chymosin has been cloned in chosen microbes, yeasts, and molds. A recombinant chymosin from *E. coli* has been created. Microbial recombinant chymosin contain no pepsin, though 5 to 50% of the milk clotting action of calf rennets might be because of pepsin (Kawaguchi et al., 1987; El-Sohaimy et al., 2010).

Expanding world production of cheddar (3% each year throughout recent years), accompanying with a diminished stock calf neonatal, the stockpile of veal rennet has been deficient for a long time, which has led to a quest for rennet substitutes. During the last 40 years, the expanding utilization of an assortment of milk-clotting catalysts has turned into a significant specialized pattern in cheese production. Although numerous proteinases can coagulate milk, just few have been viewed as pretty much adequate as rennets. These are cow, porcine, and chicken pepsins; rennet (watery concentrates produced using the fourth stomach of calves, kids, sheep, goat, and cow); microbial rennets made by *Endothia parasitica*, and *Mucor* species (*Mucor miebei* or *M. pusillus*) and *Cryphonectria parasitica*, and plant proteases (Shah et al., 2014; Kim et al., 2004a).

Chicken pepsin is the most unreasonable of pepsins and is utilized uniquely in extraordinary conditions. Bovine pepsin gives commonly palatable outcomes regarding cheese yield and quality; numerous commercial “calf rennets” contain a significant extent of bovine pepsin. Although the proteolytic specificity of the three normally utilized fungal rennets on αs1- and β-caseins is extensively different from that of calf chymosin, they by and large yield acceptable cheese and were broadly utilized in the United States before the presentation of microbial recombinant chymosin (Nelson, 1977; Green, 1977).

Milk-Clotting Enzymes from Plants

Although several properties of microbial milk-clotting enzymes such as high proteolytic activity and high resistance to heat denaturation made them suitable for cheese manufacturing, plant protease have attracted considerable attention and interest in cheese industry since they are easily available and their purification is simple. As discussed above plant protease are classified into several groups depending on the mechanism of catalysis in the hydrolytic step. Aspartic, serine and cysteine protease are the three major milk clotting enzymes from plants (Shah et al., 2014).

Aspartic protease are present in many plant species and have aspartic acid residues in their active site. They participate in many activities in plants. Examples include:

1. Cardosin from flowers of the artichoke thistle (*Cynara cardunculus*)
2. Cynarase from flowers of globe artichoke (*Cynara scolymu*)
3. A Cardosin A like enzyme from thistle (*Cynara humilis*)
4. An enzymatic extract from the thistle *Silybum marianum* flowers
5. Oryzasin from seeds of rice (*Oryza sativa*)
6. Enzymatic extract from flowers of moringa (*Moringa oleifera*)
7. Onopordosin from cotton thistle (*Onopordum acanthium*)
8. Cirsin JN703462 from flowers of common thistle (*Cirisum vulgare*)
9. Enzymatic extract from cell suspension of red star-thistle (*Centaurea calcitrapa*)

Serine proteases have in their active sites serine residues. Their main role in plants is almost the same as the aspartic proteases, with some additional features. Serine proteases are widespread in plants and belong to several taxonomic groups. They are extracted, purified and characterized from several parts of plants, especially fruits. Some of the enzymes in this group are (Di Cera, 2009):

1. Dubiumin from seeds of *Solanum dubium* (Solanum, also called bittersweet or woody nightshade) (from Wikipedia, the free encyclopedia).
2. Cucumisin from *Cucumis melo* fruits (Muskmelon is a species of melon that has been developed into many cultivated varieties. These include honeydew, cantaloupe, and Persian melon) (from Wikipedia, the free encyclopedia).
3. Lettucin from leaves of lettuce, *Lactuca sativa* (from Wikipedia, the free encyclopedia).
4. Religiosin from *Ficus religiosa* (Sacred fig is a species of fig native to the Indian subcontinent and Indochina) (from Wikipedia, the free encyclopedia).
5. Streblin from stems latex of *Streblus asper* (a tree known by several common names, including Siamese rough bush, khoi, serut, and toothbrush tree).

Cysteine proteases or thiol-proteases have a catalytic mechanism that involves a cysteine group in their active site. Plant extracts, or their purified proteases, have been extensively characterized in many studies, for their potential use as milk coagulants. Following is a list of examples of cysteine proteases from plants (Amal Ben Amira et al., 2017; Lo Piero et al., 2011):

1. Enzymatic extract from seeds of *Albizia lebbeck* (a species of *Albizia*, native to Indomalaya, New Guinea, and Northern Australia and widely cultivated in tropical and subtropical regions). It is often called lebbek tree siris, frywood, koko, and woman's tongue tree.
2. Enzymatic extract from seeds of sunflower (*Helianthus annuus*).

3. Ficin from branches latex of figs tree *Ficus carica sylvestris* and an enzyme from stem latex of *Sideroxylon obtusifolium* (*Sideroxylon* is a genus of trees in the family *Sapotaceae*. They are collectively known as bully trees).
4. Actinidin from kiwi (*Actinidia chinensis)* fruits.
5. Calotropain from latex of crown flower *Calotropis gigantean.*

Recently, we reported on application of actinidin to the production of cheese from cow's milk. Comparison of actinidin and chymosin indicated that the former could be a potential alternative to the latter in application as milk coagulant (Alirezaei et al., 2011).

In general, the major drawback of most plant rennets is the development of an increased bitterness and the appearance of cheese texture defects during storage and/or ripening. These defects are mainly due to excessive proteolytic activities and low ratios of milk-clotting activity/proteolytic activity. For this reason, the evaluation of enzyme activities and their comparison with those of commercial rennet (chymosin) is an important first step in selecting a suitable plant rennet.

Taken together, the utilization of various sorts of plant proteases in cheese indudrty impacts the hydrolysis level of the protein matrix of milk, prompting differeneces in sensory properties of cheese. The vast majority of plant rennets produce cheeses with harsh flavors because of too much proteolytic activity, which restricts their industrial application. Hence, the determination of a proper plant coagulant and the control of various gelation properties are vital to acquire a superior quality of end product.

2.3.3.3 Application of Enzymes in Meat Industry

Meat and meat product utilization (beef, pork, lamb, goat, and poultry) has expanded, especially in nonindustrial nations. The world utilization of meat products will arrive at 40 kg per capita in 2020. The platability of meat is affected by a few factors, among which meat tenderness is viewed as the main determinant for consumers. The processes associated with the transformation of muscle to meat are complex. The chemical and physical properties of muscle tissue and the related connective tissue are determinant of meat quality. Of the relative multitude of characteristics of meat quality, consumers rate tenderness as the most significant. Tenderness is a quality attribute coming about because of the interaction of actomyosin of myofibrillar proteins, the foundation of connective tissue, sarcomere length, and the bulk density of fat. Meat tendeerness likewise relies on the degree of protrolysis of muscles (Arshad et al., 2016; Marques et al., 2010).

There are multiple ways of tenderizing meat, by chemical or physical means, which basically decrease the amount of recognizable connective tissue without causing broad breakdown of myofibrillar proteins. The myofibrillar breakdown begins after initiation of the enzymatic system and involves the proteins tropomyosin, troponin T, troponin I, C-protein, connectin, desmin, vinculin, dystrophin, nebulin, and titin. The first change in meat tenderness is because of the activity of complex endogenous calpain-calpastatin system, which acts in muscle tissue after slaughter. In muscles, these proteolytic enzymes play a huge part in after death

proteolysis and meat tenderization. Cathepsins were the main enzymes utilized in meat tenderization, after which calpain was applied owing to its action in changing the Z-line thickness found in post-mortem, despite the fact that it was not at first connected with meat tenderization. Calpains are calcium-dependent proteases that break down myofibrillar proteins. Calpastastin, on the other hand, inactivates calpains, decreases the myofibrillar degradation, and hence lessens the tenderness. Calpastatin effect is finished after being inactivated by cooking. The amount of the enzymes changes among species, which affect the degree of meat tenderness, because of expanded or diminished proteolysis of myofibrillar proteins (Lian et al., 2013).

Meat tenderness relies upon the kind of muscle, pre- and post-mortem variables, and after death pH and temperature. The research for significant proteases with unique specificity for industrial applications is consistently a continued challange. Proteolytic enzymes from plant sources have gotten extraordinary attention for being active over a wide range of temperatures and pH's. Treatment by proteolytic enzymes is one of the most well-known techniques for meat tenderization. The utilization of exogenous proteases for meat tenderization is a rather modern strategy to further develop meat quality. There are few exogenous proteolytic enzymes, plant proteases (papain, bromelain, ficin, and actinidin), and proteases from *Aspergillus oryzae* and *Bacillus subtilis*, which have been considered as generally regarded as safe (GRAS) for use in the meat. Papain and other sulfhydryl endopeptidases (bromelain and ficin) are applied to muscle or meats that don't turn out to be sufficiently tendered during post-mortem aging. These enzymes are powerful in this application since they can hydrolyze collagen and elastin, connective tissue proteins that cause toughness in meat. These proteolytic enzymes are blended in the meat to break down the proteins in muscle and hydrolyze collagen and elastin, which helps in meat tenderization. Papain and bromelain are the most regularly involved plant enzymes for meat tenderization. As meat tenderizers, proteolytic enzymes are the most ideal for breakdown of collagen and elastin in connective tissue at somewhat low pH and low temperature. The structure of myosin and actin fibers is impacted by the plant proteases. The tenderness of meat is deteremined by enzyme activity assessment, myofibrillar fragmentation index, hydroxyproline estimation, and examining by electron microscopy. The ideal meat tenderizer would be a proteolytic enzyme with specificity for collagen and elastin in connective tissue, at the generally low pH of meat, which would act either at the low temperature at which meat is stored or at the high temperature accomplished during cooking (Arshad et al., 2016; Lian et al., 2013).

Papain

Papain is a nonspecific thiol protease and the significant protein constituent of the latex in the tropical plant Carica papaya. It is an important plant protease. The latex is obtained by scoring, and afterward permitting it to dry on the fruit, and a crude material is resulted. In the food industry, papain is regularly utilized for meat tenderization, production of protein hydrolysate, and the clarifying of juice and beer. Papain is also used in the baking and dairy industries (in cheese production),

and for the extraction of flavor and color compounds from plants. Papain is prepared by decersing contaminants and further extraction. The enzyme has high thermal and pressure resistances, requiring exceptional process conditions for sufficient inactivation (to accomplish 95% inactivation of papain at 900 MPa and 80 °C, 22 min of processing is required). The physiological job of papain in plants is to protect them from insects. Papain tends to over tenderize the meat surface, making it "soft". Papain is utilized at a portion of 300 units/kg to release free amino acids in dry fermented frankfurters. The three-dimentional structure for papain is reported. Wide range enzymatic activity has been shown by papain in the pH range 5.9–7.5 and the temperatures of 70–90 °C with optimum of 65–75 °C. Under these conditions, papain has superb activity in hydrolysis of myofibrillar proteins and moderate impact on hydrolysis of collagen (Istrati, 2008; Gokoglu et al., 2017).

Papain can be utilized in the fruit juice and beer clarification. In beer, an imperfection alluded to as chill-haze might be brought about by the combination (complexing) of tannins and proteins. Papain has for some time been utilized to hydrolyze protein and limit cloudiness, despite the fact that bromelain and ficin just as other bacterial and fungal proteases may now be utilized for this reason. The endopeptidase is added after aging and preceding final filtering. Papain is finally inactivated by pasturization, and unnecessary activity of papain might cause loss of foam stability. Controlled or evaluated proteolysis in brew is important, since some remaining protein is important to preserve specific quality attributes (Mosafa et al., 2013).

Bromelain

Bromelain (or bromelin) enzymes are present in huge amounts in fruits, leaves, root, and stems of the Bromeliacea family, of which pineapple (*Ananas comosus*) is the best known. The juice of various pieces of the plant contains bromelain in soluble form. Its high proteolytic action has made a wide interest in various applications, principally in tenderization, food, detergents, and the textile industry. Bromelain is commercially accessible in powdered form. It is assessed that 95% of the utilized proteases in the United States are derived from plant proteases like papain and bromelain, though microbial tenderizers are not utilized broadly. Reverse micellar extraction is utilized for isolation and purification of bromelain from pineapple. There is high bromelain recovery and purification in this strategy. This enzyme, as different proteases, breakdown myofibrillar proteins and collagen, causing overtenderization of meat. Bromelain can be utilized in beef at 10 mg/100 g meat and ideal tenderization happens after 24 h at 4 °C. Following this time span, the enzyme can be inactivated by heating at 70°. pH range for enzymatic activity for bromelain is 4–7 and optimum pH is 5–6. The range of temperature for activity is at 50–80 °C and optimum is 65–75 °C. Under these conditions, bromelain has moderate hydrolysis activity of myofibrillar proteins and good effect on hydrolysis of collagen (Arshad et al., 2016; Gokoglu et al., 2017).

Ficin

Due to the problems raised by the use of animal or microbial recombinant proteases, the use of ficin is becoming increasingly popular. Ficin is an outstanding example of proteases from plants. This enzyme can be applied for protein hydrolysis, the production of bioactive peptides, meat tenderization, milk coagulations in cheese making, or peptide synthesis. Ficin does have huge potential and brilliant prospect in the near future. Ficin is a sulfhydryl or cysteine protease commonly obtained from *Ficus carica* (fig tree) that enhances the solubility of muscle proteins (Englund et al., 1968; Morellon-Sterling et al., 2020). Ficin has a molecular weight of 44.5 kDa and shows maximal activity at pH 5.0–9.0 and optimum pH is 7.0. The enzyme is fully active at 45–75 °C with optimum temperature of 60–70 °C. Under these conditions, ficin has moderate activity in hydrolysis of myofibrillar proteins and excellent effect on hydrolysis of collagen. These properties make ficins a beneficial class of plant proteases for use. We used ficin to tenderize beef used for manufacture of sausages. Results indicated that solubility of meat proteins increased and SDS-PAGE results showed the disappearance of several protein bands in ficin-treated meat. Ficin-tenderized meat substantially improved water-holding capacity and emulsion stability. The results of this study indicated that some quality attributes of meat products can be improved by enzymatic modification of protein sources in the manufacture of meat products (Ramezani et al., 2003).

Actinidin

Actinidin is a novel sulfhydryl protease extracted from gooseberry or the kiwi fruit. Actinidin is the predominant enzyme in kiwifruit and can play a role in aiding the digestive process. It has a molecular weight of 32 kDa. It is used commercially in meat industry to tenderize meat. The ability of pre-rigor infusion of kiwifruit juice to improve the tenderness of lamb was investigated. It was shown that actinidin in kiwi fruit juice enhances proteolytic activity, resulting from the infused kiwifruit juice in carcasses, and is associated with significant degradation of the myofibrillar proteins, appearance of new peptides, and activation of m-calpain during post mortem aging. Thus, kiwifruit juice is powerful and easily prepared meat tenderizer, which could contribute efficiently and effectively to the meat tenderization process (Boland, 2013; Morton et al., 2009).

Actinidin has many applications in the food industry because of its advantages over other plant proteases such as papain and ficin. Actinidin shows mild tenderizing activity even at high concentrations, preventing surface mushiness. It has a relatively low inactivation temperature (60 °C), which makes it easier to control the tenderization process without overcooking. A group in our lab studied the effect of actinidin on beef for use in manufacture of sausage. Actinidin from kiwi fruit was partially purified by precipitation with ammonium sulfate, followed by DEAE-Sephadex column chromatography. The effect of purified actinidin on the protein solubility (nitrogen solubility index [NSI]), water-holding capacity (WHC), texture, and SDS-PAGE pattern of beef was studied and the quality attributes of a sausage product were evaluated. Actinidin significantly increased NSI and WHC of beef; the highest NSI and WHC (approximately 20% and 8% increase, respectively) was

observed when beef was incubated with 0.9 unit enzyme/g beef. Texture analysis indicated increased tenderization (10% decrease in shear force) when slices of beef were treated with actinidin at 37 °C for 2 h. SDS-PAGE results indicated appearance of several low molecular weight bands (<10 kDa) after treating beef with different levels of actinidin for 30 or 60 min. Slight changes in protein band in the range of 100–120 kDa and 13–25 kDa were also observed. Use of actinidin-tenderized beef significantly improved emulsion stability, texture, and organoleptic properties of the sausage product (Lewis & Luh, 1988). We applied actinidin to tenderize camel meat and beef and used the tenderized meats in the manufacture of emulsion type sausages (Gheisari et al., 2008). The properties of sausages made from the meat of both species were similar. Emulsion satiability, folding, texture, taste, and overall quality of sausages produced from actinidin-tenderized meat were superior than untreated samples (Aminlari et al., 2009). The actinidin has less tenderization property as compared to other traditional plant proteases and still not approved as GRAS by FDA (Lewis & Luh, 2007; Toohey et al., 2011).

Calpains

Endogenous proteolytic systems are responsible for modifying proteinases as well as the meat tenderization. Abundant evidence has shown that calpains and calpastatin (CAST) have the closest relationship with tenderness in livestock. They are involved in a wide range of physiological processes including muscle growth and differentiation, pathological conditions and postmortem meat aging (Lian et al., 2013). Meat tenderness undergoes changes after slaughtering due to the activity of the endogenous calpains and calpastatin. These calcium-dependent proteases degrade the myofibrillar proteins. The calpain system consists of three members—m-calpain, μ-calpain, and calpastatin, which is the calpain-specific endogenous inhibitor. Both m-calpain and μ-calpain are cysteine proteases, and their proteolytic activity is affected by oxidation, which can influence the quality of fresh meat. In the presence of calcium, calpains autolyze, and this autolysis is indication of their proteolytic activation during postmortem changes in muscles (Zhang et al., 2013). Calpain is an important enzyme that is chiefly used for degradation of myofibrillar proteins. It also aids in meat tenderizing and improves water holding capacity during postmortem aging.

2.3.3.4 Conclusion

In meat, tenderness is the most important factor associated with meat palatability and consumer satisfaction. Different plant proteases like papain, bromelain, actinidin, and ficin have been used for tenderization of meat and meat products. These enzymes are effective in this application because they can hydrolyze collagen and elastin, connective tissue proteins that cause toughness in meat. Antemortem application of enzyme is possible, as a fairly pure solution in saline can be injected intravenously into animals 2–10 min before slaughter, sometimes after stunning; this helps distribute the enzyme throughout the muscle tissues. Injection of inactivated papain (disulfide form) obviates any discomfort among animals, since the enzyme

becomes activated by the reducing conditions that soon prevail postmortem (Arshad et al., 2016).

2.3.4 Bioactive Peptides

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may influence health (Sánchez & Vázquez, 2017). In recent years, peptides with known sequences have been identified which have been shown to contain biological activities such as opiate-like, mineral binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, and antihypertensive actions. These bioactive peptides benefit the human physiological system through the cardiovascular, nervous, gastrointestinal, and immune systems (Choi et al., 2012). Most of the bioactive peptides are inactive in the native protein sequences and become active only when released when the parent proteins are hydrolyzed in vivo by the action of proteolytic enzymes during digestion or in vitro by intentionally added proteases (Korhonen & Pihlanto, 2006). The activity of these peptides is based on the inherent amino acid composition and sequence. Bioactive peptides usually contain 3–20 amino acid residues per molecule (Bhat et al., 2015), but in some cases may consist of more than 20 amino acids. These peptides may be used as components of functional foods or nutraceuticals because of their health-enhancing potential and safety profiles. There is increasing commercial interest in the production of bioactive peptides from various sources. Some of rich sources of bioactive peptides include milk and egg, meat of various species of animals, fish, many plants including soy bean, chickpeas, rice, and many other food-proteins and nonconventional protein sources (Mazorra-Manzano et al., 2018; Sabbione et al., 2016).

Proteolytic enzymes have the ability to modify proteins through limited or extensive cleavage, releasing free amino acids, peptides, or polypeptides with physicochemical properties different from the original protein. Proteolytic enzymes from proteolytic system of starters, proteases endogenous to food, or added enzymes (e.g., rennet) differ in their specificity and therefore in their capacity to release bioactive sequences (Korhonen & Pihlanto, 2006; Sabbione et al., 2016). Most hydrolytic processes use one protease at a time; however, a combination of two enzymes with different specificity has also been explored. Sometimes, proteins partially hydrolyzed are further treated with pepsin and trypsin to simulate gastrointestinal digestion. Other proteases widely used for bioactive peptides' production include commercial preparations such as Alcalase, Neutrase, Flavourzyme, Thermolysin derived from bacteria and fungi (Rui et al., 2012), and ficin (Shahidi et al., 2018).

We have studied the production of peptide fragments produced from goat's milk whey proteins using trypsin and ficin and evaluated the bacterial growth inhibitory activity of peptides. Goat's milk whey proteins were subjected to enzymatic hydrolysis and peptides were purified by ultrafiltration followed by reverse-phase high-performance liquid chromatography (RP-HPLC). Growth inhibitory activities of

hydrolysates ranged from 4.67% to 87.46% for *E. coli* and 3.03% to 98.63% for *B. cereus*. Among all peptide fragments, permeate containing 3 kDa peptides produced by trypsin showed maximum inhibition against Gram-positive and Gram-negative bacteria. This fraction was further purified by HPLC. Fourteen peptide fractions were collected and evaluated for their growth inhibitory activities. Two fractions showed the highest growth inhibitory activities with MIC50's of 383 ± 8 and 492 ± 10 μg/mL against *E. coli* and *B. cereus*, respectively. In a similar study, we used trypsin and ficin to generate antibacterial peptides from goat milk caseins. The peptide obtained by ficin with MW of <3 kDa showed the highest antimicrobial activity and was selected for further purification by reversed-phase high-performance liquid chromatography (RP-HPLC). Twenty-seven peptide fractions were separated, and their antimicrobial activities were evaluated. The results showed that one of the fractions (No. 14) possessed the highest activity against *Escherichia coli* and *Bacillus cereus* (Esmaeilpour et al., 2016, 2017). The authors of both papers suggested these novel antibacterial peptides can potentially replace synthetic food preservatives in food industries.

In another research project, the yeasts *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* protein hydrolysates were prepared by trypsin and chymotrypsin hydrolysis and the peptides purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The antioxidant and ACE (angiotensin-converting enzyme) inhibitory activities of the generated peptides were determined. From *K. marxianus* two new peptides, LL-9, MW 1180 Da and VL-9, MW 1118 Da, were identified. These peptides were sequenced and their functional properties studied. Both peptides exhibited significant ACE inhibitory activity (IC50 of 22.88 mM for LL-9 and 15.20 mM, for VL-9). Molecular docking studies revealed that the ACE inhibitory activities are due to interaction with the His513, His353, Glu281 and Tyr520, Lys511, Gln281 pockets of ACE by LL-9 and VL-9, respectively. In the case of *S. cerevisiae*, a fraction with molecular weight of <3 kDa exhibited the highest activity. RP-HPLC resolved this fraction into five fractions, one of which (fraction F3) with amino acid sequence of Tyr-Gly-Lys-Pro-Val-Ala-Val-Pro-Ala-Arg (MW: 1057.45 Da) exhibited ACE inhibitory ($IC_{50} = 0.42 \pm 0.02$ mg/mL) and antioxidant activities (26.25 ± 0.13 μM TE/μg protein). Taken together, the results of these studies showed that *K. marxianus* and *S. cerevisiae* proteins contain specific peptides in their sequences which can be released by enzymatic hydrolysis.

These peptides have excellent bioactive properties that can potentially replace the antioxidant and antihypertensive agents with chemical origin (Mirzaei et al., 2015, 2017).

The use of plant proteases in the production of bioactive peptides is still scarce. Cysteine proteases such as papain, ficin, and bromelain are currently the most used plant proteases. However, serine proteases such as zingibain, cucumisin, and actinidin obtained from ginger rhizome, melon, and kiwifruit, respectively, are three new emerging plant proteases which have been considered recently (Nafi et al., 2013).

The search for novel specialized proteolytic enzymes with preference for specific peptide bonds for the selective release of bioactive peptides requires further study in order to improve process efficiency. The various plant proteases, the protein substrates used, and the bioactive properties of peptides produced and their potential to prevent or treat disorders such as hypertension, diabetes, obesity, and cancer have been extensively reviewed (Mazorra-Manzano et al., 2018).

2.4 Enzymes Working on Lipids

2.4.1 Lipases

Lipases are key enzymes involved in fat digestion in vertebrates by converting insoluble triacylglycerols into more soluble products, fatty acids, and monoacylglycerols, which can easily be assimilated by the organisms' intestinal absorption. Most lipases have a basic pH optimum. Lipases act only at the oil–water interface (Schmid & Verger, 1998).

While activity of endogenous lipases is often associated with acylglycerol hydrolysis and problems with lipid degradation and/or hydrolytic rancidity (or leading to oxidative rancidity since liberated fatty acids tend to be more prone to oxidation), exogenous lipases are used for beneficial purposes. Lipases for food processing are obtained from edible forestomach tissue of calves, kids, or lambs, and animal pancreatic tissues as purified edible tissue preparations or as aqueous extracts (NAS, 1996). The porcine pancreatic enzyme is a glycoprotein composed of 450 amino acids with a calculated MW of ~50 kDa (Wong, 2003). Industrial lipases are also produced by the controlled fermentation of *Aspergillus niger* var., *Aspergillus oryzae* var., *Candida rugosa*, and *Rhizomucor miehei* as a powder or liquid (Parkin, 2017).

Currently, commercial uses of lipases involve liberating flavoring (short-chain) acids from lipids and rearranging fatty acyl groups along the glycerol backbone to create highly valued and functional triacylglycerols from low-value lipids. Typical applications in the food industry include: flavor generation in cheese. Using pregastric lipases from goat, lamb, and calf, selective hydrolysis of short-chain (C4–C8) fatty acids from triacylglycerols of milk fat can be achieved (Parkin, 2017). The lipase in papaya latex is selective for the sn-3-glycerol position, but since papaya latex contains papain, it would not be suitable in cheese. Lipases are also used for modification of lipids, manufacture of dairy products and confectionery goods, and development of flavors in processed foods. These applications are founded on selecting lipases with reaction selectivities required to yield the desired products. Selectivity of lipases involves selectivity toward fatty acylgroup, ester position along the sn-glycerol backbone, size of the glyceride (mono-, di-, or triacylated), as well as interactions among these factors, which confer characteristic stereoselectivity. The types of selectivity are exhibited by many of the commercially relevant or promising lipases from over 100 characterized sources (Shahidi et al., 2018).

Another major use of lipases is production of "structured lipids" in which the fatty acyl groups are rearranged by lipases to yield a novel distribution along sn-glycerol to create high-value lipids from low-value acylglycerol (Gunstone, 1999).

Lipases are used in baking industry and are added to bread doughs to supplement endogenous cereal grain lipases and are added as dough improvers. This functionality results in increased bread volume, more uniform crumb and air cell size, and lesser tendency to stale, hydrolysis of lipids, thereby producing mono- and diacylglycerolipids which function as emulsifying agents in the dough (Sonnet & Gazzillo, 1991; Parkin, 2017).

2.4.2 Lipoxygenases

Lipoxygenase is a naturally existing and inexpensive enzyme widely distributed in plant and animal cells and is abundantly found in legumes. Lipoxygenase is an oxidoreductive enzyme catalyzing many oxidative reactions (Song et al., 2016). The substrates of lipoxygenase in plants are mainly linoleic acid and linolenic acid. Arachidonic acid is the primary substrate for lipoxygenase in animal cells. Lipoxygenase catalyzes the dioxygenation of polyunsaturated fatty acids (PUFA), converting them to diene hydroperoxy fatty acids which further decompose to different chemicals and volatile compounds (Gigot et al., 2010). Lipoxygenase utilizes atmospheric oxygen to react with PUFA and forms potentially useful and valuable chemicals such as leukotrienes and lipoxins. The enzyme has many potential usages in the food industry; however, it is also associated with some unwanted reactions, which include color change and nutrient deterioration (Parkin, 2017). The flavor and aroma compounds produced by lipoxygenase activity on fatty acids affect the food flavor. In one aspect, the aroma compounds might improve the desirability of foods, and on the other hand, the oxidized compounds produced might give rise to off-flavor of foods. By studying and understanding the properties and reaction mechanism of LOX, food researchers could well control and utilize LOX as a natural food additive (Shi et al., 2020). In the baking industry, it functions as an effective baking conditioner and flour treatment agent. It is a potential substitute for potassium bromate and benzoyl peroxide, which are commonly used as strengthening and bleaching agents, respectively. Dough strengthening is probably through affecting disulfide cross-links within the gluten. LOX has been widely used in the food and beverage industries as a biocatalyst to produce aroma compounds at a low price and large quantity (Parkin, 2017).

It appears that in the future, the enzyme will play a very important role in the food industry. By better understanding the mechanism of action of the enzyme, controlling the undesirable activities of the enzyme by inhibiting it allows food producers to improve the quality of food.

2.4.3 Phospholipases

Mammal, plant, and microbial phospholipases are continuously being studied, experimented, and some of them are even commercially available at industrial scale for food industry. Phospholipases form a large class of enzymes with wide diversity (Mansfeld, 2009). They are generally classified as acyl hydrolases and phosphodiesterases. These enzymes are further divided into different groups depending on the site of action within the phospholipids (PL) molecule. The acyl hydrolases include the phospholipase A1 (specifically hydrolyzes 1-acyl ester bond of PL) to release lyso-phospholipids (lyso-PL) and free fatty acids (FFA), phospholipase A2 (catalyzes the hydrolysis of fatty acids at the sn-2 position of PL, releasing lyso-PL and FFA), phospholipase B (which does not discriminate between the two positional acyl ester bonds), and lysophospholipase A ½ (partially hydrolyzes PL) (Parkin, 2017). The phosphodiesterases are represented by phospholipase C and D (which cleave the phosphorus–oxygen bond between glycerol and phosphate, releasing diacylglycerol and phosphate esters). Except phospholipases A2, most of these phospholipases are not widely available at large quantities for industrial purposes. The use of secreted PLA 2 from porcine pancreas or snake and bee venoms has a very long tradition in food industry for modification of PL, such as egg yolk production for emulsification in mayonnaise, sauces or salad dressings, baking industry, or refinement of vegetable oils by degumming (Mansfeld, 2009).

The most representative examples of the use of phospholipases in food industry can be found in the production of edible oils, dairy, and baking products or emulsifying agents. Phospholipases are incorporated in processes such as the degumming of vegetables oils during refinement for removing undesirable compounds, the manufacture of cheese for yield increasing, or the production of bread as bakery improvers for reducing the inclusion of emulsifying compounds (Ramrakhiani & Chand, 2011). Lipases and phospholipases offer the opportunity to generate compounds with technological effects and emulsifier characteristics in the bread making, with the main final advantage of allowing the reduction or replacement of added emulsifiers in bakery products. On the other hand, superior emulsifying properties of released lysolipids can be obtained, leading to improved dough rheological properties (Zhao et al., 2010; Casado et al., 2012).

2.5 Miscellaneous Enzymes

2.5.1 Catalase

The enzyme catalase is known to catalyze the breakdown of hydrogen peroxide into oxygen and water. It is an oxidoreductase enzyme as it plays a crucial role in quenching the reactive oxygen species (ROS), i.e., hydrogen peroxide, often produced as a by-product of aerobic respiration. Hence, it acts as an antioxidant and protects the cell against oxidative stress (Kaushal et al., 2018).

Catalase is widely distributed in nature. It is found in all aerobic microorganisms and in all plant and animal cells. The catalase activity of mammalian cells varies. It is highest in liver and kidney and low in connective tissues. Catalase has been used in the food processing industry to determine the adequacy of blanching (70–105 °C) of vegetables and fruits to destroy microorganisms and enzymes to preserve color, texture, flavor, taste, and aroma of the products when stored frozen up to 2 years. Early in the application of blanching for stabilization of frozen foods, catalase was used especially for peas (24–26), the best on the market (Williams et al., 1986; Wong & Whitaker, 2003). In the dairy industry, catalase activity is a sensitive and easily detected indicator of contamination of milk by neutrophil granucytes. Also, the use of catalase has proven to be a very cost-efficient and also a comparatively environmental friendly method of getting rid of excess hydrogen peroxide from milk samples for cheese production. H_2O_2 is used in the cold pasteurization process of milk for cheese manufacture. The residual H_2O_2 is converted to water and molecular oxygen by catalase, thereby avoiding its toxicity. Catalase enzymes are typically obtained from bovine livers or microbial sources (Kilcawley et al., 1998).

2.5.2 Amino Oxidases

Biogenic amines and polyamines are present in variety of foods, such as fish, meat, cheese, vegetables, and wines. These are organic bases with aliphatic, aromatic, and heterocyclic structures. They are produced by decarboxylation of amino acid substrates catalyzed by enzymes from contaminating bacteria. The most common biogenic amines found in foods are histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine (Ruiz-Capillas & Herrero, 2019). The formation of biogenic amines in food can result in consumers suffering allergic reactions, characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypertension. Several methods for prevention of the production or removal of biogenic amine in food have been practiced, primarily by limiting microbial growth through chilling and freezing. However, for many socioeconomic and technological reasons, such approaches are not always practical. Therefore, alternative measures to prevent biogenic amine formation in foods or to reduce their levels once formed are needed. One approach might be application of enzymes which metabolite these amines (Naila et al., 2010).

Amine oxidase catalyzes the reaction with mono-, di-, or polyamine as substrate (Ito & Ma, 2003):

$$RCH_2NH_2 + O_2 + H_2O \rightarrow NH_3 + RCHO + H_2O_2$$

The activity of these enzymes has been detected in *Arthrobactor*, yeast *Candida boidinii*, *Asp. niger*, pea seedling, bovine plasma, and bovine lung. In human and animals, the enzyme plays an important role for the metabolism of biogenic monoamines in the central nervous system and peripheral tissues. Pea seedling amine oxidase, a copper containing diamine oxidase, catalyzes the oxidative

deamination of histamine to the corresponding aldehyde, i.e., imidazole acetaldehyde concomitant with the reduction of dioxygen to hydrogen peroxide. The antihistamine properties of this enzyme make it an attractive candidate in fish industry to combat the allergic responses elicited by consumption of fish. However, the observed variation in the efficacy of the enzyme has been attributed to the diversity and distribution of the enzyme inhibitors present in the variety of fish species (Ebrahimnejad et al., 2013; Stránská et al., 2007).

2.5.3 Phenylalanine Ammonia-Lyase

Phenylketonuria (PKU) is an autosomal recessive genetic disorder affecting on average incidence of about 1–3 case in 10,000 Caucasian live births. It is caused by deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) which is responsible for converting phenylalanine to tyrosine using molecular oxygen and tetrahydrobioptrin as necessary cofactor to perform its catalysis (Al Hafid & Christodoulou, 2015). PAH deficiency leads to hyperphenylalaninenemia, a dramatic increase in blood phenylalanine concentration (<120 to >1200 μM) with concurrent appearance of phenylalanine, phenylpyruvate, and phenylacetone in urine. High levels of accumulated phenylalanine in PKU patients are toxic to human body if left untreated and are associated with an abnormal phenotype presenting with growth failure, microcephaly, seizure, and mental retardation. Children with severe PKU can have normal cognitive development when dietary treatment is initiated in early infancy and the blood phenylalanine level is maintained at near normal or normal levels (Ding et al., 2004). The established treatment for PKU resides in a phenylalanine-restricted diet, which has known deficiencies of several nutrients and unsatisfactory organoleptic properties, making long-term compliance a major challenge (Levy, 1999).

Phenylalanine ammonia lyase (PAL) is an enzyme that catalyzes a reaction converting L-phenylalanine to ammonia and *trans*-cinnamic acid (which is excreted as hippurate in urine). PAL is found widely in plants with isoenzymes existing within many different species. It has a molecular mass in the range of 270–330 kDa. A large number of plant species were screened for PAL activity and it was found that enzyme activity was highest in grain seedlings, with maximal enzyme activity in 7-day-old red spring wheat (Goldson et al., 2008; Camm & Towers, 1973). We have studied the stability of wheat seedling PAL during storage at different temperatures and found a first order kinetic for inactivation of PLA with half-life of 30 and 18 days at -18 and 4 °C, respectively. Activity of PAL increased while germination occurred at 25 °C up to 8 days. These results can be used to develop appropriate strategies for storing PAL containing materials with retained PAL activities (Aminlari et al., 2010).

PAL has recently been studied for possible therapeutic benefits in humans afflicted with phenylketonuria. The use of phenylalanine ammonia-lyase (PAL) is an attractive alternative to the dietary treatment of PKU and both pharmacologic and physiologic proofs of principle have been established using recombinant PAL for

enzyme substitution therapy in a murine PKU model (Sarkissian et al., 1999). A large amount of active recombinant PAL from parsley *(Petroselinum crispum)* has been prepared and chemically modified to render PAL nonimmunogenic and stable in the circulation (Kim et al., 2004b). Oral therapy with phenylalanine ammonia lyase (PAL), naturally encapsulated in plant cells, may provide a potential alternative treatment for patients with PKU (Goldson et al., 2008). One significant challenge in application of PAL for PKU therapy is protecting PAL from proteolysis and/or denaturation and against immunologic responses. Different physical and chemical methods have been investigated to address this problem. These include PAL immobilization in semipermeable, microcapsules, the entrapment of PAL in silk fibroin, and the immobilization of recombinant PAL in expression bacteria such as *Escherichia coli* or *Lactobacillus lactis*, and modification with PEGylation (Bell et al., 2017; Ikeda et al., 2005).

2.5.4 Lysozyme

In recent years, consumer demand for 'natural' foods has driven development of products without additives. In order to meet this demand, much attention and interest have been directed towards identification and application of naturally made compounds such as antimicrobial agents, in food and pharmaceuticals (Branen & Davidson, 2004). Some naturally occurring proteins such as lactoperoxidase, lactoferrin, and lysozyme have received much attention and are being considered as potential antimicrobial agents to replace the currently used synthetic food preservatives (Demain, 2009).

Lysozyme, also known as muramidase or *N*-acetylmuramide glycanhydrolase, is an antimicrobial enzyme found in many different sources, from viruses to vertebrates, and has been subjected to extensive scrutiny, both as a protein model and a natural antimicrobial and pharmaceutical agent. It forms part of the innate immune system. Lysozyme is abundant in secretions including tears, saliva, human milk, and mucus. Chicken egg white has the highest content of lysozyme (it constitutes 3.5% of the total egg white proteins), from which this enzyme is purified and is commercially produced (Proctor & Cunningham, 1988). Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis of 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall. In general, lysozyme shows in vitro antimicrobial activity against some Gram-positive bacteria such as Staphylococcus aureus, *Micrococcus luteus*, *Bacillus stearothermophilus*, and *Clostridium tyrobutyricum*, but little action against Gram-negative bacteria (Cunningham et al., 1991). Gram-negative bacteria, including foodborne pathogens, resist lysozyme due to steric hindrance posed by the outer LPS layer, hence lysozyme cannot access the peptidoglycan (Ibrahim et al., 1994). Thus, modification of lysozyme, which can broaden its antibacterial properties against both Gram-negative and Gram-positive bacteria, can also increase the usefulness of lysozyme (Seo et al., 2013).

Lysozyme has received considerable interest as a food preservative (Cegielska-Radziejewska & Szablewski, 2013). In many countries, lysozyme is used as a preservative in many types of foods (including vegetables, sea foods, soy bean products, meat products, and semihard cheeses), and as a component of pharmaceutical products. In the European Union, its use in specific products such as hard cheeses and in wine making to control infection (E number E 1105 as a food additive) is allowed (Losso et al., 2000). Proctor and Cunningham (1988) and Cunningham et al. (1991) have reviewed the use of lysozyme as a food preservative.

During the last two decades, extensive researches in the authors' laboratory and others have been directed toward modification of lysozyme in order to improve its antimicrobial properties. Several types of these modifications are summarized in Table 2.4. The results of modifications of lysozyme using its conjugation with

Table 2.4 Modified lysozymes and their properties (from Aminlari et al., 2014)

Modified lysozymes	Effect on functionality
Palmitic acid	Enhanced antimicrobial against *E. coli* (WT-3301)
Short and middle chain saturated fatty acids	Enhanced antimicrobial against G-positive bacteria
Glucose–stearic acid monoester	Enhanced activity against *E. coli* and *E. tarda* (G8104)
Perillaldehyde	Enhanced activity against *E. coli* K12 and *S. aureus*
Cinnamaldehyde	Enhanced activity against *E. coli* and *S. aureus*
Glucosamine	Improved solubility at different pHs and temperatures, increased heat stability, emulsion activity and stability, and foam capacity
Caffeic acid–cinnamic acid	Antimicrobial activity against *E. coli* (ATCC 8739), decreased activity against *S. aureus* (ATCC 6538)
Dextran	Enhanced activity against *E. coli* and *S. aureus* in cheese
	Enhanced activity against *E. coli* and *S. aureus* in milk
	Treatment of bacterial isolates from cows with mastitis
	Preparation of a lysozyme–dextran nanogel
	Increased heat stability, higher emulsifying property
Galactomannan	Antibacterial emulsifier
	Emulsifier, antibacterial against G-negative pathogen *E. tarda*
Chitosan	Enhanced bactericidal action against *E. coli* K12
	Lysozyme-composite film with activity against *E. coli*, *L. monocytogenes*, and *S. faecalis*
Cellulose	Preparing a textile with potential barrier to microbial invasion
Gum Arabic	Enhanced activity against *E. coli* and *S. aureus* in mayonnaise
Xanthan gum	Enhanced activity against *E. coli* and *S. aureus*
Dextran sulfate	Enhanced activity against *E.* coli and *S. aureus*
Barley beta-glucan	Enhanced activity against *E.* coli and *S. aureus*
Tragacanth	Enhanced activity against *E. coli*, *S. typhimyrium*, *B. cereus*, and *S. aureus*
Inulin	Improved functional properties
Trypsin and ficin digestion of	Enhanced activity of peptides *E. coli* and *B. cereus* dextran-conjugated lysozyme

different small molecule and polysaccharides by application of a Maillard-based reaction, as well as modifications using proteolytic enzymes, have revealed that these types of modifications have not only increased the functional properties (such as solubility and heat stability), but also extended the antimicrobial activity of lysozyme (Aminlari et al., 2014). We have shown that it is possible to broaden the antimicrobial effect of lysozyme by several types of modifications, which consequently make these lysozyme derivatives excellent food preservatives. Table 2.3 summarizes the effect of different types of modifications on lysozyme.

2.6 Conclusions

Nature provides us with remarkably vast array of enzymes with extraordinary catalytic properties. In their natural reservoirs, these so-called "endogenous" enzymes perform essentially all biochemical reactions vital to the living organisms. When these enzymes are removed from their natural habitat, they continue to catalyze same reactions when they have access to their substrate. At this stage, they are indeed "exogenous" enzyme. Food scientists have used these exogenous enzymes as food additives for many years. In this chapter, the properties of several enzymes from plant and animal sources were presented and their application or potential application in food industry was discussed. Over the years, it has become clear that despite advances in experimental mutational studies, a quantitative understanding of enzyme catalysis will not be possible without computer modeling approaches (Frushicheva et al., 2014). With the advent of new technologies such as information technology, rational molecular design, and DNA enzyme (deoxyribozyme) technologies (Breaker, 1997; Woolcock, 2016), it is expected that new doors for application of these technologies will open to food enzymologists in the near future. The discussion is by no means complete, and with the advent of knowledge of enzymology, the endeavor will continue. For those of us who (as Arthur Korenberg, the discoverer of DNA polymerase once said) are in love with enzymes, the challenge has just began.

References

Aehle, W. (Ed.). (2007). *Enzymes in industry, production and applications* (3rd ed., pp. 1–12). Wiley-VCH Verlag GmbH and Co. KGaA.

Aiyer, P. V. (2005). Amylases and their applications. *African Journal of Biotechnology, 4*, 1525–1529.

Al Hafid, N., & Christodoulou, J. (2015). Phenylketonuria: A review of current and future treatments. *Translational Pediatrics, 4*, 304–317.

Alirezaei, M., Aminlari, M., Gheisar, H. R., & Tavana, M. (2011). Actinidin: A promising milk coagulating enzyme. *European Journal of Food Research and Review, 1*, 43–51.

Alkorta, I., Garbisu, L. M. J., & Serra, J. L. (1998). Industrial applications of pectic enzymes: A review. *Process Biochemistry, 33*, 21–28.

Amal Ben Amira, A., Besbes, S., Attia, H., & Blecker, C. (2017). Milk-clotting properties of plant rennets and their enzymatic, rheological and sensory role in cheese making: A review. *International Journal of Food Properties, 20*, S76–S93.

Aminlari, L., Mohammadi Hashemi, M., & Aminlari, M. (2014). Modified lysozymes as novel broad spectrum natural antimicrobial agents in foods. *Journal of Food Science., 79*, R1077–R1090.

Aminlari, L., Roshanzadeh, S., Jafarpoor, D., & Aminlari, M. (2010). Changes in phenylalanine ammonia lyase activity of wheat seedling during storage at different temperatures. In *First International Congress on Food Technology*, November 3–6, 2010, Antalya, Turkey.

Aminlari, M., Shekarforoush, S. S., Gheisari, H. R., & Golestan, L. (2009). Effect of actinidin on the protein solubility, water holding capacity, texture, electrophoretic pattern of beef, and on the quality attributes of a sausage product. *Journal of Food Science, 74*, C221–C226.

Aminlari, M., & Vaseghi, T. (2006). Biochemical properties and biological functions of the enzyme rhodanese in domestic animals. *Iranian Journal of Veterinary Research, 7*, 1–13.

Arshad, M. S., Kwon, J. H., Imran, M., Sohaib, M., Aslam, A., Nawaz, I., Amjad, Z., Khan, U., & Javed, M. (2016). Plant and bacterial proteases: A key towards improving meat tenderization, a mini review. *Cogent Food and Agriculture, 2*, 1261780. https://doi.org/10.1080/23311932.2016.1261780.

Autio, K., Harkonen, H., Parkkonen, T., Frigard, T., Poutanen, K., Siika-Aho, M., & Aman, M. (1996). Effects of purified endo β-glucanase on the structural and baking characteristics of rye doughs. *Lebensmittel-Wissenschaft und -Technologie, 29*, 18–27.

Bamforth, C. W. (2009). Current perspectives on the role of enzymes in brewing. *Journal of Cereal Science, 50*, 353–357.

Bell, S. M., Wendt, D. J., Zhang, Y., Taylor, T. W., Long, S., TsurudaL, Z. B., Laipis, P., & Fitzpatrick, P. A. (2017). Formulation and PEGylation optimization of the therapeutic PEGylated phenylalanine ammonia lyase for the treatment of phenylketonuria. *PLoS One, 12*, e0173269. https://doi.org/10.1371/journal.pone.0173269.

Benen, J. A. E., Voragen, A. G. I., & Visser, J. (2003). Pectic enzymes. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Bhardwaj, N., Kumar, B., & Verma, P. A. (2019). Detailed overview of xylanases: An emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing, 6*, 40.

Bhat, Z. F., Kumar, S., & Bhat, H. F. (2015). Bioactive peptides of animal origin: A review. *Journal of Food Science and Technology, 9*, 5377–5392. https://doi.org/10.1007/s13197-015-1731-5

Biely, P. (2003). Xylanolytic enzymes. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Boland, M. (2013). Kiwifruit proteins and enzymes: Actinidin and other significant proteins. *Advances in Food and Nutrition Research, 68*, 59–80.

Bourne, Y., & Henrissat, B. (2001). Glycoside hydrolases and glycosyltransferases: Families and functional modules. *Current Opinion in Structural Biology, 11*, 593–600. https://doi.org/10.1016/s0959-440x(00)00253-0

Branen, K. J., & Davidson, P. M. (2004). Enhancement of nisin, lysozyme and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lacto-ferrin. *International Journal of Food Microbiology, 90*, 63–73.

Breaker, R. R. (1997). DNA enzymes. *Nature Biotechnology, 15*, 427–431.

Camm, E. L., & Towers, G. (1973). Phenylalanine ammonia lyase. *Phytochemistry, 12*, 961–973. https://doi.org/10.1016/0031-9422(73)85001-0.

Casado, V., Martín, D., Torres, C., & Reglero, G. (2012). Phospholipases in food industry: A review. In G. Sandoval (Ed.), *Lipases and phospholipases: Methods and protocols, methods in molecular biology* (Vol. 861, pp. 495–523). Springer Science and Business Media. https://doi.org/10.1007/978-1-61779-600-5_29

Cegielska-Radziejewska, R., & Szablewski, T. (2013). Effect of modified lysozyme on the microflora and sensory attributes of ground pork. *Journal of Food Protection, 76*, 338–342.

Chahardahcherik, M., Ashrafi, M., Ghasemi, Y., & Aminlari, M. (2020). Effect of chemical modification with carboxymethyl dextran on kinetic, structural and immunological properties of asparaginase. *Analytical Biochemistry, 591*, 113537. https://doi.org/10.1016/j.ab.2019.113537

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry, 135*, 3020–3038.

Chaudhary, M., & Gupta, R. (2012). Cyanide detoxifying enzyme: Rhodanese. *Current Biotechnology, 1*, 327–335.

Choi, J., Latha, S., Hasan, A., & Anad, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*, 1–12.

Christophersen, C., Otzen, D. E., Norman, B. E., Christensen, S., & Schäfer, T. (1998). Enzymatic characterization of Novamyl[R], a thermostable alpha-amylase. *Starch-Stuttgart, 50*, 39–45.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology, 11*, 254–262.

Cunningham, F. E., Proctor, V. A., & Goetsch, S. J. (1991). Egg-white lysozyme as a food preservative: An overview. *World's Poultry Science Journal, 47*, 141–163.

Das, R., & Kayastha, A. M. (2019). β-Amylase: General properties, mechanism and panorama of applications by immobilization on nano-structures. In Q. Husain & M. Ullah (Eds.), *Biocatalysis, enzymatic basics and applications* (pp. 17–38). Springer.

Demain, A. L. (2009). Antibiotics: Natural products essential to human health. *Medicinal Research Reviews, 29*, 821–842.

Di Cera, E. (2009). Serine proteases. *IUBMB Life, 61*, 510–515.

Diamond, S. L. (2007). Methods for mapping protease specificity. *Current Opinion in Chemical Biology, 11*, 46–51.

Ding, Z., Harding, C. O., & Thöny, B. (2004). State-of-the-art 2003 on PKU gene therapy. *Molecular Genetics and Metabolism, 81*, 3–8.

Dixit, S., Upadhyay, S. K., Singh, H., Pandey, B., Chandrashekar, K., & Verma, P. C. (2013). Pectin methylesterasease of Datura species, purification, and characterization from *Datura stramonium* and its application. *Plant Signaling & Behavior, 8*, e25681. https://doi.org/10.4161/psb.25681

Domingo, C., Roberts, K., Stacey, N. J., Connerton, I., Ruíz-Teran, F., & MC, M. C. (1998). Apectatelyase from Zinnia elegans is auxin inducible. *The Plant Journal, 13*, 17–28.

Ebrahimnejad, H., Gheisari, H. R., & Khan Nazer, A. H. (2013). Pea seedling amine oxidase application: An emerging antihistamine strategy in tuna fish. *Journal of Food Processing and Technology, 4*, 242–248. https://doi.org/10.4172/2157-7110.1000242

El-Sohaimy, S. A., Hafez, E. E., & El-Saadani, M. A. (2010). Cloning and in vitro-transcription of chymosin gene in *E. coli*. *The Open Nutraceuticals Journal, 3*, 63–68.

Englund, P. T., King, T. P., Craig, L. C., & Walti, A. (1968). Studies on ficin. I. Its isolation and characterization. *Biochemistry, 7*, 163–175.

Esmaeilpour, M., Ehsani, M. R., Aminlari, M., Shekarforoush, S. S., & Hoseini, E. (2016). Antimicrobial activity of peptides derived from enzymatic hydrolysis of goat milk caseins. *Comparative Clinical Pathology, 25*, 599–605. https://doi.org/10.1007/s00580-016-2237-x

Esmaeilpour, M., Ehsani, M. R., Aminlari, M., Shekarforoush, S. S., & Hoseini, E. (2017). Antimicrobial peptides derived from goat's milk whey proteins obtained by enzymatic hydrolysis. *Journal of Food Biosciences and Technology, 7*, 65–72.

Fernandes, P. (2018). *Enzymatic processing in the food industry*. Elsevier.

Fox, P. F. (1993). Exogenous enzymes in dairy technology—A review. *Journal of Food Biochemistry, 17*, 173–199.

Frushicheva, M. P., Mills, M. J. L., Schopf, P., Singh, M. K., Prasad, R. B., & Warshel, A. (2014). Computer aided enzyme design and catalytic concepts. *Current Opinion in Chemical Biology, 21*, 56–62.

Gheisari, H. R., Shekarforoush, S. S., & Aminlari, M. (2008). Application of fresh, defrosted and actinidin-tenderized camel and cattle meat in the production of emulsion type sausages. *Advances in Food Sciences, 30*, 1–7.

Gigot, C., Ongena, M., Fauconnier, M., Wathelet, J., Jardin, P. D., & Thonart, P. (2010). The lipoxygenase metabolic pathway in plants: Potential for industrial production of natural green leaf volatiles. *Biotechnology, Agronomy, Society and Environment, 14*, 451–460.

Gokoglu, N., Yerlikaya, P., Ucak, I., & Yatmaz, H. A. (2017). Effect of bromelain and papain enzymes addition on physiochemical and textural properties of squid (Loligo vulgaris). *Food Measure, 11*, 345–353. https://doi.org/10.1007/s11694-016-9403-3

Goldson, A., Lam, M., Scaman, C. H., Clemens, S., & Kermode, A. (2008). Screening of phenylalanine ammonia lyase in plant tissues, and retention of activity during dehydration. *Journal of the Science of Food and Agriculture, 88*, 619–625.

Gomaa, A. M. (2018). Application of enzymes in brewing. *Journal of Nutrition and Food Science Forecast, 1*, 1–5.

Grahn, S., Kurth, T., Ullmann, D., & Jakubke, H. (1999). S′ subsite mapping of serine proteases based on fluorescence resonance energy transfer. *Biochimica et Biophysica Acta, 1431*, 329–337.

Green, M. L. (1977). Review of the progress of dairy science: Milk coagulants. *The Journal of Dairy Research, 44*, 159–188.

Gunstone, F. D. (Ed.). (1999). *Lipid synthesis and manufacture* (Vol. 472). CRC Press LLC.

Gupta, R., Kohli, P., & Kalia, M. (2015). Pectin methylesteraseases: A review. *Journal of Bioprocessing and Biotechniques, 5*, 1–5. https://doi.org/10.4172/2155-9821.1000227

Hebeda, R. E., Bowles, L. K., & Teague, W. M. (1991). Use of intermediate stability enzymes for retarding staling in baked goods. *Cereal Foods World, 36*, 619–624.

Hedstrom, L. (2002). Serine protease mechanism and specificity. *Chemical Reviews, 102*, 4501–4523.

Hefferon, K. (2017). Cellulase production in transgenic plants: Molecular pharming with a twist. *Advances in Biotechnology and Microbiology, 6*, 555685. https://doi.org/10.19080/AIBM.2017.06.555685

Hughes, H. A. (1999). Biosynthesis and degradation of cyanogenic glycosides. In D. Barton, K. Nakanishi, & O. Meth-Cohn (Eds.), *Comprehensive natural product chemistry* (pp. 881–895). Pergamon Press.

Ibrahim, H. R., Hl, H., Fujiki, M., Kim, M., & Yamamoto, T. (1994). Enhanced anti-microbial action of lysozyme against gram-negative and gram-positive bacteria due to modification with perillaldehyde. *Journal of Agricultural and Food Chemistry, 42*, 1813–1817.

Ikeda, K., Inada, Y., Schiltz, E., Fujii, T., Takahashi, M., Mitsui, K., Kodera, Y., Matsushima, A., Schulz, G. E., & Nishimura, H. (2005). Phenylalanine ammonia-lyase modified with polyethylene glycol: Potential therapeutic agent for phenylketonuria. *Amino Acids, 29*, 283–287. https://doi.org/10.1007/s00726-005-0218-5

Illanes, A. (2008). Enzyme production. In A. Illanes (Ed.), *Enzyme biocatalysis* (pp. 57–106). Springer.

Istrati, D. (2008). The influence of enzymatic tenderization with papain on functional properties of adult beef. *Journal of Agroalimentary Processes and Technologies, 14*, 140–146.

Ito, A., & Ma, J. (2003). Amine oxidase. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Javed, R., Nawaz, A., Munir, M., Hanif, M., Mukhtar, H., Ul Haq, I., & Abdullah, R. (2018). Extraction, purification and industrial applications of pectinase: A review. *Journal of Biotechnology and Bioresearch, 1*, 1–6.

Kaushal, Y., Singh, S. G., Raina, A., & Arya, S. K. (2018). Catalase enzyme: Application in bioremediation and food industry. *Biocatalysis and Agricultural Biotechnology, 16*, 192–199. https://doi.org/10.1016/j.bcab.2018.07.035

Kawaguchi, Y., Kosugi, S., Sasaki, K., Uozumi, T., & Beppu, T. (1987). Production of chymosin in *Escherichia coli* cells and its enzymatic properties. *Agricultural and Biological Chemistry, 51*, 1871–1877. https://doi.org/10.1080/00021369.1987.10868318

Kilcawley, K. N., Wilkinson, M. G., & Fox, P. F. (1998). Enzyme-modified cheese. *International Dairy Journal, 8*, 1–10.

Kim, S. Y., Gunasekaran, S., & Olson, N. F. (2004a). Combined use of chymosin and protease from cryphonectria parasitica for control of meltability and firmness of cheddar cheese. *Journal of Dairy Science, 87*, 274–283.

Kim, W., Erlandsen, E., Surendran, S., Stevens, R., Gamez, A., Michols-Matalon, K., Tyring, S. K., & Matalon, R. (2004b). Trends in enzyme therapy for phenylketonuria. *Molecular Therapy, 10*, 220–224. https://doi.org/10.1016/j.ymthe.2004.05.001

Klemm, D., Heublein, B., Fink, H. P., & Bohn, A. (2005). Cellulose: Fascinating biopolymer and sustainable raw material. *Angewandte Chemie International Edition, 44*, 3358–3393. https://doi.org/10.1002/anie.200460587

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Journal of Dairy Technology, 16*, 945–960.

Kumar, D., Kumar, S. S., Kumar, J., Kumar, O., Mishra, S. V., Kumar, R., & Malyan, S. K. (2017). Xylanases and their industrial applications: A review. *Biochemical and Cellular Archives, 17*, 353–360.

Laurikainen, T., Harkonen, H., Autio, K., & Poutanen, K. (1998). Effects of enzymes in fibre-enriched baking. *Journal of the Science of Food and Agriculture, 76*, 239–249.

Levy, H. L. (1999). Phenylketonuria: Old disease, new approach to treatment. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 1811–1813.

Lewis, D., & Luh, B. S. (2007). Development and distribution of actinidin in kiwifruit (*Actinidia chinensis*) and its partial characterization. *Journal of Food Biochemistry, 12*, 109–116. https://doi.org/10.1111/j.1745-4514.1988.tb00363.x

Lewis, D. A., & Luh, B. S. (1988). Application of actinidin from kiwifruit to meat tenderization and characterization of beef muscle protein hydrolysis. *Journal of Food Biochemistry, 12*, 147–158.

Lian, T., Wang, L., & Liu, Y. (2013). A new insight into the role of calpains in post-mortem meat tenderization in domestic animals: A review. *Asian-Australasian Journal of Animal Sciences, 26*, 443–454. https://doi.org/10.5713/ajas.2012.12365

Lo Piero, A. R., Puglisi, I., & Petrone, G. (2011). Characterization of the purified actinidin as a plant coagulant of bovine milk. *European Food Research and Technology, 233*, 517–524. https://doi.org/10.1007/s00217-011-1543-4

Losso, J. N., Nakai, S., & Charter, E. A. (2000). Lysozyme. In A. S. Naidu (Ed.), *Natural food antimicrobial systems* (pp. 185–210). CRC Press LLC.

MacGregor, A. W., & Morgan, J. E. (1992). Determination of specific activities of malt α-amylases. *Journal of Cereal Science, 16*, 267–277.

Mansfeld, J. (2009). Plant phospholipases A2: Perspectives on biotechnological applications. *Biotechnology Letters, 31*, 1373–1380.

Marques, A. C., Marostica, M. R., Jr., & Maria Pastore, G. M. (2010). Some nutritional, technological and environmental advances in the use of enzymes in meat products. *Enzyme Research*, 480923. https://doi.org/10.4061/2010/480923

Mazorra-Manzano, M. A., Ramírez-Suarez, J. C., & Yada, R. (2018). Plant proteases for bioactive peptides release: A review. *Critical Reviews in Food Science and Nutrition, 58*, 2147–2163. https://doi.org/10.1080/10408398.2017.1308312

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., & Aminlari, M. (2016). Antioxidant, ACE-inhibitory and antimicrobial activities of *Kluyveromyces marxianus* protein hydrolysates and their peptide fractions. *Functional Foods in Health and Disease, 6*, 425–439. https://doi.org/10.31989/ffhd.v6i7.250

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., & Aminlari, M. (2017). Production of antioxidant and ACE-inhibitory peptides from Kluyveromyces marxianus protein hydrolysates: Purification and

molecular docking. *Journal of Food and Drug Analysis*. https://doi.org/10.1016/j.jfda.2017.07.008

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., Aminlari, M., & Hosseini, E. (2015). Purification and identification of antioxidant and ACE-inhibitory peptide from *Saccharomyces cerevisiae* protein hydrolysate. *Journal of Functional Foods, 19*(Part A), 259–268.

Mizani, M., Aminlari, M., & Khodabandeh, M. (2005). An effective method for producing a nutritive protein extract powder from shrimp-head waste. *Food Science and Technology International, 11*, 49–54. https://doi.org/10.1177/1082013205051271

Morellon-Sterling, R., El-Siar, H., Tavano, O. L., Berenguer-Murcia, A., & Fernández-Lafuente, R. (2020). Ficin: A protease extract with relevance in biotechnology and biocatalysis. *International Journal of Biological Macromolecules, 162*, 394–404.

Morgan, K. R., Gerrard, J., Every, D., Ross, M., & Margy Gilpin, M. (1997). Staling in starch breads: The effect of antistaling α-amylase. *Starch-Starke, 49*, 54–59.

Morton, H. J. D., Bekhit, A. E. D., & Sedcole, J. R. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science, 82*, 324–330.

Mosafa, L., Moghadam, M., & Shahed, M. (2013). Papain enzyme supported on magnetic nanoparticles: Preparation, characterization and application in the fruit juice clarification. *Chinese Journal of Catalysis, 34*, 1897–1904.

Nafi, A., Foo, H. L., Jamilah, B., & Ghazali, H. M. (2013). Properties of proteolytic enzyme, from ginger (*Zingiber officinale roscoe*). *International Food Research Journal, 20*, 363–368.

Naila, A., Flint, S., Fletcher, G., Bremer, P., & Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *Journal of Food Science, 75*, R139–R150. https://doi.org/10.1111/j.1750-3841.2010.01774.x

Nair, I. C., & Jayachandran, K. (2019). Aspartic proteasae in food industry. In B. Parameswaran, S. Varjani, & S. Raveendran (Eds.), *Green bioprocess. Enzymes in industrial food processing* (pp. 15–30). Springer.

NAS. (1996). *Food chemical codex* (4th ed., pp. 787, 803–807). National Academy Press.

Nelson, J. (1977). Impact of new milk clotting enzymes on cheese technology. *Journal of Dairy Science, 58*, 1739–1750.

Otto, H. H., & Schirmeister, T. (1997). Cysteine proteases and their inhibitors. *Chemical Reviews, 97*, 133–171.

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., & Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry, 31*, 135–152.

Parkin, K. L. (2017). Enzymes. In S. Damodaran, K. L. Parkin, & O. R. Fennema (Eds.), *Fennema's food chemistry* (pp. 331–436). CRC Press.

Poulsen, P. B., & Buchholz, K. (2003). History of enzymology with emphasis on food production. In J. R. Whitaker, A. G. J. Varagen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Proctor, V. A., & Cunningham, F. E. (1988). The chemistry of lysozyme and it use as a food preservative and a pharmaceutical. *Critical Reviews in Food Science and Nutrition, 26*, 359–395.

Ramezani, R., Aminlari, M., & Fallahi, H. (2003). Effect of chemically modified soy proteins and ficin-tenderized meat on the quality attributes of sausage. *Journal of Food Science, 68*, 85–88. https://doi.org/10.1111/jfds.2003.68.issue-1

Ramrakhiani, L., & Chand, S. (2011). Recent progress on phospholipases: Different sources, assay methods, industrial potential and pathogenicity. *Applied Biochemistry and Biotechnology, 164*, 991–1022. https://doi.org/10.1007/s12010-011-9190-6

Rocha, G. A., Trindade, M. A., Netto, F. M., & Favaro-Trindade, C. S. (2009). Microcapsules of a casein hydrolysate: Production, characterization, and application in protein bars. *Food Science and Technology International, 15*, 407–413.

Romero-Segura, C., Sanz, C., & Perez, A. G. (2009). Purification and characterization of an olive fruit β-glucosidase involved in the biosynthesis of virgin olive oil phenolics. *Journal of Agricultural and Food Chemistry, 57*, 7983–7988.

Rui, X., Boye, J., Simpson, B. K., & Prasher, S. O. (2012). Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of different thermal and enzymatic digestion treatments. *Food Research International, 49*, 739–746.

Ruiz-Capillas, C., & Herrero, A. M. (2019). Impact of biogenic amines on food quality and safety. *Food, 8*, 62. https://doi.org/10.3390/foods8020062

Sabbione, A. C., Ibañez, S. M., Martínez, E. N., Añón, M. C., & Scilingo, A. A. (2016). Antithrombotic and antioxidant activity of amaranth hydrolysate obtained by activation of an endogenous protease. *Plant Foods for Human Nutrition, 71*, 174–182.

Saini, R., Saini, H. S., & Dahiya, A. (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry, 6*, 1865–1871.

Sánchez, A., & Vázquez, A. (2017). Bioactive peptides: A review. *Food Quality Safety, 1*, 29–46. https://doi.org/10.1093/fqs/fyx006

Sarkissian, C. N., Shao, Z., Blain, F., Peevers, R., Su, H., Heft, R., Chang, T. M. S., & Scriver, C. R. A. (1999). A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 2339–2344.

Schmid, R. D., & Verger, R. (1998). Lipases. Interfacial enzymes with attractive applications. *Angewandte Chemie International Edition, 37*, 1608–1633.

Schols, H. A., & Voragen, A. G. J. (2003). Pectic polysaccharides. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Semenova, M. V., Sinitsyna, O. A., Morozova, V. V., Fedorova, E. A., Gusakov, A. V., Sokolova, L. M., Koshelov, A. A., Bubonova, T. V., Vinetskii, Y. P., & Sinitsyn, A. P. (2006). Use of a preparation from fungal pectin lyase in the food industry. *Applied Biochemistry and Microbiology, 42*, 598–602.

Seo, S., Karboune, S., L'Hocine, L., & Yaylayan, V. A. (2013). Characterization of glycated lysozyme with galactose, galactooligosaccharides and galactan: Effect of glycation on functional properties of lysozyme. *LWT—Food Science and Technology, 53*, 44–53.

Shah, M. H., Mir, S. A., & Paray, M. A. (2014). Plant proteases as milk-clotting enzymes in cheesemaking: A review. *Dairy Science and Technology, 94*, 5–16.

Shahidi, S., Jamili, S., Mostafavi, P. G., Rezaie, S., & Khorramizadeh, M. (2018). Assessment of the inhibitory effects of ficin-hydrolyzed gelatin derived from squid (*Uroteuthis duvauceli*) on breast cancer cell lines and animal model. *Iranian Journal of Allergy, Asthma and Immunology, 17*, 436–452. https://doi.org/10.18502/ijaai.v17i5.302

Shi, Y., Mandal, R., Singh, A., & Singh, A. P. (2020). Legume lipoxygenase: Strategies for application in food industry. *Legume Science*, e44. https://doi.org/10.1002/leg3.44

Singh, G., Verma, A. K., & Kumar, V. (2016). Catalytic properties, functional attributes and industrial applications of β-glucosidases. *3 Biotech, 6*, 1–14. https://doi.org/10.1007/s13205-015-0328-z

Sinha, R., Radha, C., Prakash, J., & Kaul, P. (2007). Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation. *Food Chemistry, 101*, 1484–1491.

Song, H., Wang, P., Li, C., Han, S., Lopez-Baltazar, J., Zhang, X., & Wang, X. (2016). Identification of lipoxygenase (LOX) genes from legumes and their responses in wild type and cultivated peanut upon *Aspergillus flavus* infection. *Scientific Reports, 6*, 35245. https://doi.org/10.1038/srep35245

Sonnet, P. E., & Gazzillo, J. A. (1991). Evaluation of lipase selectivity for hydrolysis. *Journal of the American Oil Chemists' Society, 68*, 11–15.

Stránská, J., Sebela, M., Tarkowski, P., Rehulka, P., Chmelík, J., Popa, I., & Peč, P. (2007). Inhibition of plant amine oxidases by a novel series of diamine derivatives. *Biochimie, 89*, 135–144.

Tavano, O. S., Berenguer-Murcia, A., Secundo, F., & Fernandez-Lafuente, R. (2018). Biotechnological applications of proteases in food technology. *Comprehensive Reviews in Food Science and Food Safety, 17*, 412–436. https://doi.org/10.1111/1541-4337.12326

Tenkanen, M., Niku-Paavola, M. L., Linder, K., & Viikari, L. (2003). Cellulases in food processing. In J. R. Whitakerm, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Thacker, S. P., Kothari, R. M., Ramamurthy, V., & Baroda, P. (1992). Characterization of barley β-amylase for application in maltose production. *Starch/Starke, 44*, 339–341.

Thalmann, M., Coiro, M., Meier, T., Wicker, T., Zeeman, S. C., & Santelia, D. (2019). The evolution of functional complexity within the β-amylase gene family in land plants. *BMC Evolutionary Biology, 19*, 66–84. https://doi.org/10.1186/s12862-019-1395-2

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry, 103*, 1385–1394.

Toohey, E. S., Kerr, M. J., van de Ven, R., & Hopkins, D. L. (2011). The effect of a kiwi fruit based solution on meat traits in beef m. semimembranosus (topside). *Meat Science, 88*, 468–471. https://doi.org/10.1016/j.meatsci.2011.01.028

Uluisik, S., & Seymour, G. B. (2020). Pectate lyases: Their role in plants and importance in fruit ripening. *Food Chemistry, 309*, 125559. https://doi.org/10.1016/j.foodchem.2019.125559

Ulusu, Y., Şentürk, S. B., Kuduğ, H., & Gökçe, I. (2016). Expression, purification and characterization of bovine chymosin enzyme using an inducible pTOL system. *Preparative Biochemistry and Biotechnology, 46*, 596–601.

Van der Maarel, M. J. E. C., ven der Veen, B., Uitdehaag, J. C. M., Leemhius, H., & Dijkhuizem, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology, 94*, 137–165.

Verma, S., Dixit, R., & Pandey, K. C. (2016). Cysteine proteases: Mode of activation and future prospects as pharmaceutical targets. *Frontiers in Pharmacology, 7*, 107. https://doi.org/10.3389/fphar.2016.00107

Vizovišek, M., Vidmar, R., Drag, M., Fonovic, M., Salvesen, G. S., & Turk, B. (2018). Protease specificity: Towards in vivo imaging applications and biomarker discovery. *Trends in Biochemical Sciences, 43*, 829–844. https://doi.org/10.1016/j.tibs.2018.07.003

Whitaker, J. R. (1990). New and future uses of enzymes in food processing. *Food Biotechnology, 4*, 669–697.

Whitaker, J. R. (1994). *Principles of enzymology for food scientists* (pp. 391–422). Marcel Dekker.

Whitaker, J. R. (2003). Proteolytic enzymes. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Williams, D. C., Lim, M. H., Chen, A. O., Pangborn, R. M., & Whitaker, J. R. (1986). Blanching of vegetables for freezing: Which indicator enzyme to choose. *Food Technology, 40*, 130–140.

Wong, D. W. S. (2003). Lipase. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Wong, D. W. S., & Robertson, G. H. (2007). α-Amylases. In J. R. Whitaker, A. G. J. Varagen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Wong, D. W. S., & Whitaker, J. R. (2003). Catalase. In J. R. Whitaker, A. G. J. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Woolcock, K. (2016). Structure of a DNA enzyme. *Nature Structural and Molecular Biology, 23*, 97.

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins. A new hypothetical mode. *Carbohydrate Polymers, 86*, 373–385. https://doi.org/10.1016/j.carbpol.2011.05.065

Zhang, W., Xiao, S., & Ahn, D. U. (2013). Protein oxidation: Basic principles and implications for meat quality. *Critical Reviews in Food Science and Nutrition, 53*, 1191–1201. https://doi.org/10.1080/10408398.2011.577540

Zhang, X. Z., & Zhang, Y. H. P. (2013). Cellulases: Characteristics, sources, production, and applications. In S. T. Yang, H. A. El-Enshasy, & N. Thongchul (Eds.), *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers* (1st ed.). John Wiley & Sons.

Zhao, X., Shi-Jian, D., Tao, G., et al. (2010). Influence of phospholipase A2 (PLA2)-treated dried egg yolk on wheat dough rheological properties. *LWT—Food Science and Technology, 43*, 45–51.

Zhu, F., Du, B., Ma, Y., & Li, J. (2017). The glycosidic aroma precursors in wine: Occurrence, characterization and potential biological applications. *Phytochemistry Reviews, 16*, 565–571.

Check for updates

3 Role of Enzymes in Fruit and Vegetable Processing Industries: Effect on Quality, Processing Method, and Application

Memthoi Devi Heirangkhongjam, Kanika Agarwal, Aparna Agarwal, and Nidhi Jaiswal

Abstract

The significance of enzymes and their application in food processing industry is increasing rapidly. Different kinds of enzymes are extensively used based on their effective application. In fruits and vegetables processing, several endogenous enzymes and newly developed enzymes are used. Enzymes present in fruits and vegetables play a huge role in determining the texture, colour, flavour, and taste attributes of the processed products. The continued enzymatic activity in fruits and vegetables affects the storage quality, shelf life, and palatability of the product. Therefore, several processing methods such as grinding, crushing, slicing, juices, or preservation are used to prolong the shelf life and reduce wastage of fruits and vegetables. Hence, it is very important to control the stability and activity of endogenous enzymes present in fruits and vegetables during food processing. Apart from the conventional techniques of thermal processing such as blanching, heating, ohmic, and microwave, new and highly advanced processing techniques like high hydrostatic pressure (HHP), high-pressure homogenization processing (HPHP), pulsed electric field (PEF), and ultrasound processing have been introduced successfully. The major advantage of these new techniques is the use of non-thermal technology, which helps in retaining the sensory attributes and nutritional content of the product. These non-thermal processing techniques are effective at ambient or sub-lethal temperatures and minimize the adverse thermal effects on the nutritional content and quality of fruits and vegetables. Nonetheless, multiple advantages are rendered by these techniques in food processing industry over conventional thermal techniques which affect not only the enzymes, but also the texture, taste, and colour of the product compelled for further investigation and improvement.

M. D. Heirangkhongjam · K. Agarwal · A. Agarwal (✉) · N. Jaiswal
Department of Food and Nutrition, Lady Irwin College, University of Delhi, New Delhi, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_3

Keywords

Enzymes · Fruit and vegetable processing · HPP · PEF · Shelf life extension

Abbreviations

CAGR	Compound annual growth rate
HG	Homogalacturonan
HHP	High hydrostatic pressure
HPHP	High-pressure homogenization processing
HTLT	High temperature-long time
HTST	High temperature-short time
OH	Ohmic heating
PEF	Pulsed electric field
PME	Pectin methylesterase
POD	Peroxidase
PPO	Polyphenol oxidase
RG-II	Rhamnogalacturonan-II

3.1 Introduction

Enzymes play a very critical role in the growth and maturation of fruits and vegetables. They also make significant contributions during the post-harvest phase in maintaining stability of raw food materials and improving their quality attributes like aroma, colour, texture, flavour, and nutritional quality. Apart from these, enzymes also have the ability to act as catalysts in transforming raw materials to food products during processing. They are also found to have extensive applications in food processing and production by enhancing the nutritional, safety, functional qualities, and overall acceptability of ingredients and processed products. Moreover, enzymes are also known for their substrate specificity, effectiveness in catalytic reactions, and enhancement rate under controlled conditions of moisture, pH, and temperature (Berg et al., 2010).

Due to rapid growth of population and fast changing lifestyle, the production and demand of convenience and processed food products have also been gradually increasing. As per the study of Markets and Markets (2020), the fruit and vegetable processing enzyme global market is expected to touch around $41.39 billion by 2022 at 6.7% CAGR from 2016 to 2020. During this corresponding period, the Asia-Pacific region enzyme market is projected to grow at the highest CAGR due to high growth of the food and beverage industry in big emerging economies like India and China. Therefore, productive usage of the existing enzymes and the development of new enzymes have become very significant in meeting the ever-increasing demands

in food processing industries. Hence, rapid advancement in enzymology is given prime focus in the food processing sector.

Currently, new enzymes based on their sources (fungi and bacteria) and types (amylase, pectinase, xylanase, and cellulase) have been isolated, characterized, and developed commercially for productive usage either in enhancing the desirable or delaying the unfavourable characteristics of foods during processing. Nonetheless, the importance and significance of endogenous enzymes in the present scenario of food processing industries cannot be overlooked for their productive usage in meeting the desired colour, texture, flavour, appearance, and stability of foods during industrial processing.

In light of the importance of enzymes in food processing, the uses of enzymes and their application in fruit and vegetable processing industries are indispensable in order to increase the productivity and shelf life, reduce cost of production, and also develop new products.

3.2 Fruit and Vegetable Composition

Knowing the composition of fruit and vegetable is the first step in determining the benefits and drawbacks of them. Fruits and vegetables are mainly composed of cellulose (7.20–43.60%), hemicelluloses (4.26–33.50%), pectin (1.50–13.40%), lignin (15.30–69.40%), and starch (3–21%) (Dhillon et al., 2013). The primary cell walls in the young plants comprise largely of cellulose and tend to thicken and become higher in hemicelluloses and lignin as they grow (Albersheim et al., 1996). Now, let's discuss in detail the different types of composition in fruits and vegetables.

3.2.1 Cellulose

Cellulose is one of the main components in fruits and vegetables ranging from 7.20% to 43.60%. It is a linear polymer of D-glucose present in the primary cell wall. It forms microfibrils, which give a stiff shape, structure, and tensile strength to the cell wall. It also helps to form a resistance against the degradation of this polymer (Szymańska-Chargot et al., 2017). These microfibrils are coated with hemicelluloses and help in binding firmly to their surface (Carpita & Gibeaut, 1993). Cellulose is known for its diverse applications in food processing industry. It is used as an emulsifier, bulking agent, texturizer, and as a fat substitute (Grassino et al., 2016). However, the application of celluloses during the extraction process needs extra precaution in order to avoid breaking down the cellulose network in maceration processes as it decreases the quantity of juice production.

3.2.2 Hemicellulose

Hemicellulose is another major component in fruits and vegetables with a variable range of 4.26–33.50%. It is a matrix polysaccharide often present along with cellulose in the cell walls and comprises of xyloglucan, arabinoxylan, glucomannan, and galactoglucomannan. Xyloglucan is the most abundant hemicellulose with similar structure as D-glucose backbone in cellulose. Hemicelluloses are bound via hydrogen bonds, forming a network of cross-linked microfibrils between pectin and cellulose. Such interconnections play a major role in the integrity of the pectin cross-linking network. Vincken et al. (1994) demonstrated that the key structure in the apple cell wall is xyloglucan and it helps in breaking down cell-wall embedded in cellulose if it gets hydrolysed. In apple cell wall matrix, xyloglucan accounts for 24% of the total amount of sugar, whereas the cellulose–xyloglucan complex accounts for 57% approximately. In food processing industries, hemicelluloses are primarily used as enhancing agents in viscosity, stabilizing, and gelling. Moreover, it is also widely used in technical and pharmaceutical fields as coatings, films, gel materials, and adhesives (Wang et al., 2010).

3.2.3 Pectin

Pectin is one of the least present components in fruits and vegetable ranging from 1.50% to 13.40%. It forms a class of complex polysaccharides commonly found in the cell walls of higher plants. It provides structure and firmness to the plant tissue in the primary cell wall and middle lamella component involved in intercellular adhesion (Thakur et al., 1997). There are three pectic polysaccharides, namely, homogalacturonan, rhamnogalacturonans, and substituted galacturonans isolated from primary cell walls. Homogalacturonan (HG) is a homopolymer, a linear chain of 1,4-linked α-D-galactosyluronic residues, known for its gel forming ability. In HG, some of the carboxyl groups are methylesterified at the C-2 and C-3 positions. Similarly, the backbone of Rhamnogalacturonan-II (RG-II) is composed of 1,4-linked D-galactosyluronic residues like HG, with a non-saccharide and an octa-saccharide side chains attached to C-2 and two disaccharides attached to C-3 of the backbone residues. RG-II is significantly used in winery and fruit juice industries because of its exceptional quality of binding heavy metals, thereby reducing the toxicity level in the final product. It also has immunomodulating activities (Grassin & Coutel, 2009). Galarturonan fractions are generally separated from other high molecular weight pectin fractions by degrading purified pectins specifically present in the galacturonan backbone either enzymatically or chemically. In fact, as explained above, the different types of pectins perform certain specific functions, but pectins in general are largely used as gelling and thickening agent in dairy and baking. Also, pectins are being widely used as a carrier of drug delivery system in cosmetic and pharmaceutical industries (Kollarigowda, 2015).

3.2.4 Starch

Starch is another main component in fruits and vegetables which is known very commonly. It is a polysaccharide composed of amylose and amylopectin. Amylose has linear chains of about 840–22,000 units of α-D-glucopyranosyl residues linked by 1→4-α-D-glucan, whereas amylopectin is usually comprised of highly branched α-1→6 and α-1→4 glucosidic linkages. Starch is a form of carbohydrate or energy reserve mostly found in storage organs, seeds, unripe fruits, and vegetables. The most important quality of starch is its water holding capacity which depends on the specific shape and size of each granule. For example, a potato granule can hold approx. 5–100 μm water/glucose unit (Jobling, 2004). Moreover, each granule contains 'blockets' of amylopectin which are crystalline and amorphous in nature. They are responsible for absorption of water, swelling, lose crystallinity, and seep amylose. However, the swelling capacity tends to be lower with higher amylose content and thus results in reduction in gel strength (Li & Yeh, 2001). Therefore, because of its inconsistent natural properties due to the vagaries of weather and agricultural conditions, most of the suppliers constantly try to make uniform starches as functional ingredients. In food processing industry, the usage of starch is manifold. It acts as an innate natural ingredient with different added functionalities like an emulsifier, stabilizer, water binder, thickener, and gelling agent.

3.2.5 Lignin

Lignin is one of the major components present in fruits and vegetables ranging from 15.30% to 69.40%. It represents a class of natural aromatic polymers of 4-hydroxyphenylpropanoids units connected by ether and carbon-carbon linkages. Lignins are generally considered a part of dietary fiber. They are mainly present in cereals, fruit, and vegetables, in which wheat bran has the richest source of lignin. Lignins have efficient antioxidant properties, mainly scavenging superoxide and hydroxyl radicals. It also inhibits the activity of xanthine oxidase, glucose-6-phosphate dehydrogenase, and non-enzymatic and enzymatic lipid peroxidation (Lu et al., 1998). In addition to this, its concentration and composition influence the adsorption capacity of cell walls. Therefore, due to these properties, lignins are considered as efficient adsorbers of hydrophobic heterocyclic aromatic amines (Funk et al., 2006). Although lignin has many applications in other different industries, in food industry it is mainly used as an additive, provides roughage to foods, and as sequestering agents.

3.3 Enzymes Used in Industrial Processing of Fruits and Vegetables

As discussed, the role of enzymes in the overall natural growth, maturation, and ripening of fruits and vegetables is very critical. This very importance of enzymes remains the same or even more in the industrial processing of fruit and vegetables. Another important positive aspect for enzymes is their ability in keeping the quality of fresh fruits and vegetables post-harvest and during storage conditions, which ultimately plays a very pivotal role in the industrial food processing.

In industrial processing, the ripening process with the application of enzymes is a major step, wherein several changes like alterations in the cell wall, middle lamellae, and membrane occur resulting in softening of tissues. Naturally, most of the enzymes in fruits and vegetable tissues are important for the maintenance of metabolism; however, they are also associated with undesirable effects on colour, flavour, odour, texture, and nutritional value. For example, in some vegetables, flavour and odour development is affected by lipoxygenase, lipase, and peroxidase (Fleuri et al., 2016). Furthermore, phenol oxidases result in discolouration and unfavourable effects on the taste and nutritional quality of fruits and vegetables. Also, fruits and vegetables containing pectic substances and α-amylases have major effects on their textural integrity (Berg et al., 2010; Fleuri et al., 2016).

With the advancement in enzymology, different types of new enzymes have been developed and are being used in improving the quality of products, development of new products, and in processing aids such as peeling, extraction of juice, and clarification, thereby increasing the efficiency in processing operation. For example, amylases, cellulases, and pectinases facilitate maceration, liquefaction, and clarification in processing of fruit juice, and hence they are cost-effective and increase the yield. Moreover, different enzyme extracts from plant tissues, bacteria, yeast, and fungi are applied in fruit and vegetable processing industries for the same purpose (Leadlay, 1993). In Table 3.1, some of the important microorganism origin enzymes used in industrial processing and its application are summarized. In Table 3.2, different types of enzymes, product type, and its application in fruit and vegetable processing are highlighted.

Enzymes are classified broadly into four types: Pectinases, Cellulase, Xylanase, and Amylase. In the proceeding paragraphs, we will be discussing in brief the different types of enzymes in fruit and vegetable processing industry.

3.3.1 Pectinases

Those enzymes whose primary role is to break down pectin, a structural heteropolysaccharide found in primary cell walls of fruits and vegetables, cereals, and fibers, are known as pectinases (Singh et al., 2003). The pectin substances are of high molecular weight acidic heteropolysaccharide primarily composed of α-(1–4)-linked D-galacturonic acid residues (Kavuthodi & Sebastian, 2018). Pectic acids are water-soluble substances having variable degrees of methyl ester groups and for

Table 3.1 Enzymes derived from microorganisms and their application in fruits and vegetable processing

Enzyme	Microorganism	Action	Application
Pectinase	*Aspergillus* spp., *Penicillium funiculosum*	Pectin hydrolysis	Degradation of pectins, increases the overall juice production, fruit juice clarification
Pectinesterase	*Aspergillus* spp.	Remove methyl groups from galactose units of pectin	Increase firmness of vegetables and also used with pectinase depectinisation technology
Protopectinase	*Kluyveromyces fragilis*, *Galactomyces reesei*, *Trichosporon fragilis*, *Bacillus subtilis*	Catalyse pectin solubilization	Clarification and reduction of viscosity in fruit juices
Hemicellulase	*Aspergillus* spp., *Bacillus subtilis*, *Trichoderma reesei*	Hemicellulose hydrolysis	Helps extraction of fruit juices, vegetable oils, and aromatic compounds, acts on hydrolysis of soluble pectin and cell wall components with pectinases, lowers viscosity and texture
α-Amylase	*Aspergillus* spp., *Bacillus* spp., *Microbacterium imperiale*	Random hydrolyses α-1,4 bounds to rupture starch and produce maltose	Hydrolysing starch to reduced viscosity, liquefying adjunct, helps in sugar production, for softness and increases volume of fruit juices and vegetables
Glucoamylase	*Aspergillus niger*, *Rhizopus* spp.	Hydrolyse dextrin from starch in glucose	Fruit juice extraction and also used for corn syrup and glucose production
Glucose isomerise	*Streptomyces rubiginosus*, *Streptomyces lividans*, *Actinplanes missouriensis*, *Bacillus coagulans*	Conversion of glucose to fructose	Helps in high-fructose corn syrup production (beverage sweetener)
Cellulase	*Trichoderma* spp., *Aspergillus niger*	Hydrolyses cellulose	Liquefaction of fruit in juice production

Note: Adapted from "Enzymes in food and beverage processing", Fleuri, L. F., Delgado, C. H. O., Novelli, P. K., Pivetta, M. R., Do Prado, D. Z., & Simon, J. W., 2015, p. 257, London, New York: CRC Press, Taylor & Francis Group

neutralization, which form gels with the addition of sugars and acids under favourable conditions (Guo et al., 2014). Pectic substances are classified into protopectins, pectic acids, and pectin which are partially soluble in water (Uneojo & Pastore, 2007). These substances are generally degraded by the enzyme pectinases. They are further classified into different sub-types, namely,

Table 3.2 Enzymes used in fruit and vegetable processing

Name of fruit	Product type	Enzyme	Application
Apple (*Pyrus malus* L.)	Juice	Amylase	Decrease starch concentration to improve durability
Banana (*Musa sapientum* L.)	Juice	Pectinase	Decrease turbidity and viscosity
Blueberry (*Vaccinium myrtillus* L.)	Juice	Pectinase	Improve juice yield and anthocyanin level
Citrus (*Citrus sinensis* L. Osbeck)	Juice	Pectinase and cellulase	Decrease the turbidity of juice
Cherry (*Prunus avium* L.)	Juice	Pectinase and protease	Decrease turbidity and increase stability
Cloudy ginkgo (*Ginkgo biloba* L.)	Juice	Amylase and protease	Reduce hydrolysis time to improve stability
Grape (*Vitis vinifera* L.)	Juice	Pectinase	Decrease turbidity and soluble solids
Lemon (*Citrus sinensis* L. Osbeck)	Soft drink punch	Pectinase	Clarification of peel extract to produce soft drinks
Pineapple (*Ananas comosus* L.)	Juice	Pectinase and cellulase	Improve soluble solids and aromas
Pomegranate (*Punica granatum* L.)	Juice	Pectinase	Improve concentration of antioxidants and decrease turbidity
Carrot (*Daucus carota* L.)	Juice	Pectinase	Improved nutritional properties as the content of polyphenols and flavonoids
Date syrup (*Phoenix dactylifera* L.)	Syrup	Cellulase and pectinases	Reduce turbidity and increase the extraction of soluble solids
High-fructose corn syrup (HFCS—*Zea mays* L.)	Syrup	Amylase	Conversion of glucose to fructose
Olive oil (*Olea europaea* L.)	Oil	Pectinase, cellulase, and hemicellulase	Oil extraction from olive residue and improve soluble solids on oil extraction
Sunflower oil (*Helianthus annuus* L.)	Oil	Pectinase	Improve oil yield

Note: Adapted from "Enzymes in food and beverage processing", Fleuri, L. F., Delgado, C. H. O., Novelli, P. K., Pivetta, M. R., Do Prado, D. Z., & Simon, J. W., 2015, p. 258, London, New York: CRC Press, Taylor & Francis Group

methylesterases (based on mechanical action), polygalacturonases, and lyases (based on their mode of action).

Then, polygalacturonases are further sub-classified into endo-polygalacturonases (E.C. 3.2.1.15) and exo-polygalacturonases (E.C. 3.2.1.67). Similarly, lyases are also sub-classified into three types, namely, pectin methylesterases (E.C. 3.1.1.11),

pectatelyases (E.C. 4.2.2.9 and E.C. 4.2.2.2), or pectin lyases (E.C. 4.2.2.10) (Kc et al., 2020).

As per a recent study, it is reported that pectinases from microbial origin account for about 25% of global industrial enzymes market which is projected to reach USD 6.3 billion by 2021 (Oumer, 2017; Oumer & Abate, 2018). This clearly highlights the significance of microorganism origin enzyme as a reliable source of industrial enzyme production. Pectinases or pectinolytic enzymes are also naturally produced by many other organisms like insects, bacteria, nematodes, and protozoans (Khairnar et al., 2009). Some of the other commonly used microorganisms for extensive production of pectinases are *Aspergillus* spp., *Bacillus* spp., *Erwinia* spp., and *Penicillium* spp. (Oumer, 2017).

In food processing industries, especially in fruits and vegetables processing, pectinases have multiple usage. They are mainly used during processing of citrus juice. Moreover, pectinases are also generally recommended for use with the combination of other enzymes such as cellulases and hemicellulases. Such combinations of enzymes are generally used in facilitating the process of maceration, liquefaction, and clarification. It ultimately helps in increasing the extraction yield and enhancing the concentration of acids, colourants, and flavourings (Oumer & Abate, 2018). Apart from these, pectinases are also widely used in wine clarification, concentration, and fermentation of tea, cocoa, and coffee. Further, these enzymes are also used regularly in pickling, preparation of jam and jellies, syrups, starches, and vegetable oil extraction (Kubra et al., 2018).

3.3.2 Cellulases

Cellulases are enzymes that promote the process of hydrolysis of "cellulose", a fibrous, tough and water-insoluble polysaccharide and a homopolymer comprising of several glucose units joined by β-1,4 linkages. They act synergistically as a biocatalyst to release the sugar molecules, which helps in converting into ethanol and organic acids (Fleuri et al., 2016). Cellulases are generally produced as a multi-enzyme system comprising of glucosidase (β-D-glucoside gluco-hydrolase, E.C. 3.2.1.21), β-1,4-endoglucanase (1,4-β-D-glucan 4-glucan hydrolase, E.C. 3.2.1.4), and cellobiohydrolase or exoglucanase (1,4-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91) (Jecu, 2000). Cellulases are produced by bacteria and fungi (Sharma et al., 2017). However, for commercial preparation of cellulases, different filamentous fungi such as *Trichoderma reesei* (Megazyme) and *Aspergillus niger* (Cellulocast from Novozyme) are used. Some other common cellulases producing fungi are *Aspergillus*, *Chaetomium*, *Fusarium*, *Humicola*, *Neocallimastix*, *Penicillium*, *Piromonas*, *Thermoascus*, *Trichoderma,* etc. (Singhania et al., 2010; Bansal et al., 2011).

In food processing industry, majorly in beverage industry, cellulase enzymes are extensively used in the production of fruit juices and wine processing. It facilitates the extraction of juice and maceration process by breaking the cellulose chains present in the plant cells. It also helps in extracting pigments and flavouring

substances present in the grape skin. Besides releasing the flavouring substances and improving the aroma and flavour of beverages, it also breaks the unpleasant-tasting compounds present in the fruits and vegetables (Juturu & Wu, 2013).

3.3.3 Hemicellulase and Xylanase

Hemicellulase is an enzyme complex that breaks down the backbone of xylan and arabinose side chains and releases pentoses (xylose and arabinose) (Yang et al., 2017). Similarly, xylanases (endo-1,4-β-D-xylanohydrolase; E.C. 3.2.1.8) are hydrolytic enzymes involved in depolymerization of xylan. They are usually present in superior plants, such as cereals, grasses, and trees that present noncellulosic polysaccharides, such as D-glucose, L-arabinose, D-xylose, D-mannose, D-galactose, D-glucuronic, and D-galacturonic acid (Cunha & Gandini, 2010). These enzymes also degrade hemicellulose polymers, including acetyl xylan esterase (E.C. 3.1.1.72), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C. 3.2.1.55), arabinase (endo-α-L-arabinase, E.C. 3.2.1.99), endo-xylanase (endo-1,4–βxylanase, E.C. 3.2.1.8), feruloyl esterase (E.C. 3.1.1.73), α-glucuronidase (α-glucosiduronase, E.C. 3.2.1.139), and β-xylosidase (xylan-β-1,4-xylosidase, E.C. 3.2.1.37) (Juturu & Wu, 2013). Polymers such as xylan and arabinoxylan are completely hydrolysed by the synergistic action of several xylanolytic enzymes: endo-1,4-β-D-xylanases. The hydrolysis of these polymers degrades the β-D-xylan linkages; β-D-xylosidases release a xylose monomer from the cleavage of the non-reducing end of xylo-oligosaccharides and xylobiose (Terrasan et al., 2010).

Currently, commercial xylanases are produced on a large scale in many countries, such as United States, Japan, Finland, Germany, Ireland, Canada, and Denmark, by fermentation processes using bacteria, yeast, and fungi (Polizeli et al., 2005). Substantial increase in the production of xylanases has been observed after the development of improved microbial strains and efficient fermentation techniques and recovery systems. It has several applications in food industries, agriculture as well as in human health.

Furthermore, during processing of beer, the cellular wall is generally hydrolysed releasing long chains of arabinoxylans which in turn increase the viscosity of beer rendering it “muddy” in appearance. Then, xylanases are added to hydrolyse arabinoxylans in order to lower oligosaccharides, thereby reducing the viscosity and consequently improving its appearance (Dervilly et al., 2002). Xylanases in combination with amylases, cellulases, and pectinases provide multiple advantages such as increase yield of juice, stabilization of fruit pulp, and increased recovery of flavours and aromas, essential oils, mineral salts, vitamins, etc.

3.3.4 Amylases

Amylases are enzymes that act as catalysts in the hydrolysis of starch into sugars such as glucose and maltose (Sundarram & Murthy, 2014). These enzymes are

extensively found in plant, animal, and microbial kingdoms. Amylases are classified into exo-amylases and endo-amylases based on their action. Exo-amylases are involved in hydrolysis of α-glucan into maltose and glucose, whereas endo-amylase hydrolyses α-glucan-forming oligosaccharides. Starch which is a polysaccharide that is an essential factor of structure, consistency, and texture in foods is hydrolysed by β-amylase. Moreover, the non-reducing terminals in starch polysaccharides and malto-oligosaccharides are also hydrolysed by β-amylase (Fleuri et al., 2016). Such enzymes are responsible for the degradation of starch and its related polymers to yield products characteristic of individual amylolytic enzymes.

Amylase enzymes have some common types, namely, amylolytic, α-amylase, β-amylase, and glucoamylase. These enzymes are largely present in a wide range of organisms, including plants, animals, and microorganisms. *Aspergillus* spp. is the most extensively used fungi for the production of amylolytic enzymes. The major sources of α-amylase enzymes are *A. niger*, *Aspergillus oryzae*, *B. circulans*, *Bacillus amyloliquefaciens*, *B. subtilis, B. licheniformis,* and *B. stearothermophilus*. β-Amylase enzymes are obtained from species like *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. However, out of these enzymes, the production of α-amylases accounts for about 30% of enzymes in world market for their extensive use in food industrial processes, such as in baking, brewing, fruit juices, syrups, starch, etc. It is also widely used in the production of drugs and other pharmaceutical products (van der Maarel et al., 2002).

3.4 Significance of Enzymes in Food Quality

In recent years, the significance of enzyme application in food processing industries has been increasing. It is due to the diversified role played by them in increasing the rate of biochemical processes. But, sometimes enzymes are also liable to unfavourable biochemical and physiological changes in fruits and vegetables. These changes alter the colour, texture, aroma, and flavour in them. Therefore, it is important to understand and characterize the interrelationships between the quality components and the associated enzymes. It also helps in determining the optimal post-harvest handling procedures and processing techniques to provide high-quality products to consumers.

3.4.1 Enzymatic Reaction on Colour of Fruits and Vegetables

The colour of a product is the primary assessment factor of quality more than any other factor. So, maintaining the natural colour of food is very critical. The colour of food products is affected during pre-harvest and post-harvest factors which are coupled by enzymatic reactions. The main enzymes responsible for changes of colour in fruits and vegetables are polyphenol oxidase (PPO), peroxidase (POD), and β-glucosidase (Zabetakis et al., 2000). Among the various processing changes, enzymatic browning causes major problem in food processing (Terefe et al., 2014).

It is due to the oxidation of phenolic compounds to quinones via polymerization reactions resulting in the production of dark colour compound called melanin (Marshall et al., 2000). Either to inhibit or delay this particular phenomenon, the fruits and vegetables are treated with anti-browning agents prior to further processing. The most commonly used anti-browning agents are citric acid, ascorbic acid, and calcium chloride. They can be used either singly or in combination with other chemicals. Ascorbic acid and citric acid are extensively used as anti-browning agents since they act as a reducing agent on the enzymatic reaction of PPO. Similarly, calcium chloride ($CaCl_2$), also known for PPO inhibitors, reduces enzymatic browning by acting on pH and as a chelating agent. However, combined treatments were found to be more effective to prevent the browning effect.

Polyphenol oxidases (PPOs) are a group of copper containing enzymes, capable of catalysing the oxidation of several phenols to *o*-quinones (Oliveira et al., 2011). These *o*-quinones in turn react with molecules which undergo non-enzymatic secondary reactions, resulting in the formation of melanin, brown complex polymers, and cross-linked polymers with protein functional groups (Rolff et al., 2011). PPOs are abundantly present in various fruits and vegetables, such as, apple, peach, banana, potato, mushroom, coffee bean, microorganisms, etc. (Eisenmenger & Reyes-De-Corcuera, 2009). Conversion of phenolics substrates into *o*-quinones occurs in two oxidation steps. In the first step, hydroxylation of *ortho*-position adjacent to an existing hydroxyl group, “monophenolase” or “monophenol oxidase”, is generally referred to as cresolase or hydroxylase activity. The second step is the oxidation of *o*-dihydroxybenzenes to *o*-benzoquinones, “diphenolase activity” or “diphenol oxidase”, referred to as oxidase or catecholase activity (Yoruk & Marshall, 2003).

The oxidation of phenolics compounds by polyphenol oxidase is the main reason for most of the enzymatic browning in foods occurred during harvesting, handling, storage, and processing (Eisenmenger & Reyes-De-Corcuera, 2009). It was reported that the PPO activity in apple, tomato, and tobacco is encoded by multiple genes and regulates tissue damage (Kim et al., 2001). In another study, expression of PPO response was observed in both damaged and non-damaged leaves (Constabel & Ryan, 1998). The expression of PPO in peaches was observed for 48 h of storage and it was found that there was a slight or gradual decrease in the PPO activity in intact tissues. On the other hand, in damaged tissues, there was a rapid increase in PPO activity (Tourino et al., 1993). In peaches, enzyme inhibitors such as ascorbic acid, sodium metabisulfite, β-mercaptoethanol, and cysteine were found to be effective against PPO activity (Belluzzo et al., 2009).

Furthermore, enzymatic browning can also be controlled by several processing techniques, such as freezing and heating coupled with natural and synthetic inhibitors (Marshall et al., 2000). However, many PPO inhibitors are associated with off-flavours, food security, and sustainability. For example, sulphites as PPO inhibitors are reported to cause allergies and thus seek natural alternatives (Loizzo et al., 2012). 4-Hexylresorcinol is a natural compound; when used along with ascorbic acid, cysteine, or kojic acid, it resulted in the reduction in PPO activity and changes colour in mango puree and apple juice (İyidoğan & Bayındırlı, 2004).

Kojic acid is another natural compound; it chelates metal ions such as Fe^{3+} and Cu^{2+} in addition to impounding of free radicals (Kim & Uyama, 2005).

Proteases are another group of enzymes that catalyse the hydrolysis of proteins which are degraded to peptides and amino acids (Omaña-Molina et al., 2013). It inhibits PPO activity through proteolysis or binding to specific sites. According to a study report, it was found that the browning reaction was decreased in apple when papain was used (Labuza et al. 1992). Also, when pineapple juice and high-pressure technique were applied, the browning reaction was reduced in apple slices. Such inhibitory effect is due to the presence of bromelin, sulfhydryl groups, citric, or malic acid (Perera et al., 2010). Similarly, the prevention of browning effect was also observed in peeled banana when pineapple juice was used (Chaisakdanugull et al., 2007). However, in some studies, bromelin was found to be ineffective in preventing browning of apple juice (Tochi et al., 2009).

Peroxidases (POD) are generally isolated from plants, animals, and microorganisms. These are oxidase enzymes which use hydrogen peroxide as a catalyst in the oxidation reaction of polyphenols, aminophenols, monophenols, and diphenols (Fatibello-Filho & Vieira, 2002). They are heat-stable and hence used as a parameter to increase the efficiency in bleaching (Aguero et al., 2008). POD lead to undesirable changes in colour, flavour, texture, and nutritional values in foods (Gonçalves et al., 2007). The browning of sugar cane juice is due to the presence of enzymes, POD and PPO, which oxidize phenolics compounds (Qudsieh et al., 2002). The enzymatic activities of POD and PPD enzymes were studied in different cultivars of grapes. In these studies, the POD extracts showed similar activity in both the soluble and bound fractions, and highest PPD activity was observed in cultivar Ruby. PPD and POD activities in cultivars Benitaka and Ruby decrease when juice extracts were treated with higher temperature and longer duration. It was observed that enzyme inactivation was achieved at 85 °C with 10 min exposure time, but the thermal treatments were not sufficient to inactivate the enzymes completely. For example, the thermal treatment in jam, jellies, and juices causes reduction in PPO and POD activities, but not sufficient to inactivate the enzymes (Freitas et al., 2008). Similar activities were also reported in processing of guavas (Zanatta et al., 2006).

In other studies, it was shown that apple peel from Fuji and Gala cultivars when compared to its pulp had elevated enzymatic activity both for PPO and POD. When heat treatment was done in the concentrated extracts of pulp and peel, there was a decline in PPO enzyme activity and total inactivation was also achieved after 10 min of heat treatment at 75 °C. However, such case of total inactivation was not observed for POD enzyme activity (Valderrama et al., 2001). Studies have shown that the enzymatic activity of PPO in fresh broccoli was higher compared to bleached broccoli. In case of POD, the activity was found to be lower in bleached broccoli, indicating that bleaching was partially effective in denaturation of these enzymes (Lopes & Clemente, 2002).

3.4.2 Enzymatic Reaction on Texture of Fruits and Vegetables

Texture of fruits and vegetables is an important indicator in determining the quality of the product. It is dependent on the cellular structure of cell walls. Any changes in the composition and structure of cell wall polysaccharides result into changes in the firmness (Alkorta et al., 1998). It influences the keeping quality such as storage time, handling damages, and consumer acceptance of fruits and vegetables after harvesting. Degradation in the textural quality may be due to several factors such as growth of microorganisms, infestation by insects, rodents, and other environmental conditions.

The texture quality of fruits and vegetables is also affected by a number of enzymes. Pectin which is an important cell wall polysaccharide helps in maintaining the texture by avoiding the breakdown of cellular structure. The enzymatic effect on the texture of fruits and vegetables happens when enzymes such as pectinases, cellulases, and hemicellulases break the cell walls. So, the inactivation of these enzymes during processing would help in preserving the texture of the food. Generally, pectin is used as a gelling and texturizing agent. It also improves the taste and appearance of processed foods like jams, jellies, and marmalades (Alkorta et al., 1998). However, pectins also have some major disadvantage in food processing, especially in juice processing. Soluble pectins in fruits are responsible for cloudiness or haziness in different fruit juices. Hence, the texture of such final juice product may or may not have positive feedback from consumers. But, these days, cloudy juices from tropical fruits are generally accepted and their market is growing. Nonetheless, clear fruit juices from apples, grapes, oranges, etc. are still the preferred choice of the consumers as they have appealing texture. Therefore, the enzymatic application on such hazy or cloudy juices becomes important for improving their texture and ultimately increasing consumer acceptability in the market. In order to overcome this issue, clarification process is conducted for the degradation of pectins by the application of enzyme pectinases (Alkorta et al., 1998). Different types of pectinases are used in this process and some names are pectin lyase, pectin esterase, and polygalacturonase (Kant et al., 2013). But, the pectinolytic enzymes from A. niger are commonly used in fruit and vegetable processing to improve the texture of the final products (Dinu et al., 2007).

Transglutaminases is another important enzyme which has been used extensively for food texture and new product development in food processing industries. The enzyme helps in the catalytic cross-linking reactions between proteins which transfer the glutamine residue, namely, γ-carboxyamide of one protein to the ε-amino group of lysine residue of the same protein or another protein (Nandakumar & Wakayama, 2015). This results in the improvement in food properties, such as firmness, elasticity, viscosity, and water-binding capacity. The main microbial sources of enzymes for improving the texture in food are *Bacillus* sp., *Streptomyces* sp., and *Corynebacterium* sp. (Zhang et al., 2012; Placido et al., 2008). Glutaminase is another enzyme that helps in texture by improving the protein properties, thereby increasing its solubility, gelation, emulsification, etc. (Nandakumar & Wakayama, 2015).

3.4.3 Enzymes in Flavour and Aroma Production

Taste and flavour are the two major attributes in determining the overall quality of various fruits and vegetables. Taste is determined by the contents of sugar, tannins, phenols, organic acids, and other compounds. Analysis of flavour compounds has given us an inclusive knowledge on the chemical compounds responsible for flavour sensations of fruits and vegetables. However, different enzymes have multiple effects on these attributes of fruits and vegetables. Enzymes such as peroxidases and lipoxygenases are responsible for off-flavour formation in fruits and vegetables (Bhowmik & Dris, 2004). The enzyme peroxidases are responsible for deterioration in colour, flavour, texture, and loss in nutritional qualities in raw or processed fruits and vegetables. The off-flavour is often linked with the oxidation of phenolic compounds and indigenous lipids. It was observed that enzyme inactivation was achieved at 70 °C with 15 min exposure time (Sessa & Anderson, 1981). Therefore, inhibition of such enzymes during processing is a must in order to retain or improve the aroma and flavour.

Another enzyme that has a major effect in flavour and aroma is lipogenase. This enzyme produces free radicals and conjugated unsaturated hydroperoxy acids by catalytic oxidation of polyunsaturated fatty acids. Then, these free radicals interact with other constituents like proteins, vitamins, phenolics, etc. present in fruits and vegetables which helps in enhancing the flavour and aroma of the product. However, these aromatic compounds may produce off-flavours in *Brassicaceae* family. According to the reports of Sheu and Chen (1991), increase in colour losses and development of off-flavour were observed in broccoli and asparagus during storage of non-blanched and under-blanched products.

Furthermore, the volatile compounds such as aldehydes, alcohols, ketones, esters, lactones, etc. are related to flavour and aromatic characteristics in foods (Beaulieu & Baldwin, 2002). For example, the compounds present in alcohols and aldehydes are extensively used as food additives due to the aroma referred to as ‘green touch’, a characteristic observed in freshly harvested fruits (Schwab et al., 2008). These volatile compounds are synthesized using various substrates like amino acids, fatty acids, and carotenoids (Goff & Klee, 2006). The main enzymes involved in the synthesis of volatile compounds from fatty acids are lipoxygenase, alcohol dehydrogenase, hydro peroxide lyase, and (3*Z*): (2*E*)-enal isomerise (Schwab et al., 2008). These enzymes are used in the extraction from fruits and vegetables such as banana, soy, tomato, and olive where they subsequently react with fatty acids to produce volatile compounds (Akacha & Gargouri, 2009).

In order to improve the taste and flavour in citrus fruits, enzymes limoninase and naringinase can be enzymatically tailored by degrading the bitter taste compounds such as limonin and naringin (Ribeiro et al., 2010). The formation of limonin can also be prevented by using limonoate dehydrogenase, as it catalyses the oxidation of its precursor lactone-A-ring to 17-dehydrolimonoate, a non-bitter derivative which cannot be changed into limonin (Merino et al., 1997). Bitter compounds in citrus fruits can be reduced by using adsorbing polymers, such as Amberlite XAD-16HP and Dowex Optipore L285 resins. Besides acting as a debittering agent, Dowex

Optipore L285 can also induce other modifications in juice processing like reduction of total titratable acidity (TTA), increasing total soluble solids (TSS), the ratio of TSS to TTA, pH, etc. (Kola et al., 2010).

3.5 Application of Enzymes in Fruit and Vegetable Processing

The significance of enzymes and its application in food processing industry is increasing rapidly. Different kinds of enzymes are used extensively based on their effective application. In fruits and vegetables processing as well, several endogenous enzymes and newly developed enzymes are used because of the following advantages:

- Low temperature requirements
- Low energy requirements during processing
- Increased product yield especially in juice processing. They make the juice stable without the addition of additives
- Less by-product formation during processing
- Improved product quality (Grassin & Coutel, 2009)

In the following paragraphs, detailed analyses are discussed on the use of enzymes in different fruit and vegetable processing techniques.

3.5.1 Fruit Firming

Texture is one of the important quality attributes of fruits and vegetables (Pan et al., 2014; Grassin & Coutel, 2009; Guillemin et al., 2008; Jensen et al., 2004; Degraeve et al., 2003). However, the texture of processed fruits and vegetables, especially the softer ones, such as strawberry, raspberry, and tomatoes, is adversely affected by thermal processing treatments like blanching, sterilization, freezing or pasteurization, and other mechanical method which may result in softening (Grassin & Coutel, 2009; Guillemin et al., 2008; Degraeve et al., 2003).

The texture and structural integrity of fruits and vegetables depend on the composition of cell walls present in them. These cell walls are composed of an interlinked fibrous structure of cellulose embedded in a matrix of pectin, hemicelluloses, and celluloses (Guillemin et al., 2008; Sila et al., 2008). The enzyme pectin methylesterase (PME) bound to the cell wall is a pectin degrading enzyme which results in demethoxylation of pectin (de-esterification of the methylated carboxy groups of polygalacturonic pectin), releasing methanol and forming carboxylated pectin. This carboxylic acid is said to interact with calcium, resulting in firmness of fruits by strengthening the cell wall (strong pectate network with added calcium) (Pan et al., 2014; Sila et al., 2008; Guillemin et al., 2008; Degraeve et al., 2003). This process is known as chelation, and it helps to overcome the

adverse effects of thermal and mechanical processing treatments resulting in reduced fruit damage and disintegration (Grassin & Coutel, 2009; Degraeve et al., 2003).

Studies were conducted as early as 1965 and 1972 to assess the role of PME in increasing the firmness of canned tomatoes and potatoes, respectively. In both the studies, heat treatments such as blanching in case of tomatoes and preheating of potato slices in water were used to assess the role of PME with firmness (Bartolome & Hoff, 1972; Hsu et al., 1965).

As per a study conducted on peaches, vacuum infiltration of blanched peaches with PME of citrus fruit for 2 h along with calcium chloride increased the firmness of peaches (Javeri et al., 1991). Another study also demonstrated that vacuum impregnated PME (*Aspergillus niger*) along with calcium chloride increased the firmness in pasteurized fruits, namely, apples, strawberries, and raspberries (Degraeve et al., 2003). Similarly, a study demonstrated enzymatic firming of red pepper by using exogenous pectinesterase (Rheozyme from Novozyme A/S) and retention of texture even after freezing or heating (Jensen et al., 2004). Thus, many fruits and vegetables may be processed by using PME and calcium, thereby retaining the texture of the products (Grassin & Coutel, 2009).

3.5.2 De-Skinning (Enzymatic Peeling)

De-skinning means removal of peel/skin of fruits and vegetables. It was initially done mostly by different methods such as manually, chemical, steam, mechanical, or freeze method. But, these methods involved high labour cost, high water consumption, and peeling losses (Toushik et al., 2017; Pretel et al., 2007a, 2010). Now, considering their disadvantages, these methods have been replaced almost by enzymatic peeling in processing industry. In this method, the product is infused in enzyme preparation of pectic substances such as pectinases, hemicellulases, and cellulases (Berry et al., 1988; Bruemmer et al., 1978). Once infused, the vacuum is applied which helps the enzymatic solution to enter the intercellular spaces (Pretel et al., 2008). In the process, cellulase releases pectin from albedo, while pectinases break down the cell wall by hydrolysis of polysaccharides (Ben-Shalom et al., 1986).

Nowadays, many commercial enzymatic preparations are available and pectolytic preparation (mixture of pectinases, cellulase, and hemicellulase) is one of them. It is obtained from *Aspergillus species* fungi. It has been observed that the enzymatic solution easily diffuses when there are large intercellular spaces in albedo, resulting into easy peeling (Baker & Bruemmer, 1989). However, many studies have found that the efficiency of enzymatic peeling effect depends on the treatment methods such as heat (scalding), vacuum infusion, temperature, and pH (Pagán et al., 2005; Suutarinen et al., 2003; Toker & Bayındırlı, 2003; Rouhana & Mannheim, 1994). Details of some recent studies are given in Table 3.3.

Furthermore, the role of enzymes in the peeling process of vegetables such as potato, carrot, onion, and Swedish turnips was examined by Suutarinen et al. (2003). It was observed that enzymes did help in peeling of carrot and onions; however, potato and Swedish turnips could not be peeled properly due to the presence of cutin

Table 3.3 Studies assessing role of enzymes in enzymatic peeling

Reference	Fruit/ vegetable	Pre-processing condition	Enzymatic solution	Result
Prakash et al. (2001)	Grapefruit	Scalding, 1–4 min	Polygalacturonase, cellulase and pectin methylesterase, incubated for 12 min	Peeling improved
Suutarinen et al. (2003)	Potato, carrot, Swedish turnip, onion		Cellulase, polygalacturonse	Difficulty in removing peel of potato and Swedish turnips
Liu et al. (2005)	Mandarins		Pectinase, polygalacturonse, and cellulase, 20 min	Peeling efficiency improved
Pretel et al. (2007b)	Orange, Thomson, and Mollar	Hot water bath (45 °C)	Pectinase, polygalacturonse, and cellulase, 40 min	Peeling improved
Pretel et al. (2007a)	Orange, Sangrina	Hot water bath (45 °C)	Pectinase, polygalacturonse, and cellulase, 30 min	Peeling improved

or suberin in vegetable surface (Suutarinen et al., 2003). As per a study by Toker and Bayindirli, it was observed that the enzymatic peeling was easier at relatively moderate temperatures such as 44–47 °C for nectarines, 41–46 °C for peaches, and 45 °C for apricots with a pH ranging from 3 to 4.1 (Toker & Bayındırlı, 2003). The steps involved during enzymatic peeling are illustrated in Fig. 3.1.

The first step is selection of fruits or vegetables and washing them with water. At times, hot water treatment or scalding is done prior to enzymatic peeling to improve the peeling process and have a high quality end product (Pretel et al., 2008, 2010). A study on grapefruit as early as 1974 demonstrated dipping the fruit in a water bath of 60 °C for 30–35 min resulted in a good quality product (Bruemmer et al., 1978). The advantage of scalding is that it reduces the viscosity of pectin as well as enhanced the ability of the peel to absorb the enzyme solution, thereby helping in enzymatic peeling process (Pretel et al., 2008) (Fig. 3.1).

Therefore, enzymatic peeling has wide range of applications in fruits and vegetables such as grapefruit, orange, mandarin, apricot, peaches, potato, carrot, and Swedish turnips (Suutarinen et al., 2003; Toker & Bayındırlı, 2003). This method is a significant alternative in food processing industry because of its advantages in improving the quality of products, reducing wastage, minimal water use, reduction in contamination, and being cost-effective (Toker & Bayındırlı, 2003).

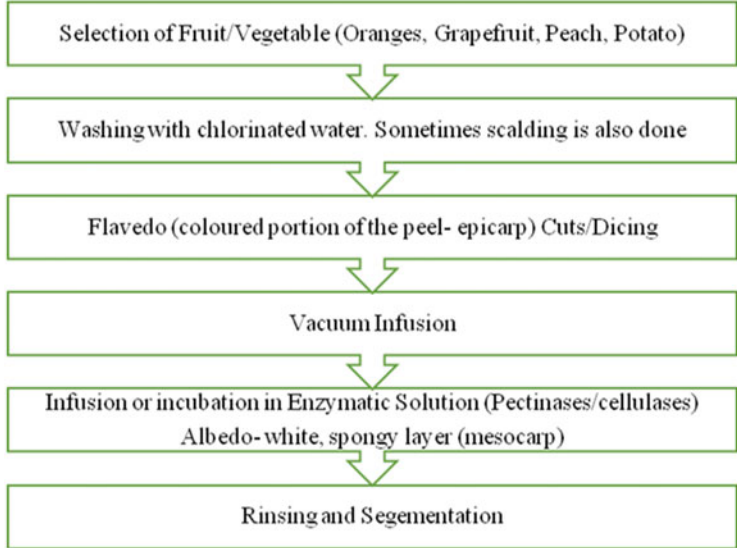


Fig. 3.1 Steps in enzymatic peeling. (Adapted from Pretel MT, Sánchez-Bel P, Egea I & Romojaro F. (2010). Enzymatic peeling of citrus fruits. In Bayindirli (Ed.), Enzymatic processing of fruits and vegetables: Chemistry and engineering applications (pp. 145–174). CRC Press, Taylor and Francis Group)

3.5.3 Application of Enzymes in Fruit and Vegetable Juice Processing

The natural liquid substance contained in fruits and vegetables is commonly known as juice. It can be obtained or prepared directly by extraction, pressing, or diffusion from fruits or vegetables and such juices are meant for direct consumption. They can be broadly categorized as juices without pulp (clarified/cloudy) or juices with pulp such as purees, nectars, and pulps (Ceci & Lozano, 2010; Cautela et al., 2010). In juice processing, different enzymes are extensively used in fruit and vegetable for higher yield, clarification, and improvement in filtration, resulting in higher quality of juices (Fleuri et al., 2016). Some of the commonly used enzymes in fruit and vegetable juice processing are pectinases, hemicellulases, and cellulases. In juice processing of fruits and vegetables, multiple steps are involved and below are the standard procedures. The steps involved in fruit juice processing are also illustrated in Fig. 3.2.

3.5.3.1 Selection of Fruit, Followed by Washing and Peeling

Enzymatic preparations can be used for peeling of fruits such as oranges, lemon, or vegetables such as pumpkin and beet (Toushik et al., 2017) as discussed in Sect. 3.4.2. This is followed by juice extraction.

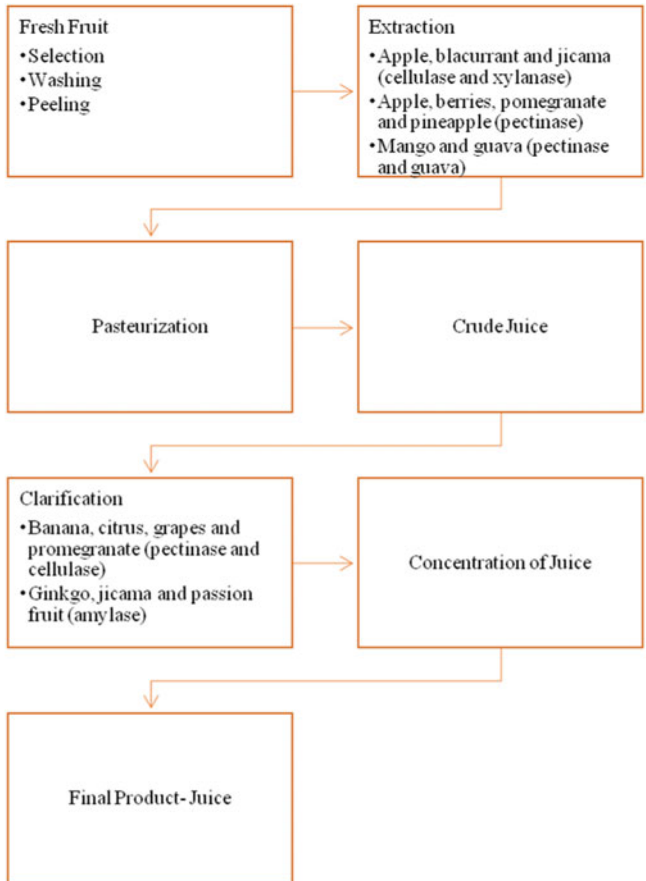


Fig. 3.2 Steps in juice processing. (Adapted from Fleuri, L. F., Delgado., C. H. O., Novelli, P. K., Pivetta, M. R., do Prado, D. Z., & Simon, J. W. (2016). *Enzymes in food and beverage processing* (pp. 255–280). CRC Press, Taylor and Francis Group)

3.5.3.2 Juice Extraction

Juice can be extracted by a number of methods—centrifugation, diffusion, extraction, and ultrafiltration. Enzyme preparations help in breakdown of cell wall and release the juice contained within the structure in fruits such as apples, grapes, berries, pears, and citrus fruits (Ribeiro et al., 2010). Among apples, the activity of endogenous enzymes is low; therefore, commercial pectinases from *Aspergillus* species are added so they can be macerated before pressing (Aehle, 2007). The extracted juice is pasteurised for microbial stability (Grassin & Coutel, 2009). A study on processing of blackberry juice observed that application of pectinolytic enzymes during pressing resulted in an increase in juice extraction as high as 81.73% in three cultivars as compared to 53.79% in the control arm (Granada et al., 2001).

Another important aspect in juice processing is the discolouration of juices during pasteurization due to presence of anthocyanins. This issue can be prevented by the use of β-glucosidase which hydrolyses anthocyanins into anthocyanidins, thereby minimizing discolouration due to its low colour intensity and solubility (Villena et al., 2007; Palma-Fernandez et al., 2002).

3.5.3.3 Clarification and Fining

Turbidity in juices is due to scattering of light caused by insoluble substances such as cell fragments that come from pulpy tissue (pectin from cell walls) in suspension. It is usually undesirable except when present in citrus fruits (Ribeiro et al., 2010). Pectin is the cause of turbidity in juices especially apple juice (Aehle, 2007). Another reason for the haziness in extracted juices is due to presence of starch in unripe fruits (Ceci & Lozano, 2010). At this stage, the role of enzymes becomes critical as enzymes like pectinase help in reducing turbidity by attracting pectin and forming clusters (aggregation of cloud particles). Then, these particles usually settle down and can be easily removed by filtration or centrifugation (Uneojo & Pastore, 2007). Apart from enzymes, flocculating or fining agents such as gelatin and bentonite may be added to aid in clarification process (Ceci & Lozano, 2010). Similarly, citrus juices, especially lemon juice, are also clarified by pectinolytic enzyme preparations (Fleuri et al., 2016).

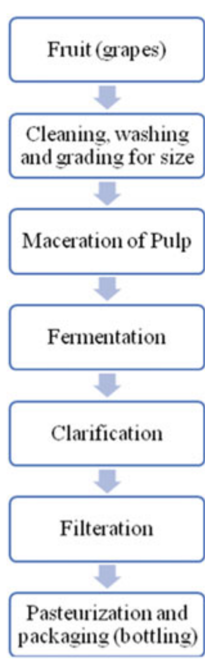
Apple juice contains about 15% of starch which results in turbidity, slow filtration rate, and gelling after concentration. The addition of amylase along with pectinase hydrolyses this starch and removes the haze or cloudiness (Ceci & Lozano, 2010; Carrín, 2004). Sometimes, haziness occurs in juice, so adding of fining agents can prevent post-bottling haziness, thereby optimizing fining and ultrafiltration process. Similarly, araban present in certain fruits may be responsible for post-concentration haze in juice, and to prevent such haze formation, arabanase is added to the juice (Ceci & Lozano, 2010).

3.5.4 Role of Enzymes in Wine Processing

The role of enzymes in the production process of alcoholic beverages such as brewing of beer and winemaking is very important (Toushik et al., 2017; Gómez-Plaza et al., 2010; Aehle, 2007). Different enzymes, namely pectinases, amylases, glucanases cellulases, glycosidases, lyzozymes, and ureases, are used. They help in improving the quality and yield of the product (Toushik et al., 2017; Gómez-Plaza et al., 2010; Aehle, 2007).

3.5.4.1 Enzymes Used in Processing of Wine

Wine is a type of alcoholic beverage made from fermentation of fresh grapes (Bruchmann & Fauveau, 2009). Pulp and skin of the grapes account for about 75–85% and 6–7%, respectively. The cell wall of grapes is composed of hemicelluloses, pectin, and structural proteins like chitinase. Also, the cellulose and pectin present in cell walls provide rigidity to the grapes, thus hindering juice



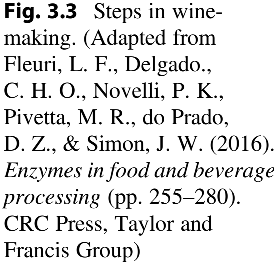


Fig. 3.3 Steps in wine-making. (Adapted from Fleuri, L. F., Delgado., C. H. O., Novelli, P. K., Pivetta, M. R., do Prado, D. Z., & Simon, J. W. (2016). *Enzymes in food and beverage processing* (pp. 255–280). CRC Press, Taylor and Francis Group)

extraction, clarification, and filtration (Claus & Mojsov, 2018). Moreover, the skin and cell walls of grapes contain several essential compounds such as anthocyanins and tannins. These compounds are responsible for colour and structure of wine (Gómez-Plaza et al., 2010; Bruchmann & Fauveau, 2009).

The natural enzymes present in grapes and those obtained from yeast, fungi, and bacteria collectively play an important role in wine making process (Gómez-Plaza et al., 2010). Usually, the manufacturers extend the action of endogenous enzymes by adding exogenous enzymes during production (Gómez-Plaza et al., 2010). The commonly used commercial enzymes include pectinases, glucanases, glycosidases, lysozymes, and ureases (Gómez-Plaza et al., 2010). The application of these exogenous enzymes and yeast strains during processing increases the rate of production, yield, and quality of the wine (Toushik et al., 2017).

The standard steps in wine processing are illustrated in Fig. 3.3.

These steps can be broadly categorized in three phases, namely pre-fermentation, fermentation, and post-fermentation.

Pre-fermentation

This phase involves crushing the fruit and extraction of juice. In red wine preparation, the skin is not separated, while it is separated in white wine. During the processing of red wine, pulp, skin, and seeds of grapes are kept together after crushing and during fermentation in order to extract colour and flavour (Byarugaba-Bazirake, 2008). The colour of red wine is due to the presence of anthocyanins and tannins in grape skin and seed (Gómez-Plaza et al., 2010). In

this phase, exogenous enzymes derived from *Aspergillus* species such as pectinases, cellulases, and hemicellulases (xylanases and galactanases) are employed, which help in expediting the extraction of juice and colour control (Espejo, 2020; Toushik et al., 2017; El Darra et al., 2016; Kelebek et al., 2007; Bautista-Ortín et al., 2005). These macerating enzymes also modify the stability, taste, structure, and mouth feel of red wine (Gao et al., 2019).

Furthermore, maceration of the cell walls by pectinases results in the liberation of liquid and other phenolic compounds (Espejo, 2020; Gao et al., 2019). It has been observed in different studies that wines prepared by enzyme treatment tend to have phenolic and tannin content, as well as colour intensity (Romero-Cascales et al., 2008; Revilla & Gonzalez-San Jose, 2003). Sometimes, maceration is accompanied by pressing (wine press extract must or wine from crushed grapes), resulting in better yield (Gómez-Plaza et al., 2010). Another important attribute in wine making is its aroma; reductases such as glycosidases and polyphenol reductase help in aroma extraction and polyphenol reduction, respectively (Espejo, 2020). Glycosidases improve the aroma of wines by releasing aromatic compounds from their non-aromatic precursors or non-odouriferous sugar compounds (Zhu et al., 2014). As per a study, treatment of Albarino wine with pectinolytic preparations and glycosidase resulted in an improved aroma (Armada et al., 2010). However, in industrial processing, usually exoglycosidases and β-glucosidase from *Aspergillus niger* are used (Gómez-Plaza et al., 2010).

Fermentation

In this phase, yeast is added to the must in red wine to initiate fermentation (Byarugaba-Bazirake, 2008). During the process, the sugars present in the juice are converted into alcohol and release carbon dioxide. In this period, pectin plays an important role by preventing the diffusion of intercellular components such as phenolic and aroma compounds into the must (Claus & Mojsov, 2018; Bruchmann & Fauveau, 2009).

Post-fermentation

This phase involves different steps such as clarification, filtration, and microbial stabilization with the help of different enzymes. Proteases are used at the time of clarification, while pectinase and β glucanase are used during filtration and lysozyme for microbial stabilization (Espejo, 2020). After pressing, the grape must is quite turbid and is rich in solid particles (Armada & Falqué, 2007). Thus, clarification is an important step to improve the quality of wine, especially in white wine (Gómez-Plaza et al., 2010). Moreover, activated carbon, bentonite, and pectinase may be added to aid in clarification or physical removal of suspended matter in the must (Gómez-Plaza et al., 2010). Commercial enzymes that help in clarification are:

(a) Pectin lyase which results in destabilization of the cloud, thereby reducing viscosity.
(b) Pectin methylesterase (PME) results in demethylation of pectin, aiding polygalacturonase in hydrolysis of pectin and leading to cloud flocculation

Table 3.4 Enzymes and their roles in wine processing

Step	Enzyme	Action
Clarification	Pectinase	Removal of suspended particles
Maceration	Pectinases, hemicellulase, cellulase	Degradation of pectin
Maturation	Pectinases and glucanases	Ageing results in mouth feel and is done at the end of fermentation
Filtration	Pectinases and glucanases	Glucanases are applied to the must to shorten the glucan chains preventing problems during filtration
Addition to wine/ must	Urease	Elimination of urea and preventing the formation of ethyl carbamate
Addition to wine/ must	Lysozyme	Elimination of bacterial
Late phase of fermentation (white wine)	Beta glucosidase	Increased aroma by splitting glucose residue from odourless precursors
Stabilization/haze formation	Proteases	Stabilization of wine, reducing betonite demand

Note: Adapted from Gómez-Plaza et al., 2010. Use of enzymes in wine production. In Bayindirli (Ed.), Enzymatic processing in fruits and vegetables: Chemistry and engineering applications (pp. 215–243). CRC Press, Taylor and Francis Group

and clarification of must (Gómez-Plaza et al., 2010; Bruchmann & Fauveau, 2009).

Furthermore, during ageing or maturation, cellulases and β-glucanases are used to improve the mouthfeel (Espejo, 2020; Gómez-Plaza et al., 2010). Other than the above mentioned advantages of the macerating enzymes, studies have also observed that pectinases are found to improve haze condition, increase pressability of grapes to reduce filtration time, and increase must volume (Sharma et al., 2017; Garg et al., 2016). Table 3.4 illustrates different enzymes and their actions during wine processing.

Other important enzymes used in commercial wine production are cinnamyl esterase, cinnamyl decarboxylase, glucanase, urease, and lysozyme. The yeast-derived enzymes, cinnamyl esterase and cinnamyl decarboxylase, may be present in commercial pectin preparations as a side activity and can cause off-flavours due to volatile vinyl phenols (Bruchmann & Fauveau, 2009). At times, grapes infected with *Botrytis cinerea* result in formation of glucans. These glucans prevent the natural sedimentation of cloud particles and cause filtration problems as well. Thus, glucanase enzyme is added to prevent the development of such glucans during wine making process (Gómez-Plaza et al., 2010). Urease is another enzyme that is added occasionally during wine preparation. This enzyme hydrolyses urea into ammonia and carbon dioxide and prevents the formation of ethyl carbamate in wine which is carcinogenic at high concentration (Gómez-Plaza et al., 2010).

Lysozyme is used in winemaking to prevent or delay malolic fermentation and sulphur dioxide is added to the must or wine (Gómez-Plaza et al., 2010).

3.6 Effect of Processing Methods on Enzymes and Vegetables

Enzymes present in fruits and vegetables play a huge role in determining the texture, colour, flavour, and taste attributes of the processed products (Oey, 2010). The continued enzymatic activity in fruits and vegetables affects the storage quality, shelf life, and palatability of the product. Therefore, several processing methods such as grinding, crushing, slicing, juices, or preservation (pickling) are used to prolong the shelf life and reduce wastage of fruits and vegetables (Oey, 2010). Hence, it is very important to control the stability and activity of endogenous enzymes present in fruits and vegetables during food processing (Oey, 2010).

Apart from the conventional techniques of processing such as blanching, heating, or freezing, new and highly advanced processing techniques like high hydrostatic pressure (HHP), pulse electric field (PEF), and ultrasound have been introduced successfully. The major advantage of these new techniques is the use of non-thermal technology, which helps in retaining the sensory attributes and nutritional content of the product (Oey, 2010; Jaiswal & Sharma, 2016). In HHP technique, it employs high pressure range of 100–600 MPa, resulting in enzyme and microbial inactivity which may affect the shelf life of the products (Briones-Labarca et al., 2015). In PEF technique, being a non-thermal processing, it uses a series of short and high voltage pulses to inactivate microbes or enzymes (such as peroxidases and polyphenol oxidases) in food (Segovia et al., 2015). However, in ultrasound processing technique, high frequency short pulses are used for inactivation (Jaiswal & Sharma, 2016). These non-thermal processing techniques are effective at ambient or sub-lethal temperatures and minimize the adverse thermal effects on the nutritional content and quality of fruits and vegetables (Tiwari et al., 2009).

However, these non-thermal processing techniques still have several drawbacks, such as high equipment and processing cost, tedious to operate, hazardous, requires stringent process control operations, etc. (Jaiswal & Sharma, 2016). Nonetheless, multiple advantages are rendered by these techniques in food processing industry over conventional thermal techniques, which affect not only the enzymes, but also the texture, taste, and colour of the product compelled for further investigation and improvement (Oey, 2010).

3.6.1 High-Hydrostatic Pressure (HHP) Processing

HHP, also known as High-Pressure Processing (HPP), is based on the Le Chatlier principle, i.e. pressure is equally applied in all directions (Augusto et al., 2018; Terefe et al., 2016; Keenan et al., 2012). As the name implied, this technique uses very high pressure for application of uniform pressure to a product or food, leading to the inactivation of certain microbes and enzymes in the food (Augusto et al.,

2018). This technique can be applied in solid, semi solid liquid, or particulate food products (Augusto et al., 2018).

The high pressure (HP) may increase the shelf life of fruits and vegetables, especially when combined with temperature by resulting in enzyme activity or inactivity and pressure-induced gel formation (Guerrero-Beltrán et al., 2005). In HHP technique, enzyme inactivation may be affected by the type of food, pH, temperature, and duration of treatment (de Castro Leite Júnior et al., 2017; Guerrero-Beltrán et al., 2005).

The HHP unit consists of a pressure vessel, pressure generator, temperature control, and pressure handling system. The HHP processing can be of two types—batch (closed vessel system) or semi-continuous (number of closed vessels). In this process, the hydrostatic pressure at a given point is transformed rapidly and uniformly in all directions. The intensity of HHP is determined by the process parameters like pressure, treatment duration, and temperature. The entire HHP processing cycle takes a few minutes and it can be performed at pressures as high as 1400 MPa and temperatures between less than 0–150 °C (Oey, 2010) (Augusto et al., 2018; Oey, 2010). The maintenance of a product inside a vessel at a high-pressure results in molecular changes affecting the enzymes (de Castro Leite Júnior et al., 2017).

Several studies have been conducted to determine the effect of HHP (at times combined with thermal processing) on enzyme activity in various fruits and vegetables as shown in Table 3.5. Enzymes like PME, POD, and PPO are considered baroresistant with POD and PME being the most and least resistant, respectively (Augusto et al., 2018). Hence, some studies have found that PPO and POD are not affected substantially by HHP (Augusto et al., 2018).

3.6.2 High-Pressure Homogenization Processing (HPHP)

HPHP, also called Ultra High-Pressure Homogenization, is a non-thermal processing technique, especially used in fruit juice processing. In this method, the fluid is pressurized with high or ultra-high pressure to flow through a narrow valve and the shear stress distribution across the product helps to inactivate microbes and enzymes present in the fluid (Augusto et al., 2018; de Castro Leite Júnior et al., 2017). It is a technique which can be used only in fluids. HPH is a continuous process and is often called dynamic high-pressure processing (Augusto et al., 2018; de Castro Leite Júnior et al., 2017). The high shear and sudden drop in pressure, turbulence, and caviation result in changes in processed foods (de Castro Leite Júnior et al., 2017).

In HPH, factors such as enzyme structure, fluid, equipment, media, and process parameters determine its effect on enzyme activity (Augusto et al., 2018). To ascertain its efficacy, HPH was used to treat oranges and PME activity was measured at five pressures (0–250 MPa) and three inlet temperatures (22, 35, 45 °C). A reduced activity of 70% can be achieved at 45 °C and 250 MPa (Welti-Chanes et al., 2009). Similarly, HPH has also been found to result in complete inactivity of

Table 3.5 Effect of HHP on enzyme activity

Reference	Fruits/ vegetables	Enzyme	Processing	Enzyme stability after application of HHP processing
Andreou et al. (2016)	Tomato juice	PME	800 MPa, 15 min, 65 °C	70% reduction in enzyme activity
		PG	500 MPa, 10 min, 55 °C	Complete inactivation of enzyme activity
Terefe et al. (2016)	Pear	POD	600 MPa, 3 min, 40–100 °C	Enzyme activation by 23% at 40 °C, while reduction by 92% at 100 °C
		PPO	600 MPa, 3 min, 100 °C	Reduction in activity by 90%
		PME	600 MPa, 3 min, 100 °C	Reduction in activity by 83%
Marszałek et al. (2015)	Strawberry puree	POD	500 MPa, 15 min, 50 °C	Reduction of about half of enzyme activity
		PPO	500 MPa, 15 min, 50 °C	Reduction of 72% of enzyme activity
Castro et al. (2008)	Green bell pepper	POD	100–200 MPa, 10 and 20 min, 18–20 °C	70% of reduction in enzyme activity
	Red bell pepper	POD	100–200 MPa, 10 and 20 min, 18–20 °C	40% of reduction in enzyme activity
	Green bell pepper	PPO	100–200 MPa, 10 and 20 min, 18–20 °C	50% reduction in enzyme activity
	Red bell pepper	PPO	100–200 MPa, 10 and 20 min, 18–20 °C	No effect

the enzyme pectate lyase at pressures more than 150 MPa (Calligaris et al., 2012). Therefore, it is not recommended to use HPH independently and preferably; it needs to be coupled with other processing methods for microbial or enzyme inactivation (Augusto et al., 2018; de Castro Leite Júnior et al., 2017; Calligaris et al., 2012; Welti-Chanes et al., 2009).

3.6.3 Ultrasound Processing (Ultrasonication)

In ultrasound processing technique, ultrasonic waves are employed with frequencies above the hearing range of humans. The ultrasonic waves are of two types: high frequency ultrasound (2–20 MHz) corresponding with low sound intensity (0.1–1 W/cm^2) and power ultrasound (20–100 kHz) with high sound intensity (10–1000 W/cm^2) (Feng et al., 2008). These techniques are used in processing industry for improving the shelf life of fruits and vegetables by inactivating the enzymes like PME, PPO, and POD present in the food (Bourke et al., 2010). But,

when the technique, high power ultrasound with low frequency, is combined with temperature/heat, the process is known as thermosonification or pressure (manosonification) (Bourke et al., 2010). The combination of heat and ultrasound helps to ensure product stability and inactivation of enzymes and microbes, thereby resulting in retaining the quality and extending the shelf life of fruits and vegetables, especially juices (Rojas et al., 2017; Saeeduddin et al., 2015). The inactivation of microorganisms or enzymes in this technique may be due to physical factors such as caviation or mechanical effects (Bourke et al., 2010; O'Donnell et al., 2010).

Ultrasonic processing in fruits and vegetables focuses on the inactivation of endogenous enzymes which are more resistant to heat treatments (Feng et al., 2008). Studies have It has been observed that ultrasonic processing either in combination with heat or pressure has minimal effect on quality of fruit juices such as orange juice, guava, and strawberry juice (Bourke et al., 2010). Further, it has been observed that high ultrasound and longer processing times may be required for enzyme inactivation as the pulp complicates the inactivation process (Rojas et al., 2017). Hence, ultrasound technique is usually combined with a thermal process to increase the rate of inactivation of enzymes in juices (Chen et al., 2019; Rojas et al., 2017; Anaya-Esparza et al., 2017; Abid et al., 2014). PME was also found to be inactivated in lemon juice (Knorr et al., 2004) and mousambi (Siwach & Kumar, 2012) by using ultrasonic processing along with heat. Likewise, peroxidase enzyme present in watercress was also found to be inactivated by the use of thermosonication (Cruz et al., 2006).

3.6.4 Pulsed Electric Field (PEF)

In PEF processing technique, electrochemical effects and ohmic heating are responsible for changing the structure and function of enzymes present in the food. At times, large specific energy inputs are required to inactivate enzymes (Poojary et al., 2017). Enzyme activity can be affected by a number of factors such as properties of the enzyme, treatment parameters, processing conditions as well as the condition of the medium (Poojary et al., 2017).

The PEF unit consists of a treatment chamber, pulse generator, a pulse monitoring system, and a temperature monitor. It can be conducted in two ways—continuous or batch processing. The unit also contains a fluid handling system for liquid foods in continuous PEF processing (Min et al., 2007). The design of the treatment chamber plays a key role in distribution of uniform temperature inside the PEF chamber (Oey, 2010). In PEF, the duration of processing takes substantially lesser time (micro to milli seconds) as compared to other methods (Oey, 2010).

However, in PEF processing, the activity on enzyme may either limit its activation/inactivation or may not be affected at all (Poojary et al., 2017). In this process, multiple factors such as food matrix, pH, enzyme dissolving medium, and certain treatment conditions affect the enzyme treatment in fruits and vegetables (Poojary et al., 2017; Oey, 2010). Two important parameters that affect the intensity of PEF processing are electric field intensity and the total duration of treatment per energy

(Oey, 2010). A higher electric field and treatment duration may result in enzyme inactivation, while a lower electric field and duration may result in enzyme activation (Poojary et al., 2017). Also, increasing pulse width lowers enzyme activity (Poojary et al., 2017).

Furthermore, several studies have been conducted to assess the effect of PEF processing on various food enzymes such as polyphenol oxidase, lipoxygenase, peroxidase, and pectin methyl esterase (Poojary et al., 2017; Aguiló-Aguayo et al., 2009; Noci et al., 2008; Espachs-Barroso et al., 2006) and are given in Table 3.6. The findings from these studies indicate that a longer treatment duration, higher electric field intensity, and pulse width result in enzyme inactivation.

3.6.5 Thermal Processing

In spite of advancement in alternative non-thermal treatments in food processing industry, thermal treatments are still widely considered the most cost-effective and simple method to ensure enzymatic and microbial inactivity in foods (Rawson et al., 2011). However, these treatments have many drawbacks as well, such as slow in conduction and convection of heat transfer, loses in nutritional and functional properties, reduction in sensory attributes, etc. (Baysal & Icier, 2010; Gonzalez & Barrett, 2010). In addition, food matrix, complexity of products, and the microorganism also affect the efficacy of thermal processing (Chen et al., 2013). As a result, optimization of thermal treatment becomes important to preserve the sensory attributes, nutritional quality, and safety of the product.

The common thermal treatments (conventional and non-conventional) employed during processing of fruits and vegetables are High temperature-long time (HTLT), High temperature-short time (HTST), and Ohmic and Microwave heating (Lee et al., 2015). Brief explanations of these treatments are discussed below.

High temperature-long time (HTLT) is a conventional thermal treatment. In this, the processing of juice and beverages is done at temperature 80 °C with >30 s holding time (Miller & Silva, 2012). It inactivates or reduces the enzymatic activities of polyphenoloxidase (PPO), pectin esterase (PE), peroxidase (POD), etc. (Marszałek et al., 2017). During the treatment, it was observed that antioxidant activity and PME enzymatic activity were reduced to 75% and 83% in mombin juice and litchi beverage, respectively (Swami Hulle & Rao, 2016; de Carvalho et al., 2015).

High temperature-short time (HTST) is another conventional thermal treatment used in food processing. It is carried out at temperature ≥ 80 °C with ≤ 30 s holding time. Here, priority is given for destruction of microorganism than nutrient degradation (Achir et al., 2016). For example, HTST treatment reduces PME and PPO activity by 95.3% and 90.9% in apple juice, respectively (Aguilar-Rosas et al., 2013). In another study based on apricot nectar, complete inactivation of POD, PPO, and PME was observed (Huang et al., 2013).

Ohmic heating (OH) is a common non-conventional thermal treatment. This treatment is primarily used in fruit juices processing for its effectiveness. Fruit juices

Table 3.6 PEF and effect on enzyme activity

Reference	Fruits/ vegetables	Enzyme	Processing	Enzyme stability after application of HP processing
Aguiló-Aguayo et al. (2009)	Tomato juice	Lipoxygenase	Electric field: 35 kV/cm for 1000, pulse width: 1–7 μs, frequency: 50–250 Hz	An increase in frequency or pulse width reduced enzyme activity
	Strawberry juice	Polygalacturonase	Electric field: 35 kV/cm for 1000 μs, treatment time: 1700 μs, frequency: 100 Hz, pulse width: 4 μs	Enzyme activity inactivated by 26%
	Strawberry juice	PME	Electric field: 35 kV/cm for 1000 μs, treatment time: 1700 μs, frequency: 100 Hz	Enzyme inactivation
Noci et al. (2008)	Apple juice	PPO	Electric field: 40 kV/cm, pulse width: 1 μs, treatment time: 6000 μs, frequency: 15 Hz, no. of pulses: 100	About 50% reduction in enzyme activity
Espachs-Barroso et al. (2006)	Carrot	PME	Pulses of 13.2–19.1 kV/cm for 40 μs, time: 1.6 ms, frequency: 0.5–5 Hz	PME inactivation—45% banana, 83% carrot, 87% orange and tomato. Enzyme inactivity increased with increased treatment time and electric field and pulses
	Banana			
	Tomato			
	Orange			

that contain water and ionic salts in higher amount are found to be more effective with this treatment (Miller & Silva, 2012). In other foods, this process has many advantages like uniform and rapid heating which ultimately helps in nutritional and sensory attributes of the processed products. As per the study by Somavat et al. (2013), *B. coagulans* (ATCC 8038) in tomato juice when treated with OH at 60 Hz and 10 kHz expedites the inactivation process as compared to conventional treatment (Somavat et al., 2013). However, microwave heating is another common

non-conventional treatment which is mainly used in urban areas. Even though it is a thermal treatment, minimal thermal exposure is required for enzymatic inactivation (Arjmandi et al., 2016).

3.7 Regulatory Aspects of Food Enzymes Used in Fruit and Vegetable Processing

The regulation of enzymes for its application in food industry is very complex and differs from county to country. The European Union (EU) classifies food enzymes as processing aids of food additives. Majority of the enzyme preparations used in food processing are categorized as processing aids by European Union since they have a role in the technological aspect of food processing stage and not in the final product (Aehle, 2007). The processing aid may not be labelled (Bruchmann & Fauveau, 2009). The enzyme lysozyme used in wine processing is considered as a food additive (Bruchmann & Fauveau, 2009) since it has a role to play in the final product and is regulated under Food Additives Directive (95/2). A vertical legislation of EU on fruit juices (Council Directive 93/77/EEC) allows pectinolytic, proteolytic, and amylolytic enzymes, while another regulation (Council Regulations 82/87/EEC) allows only pectinolytic enzymes on wines (Aehle, 2007). The Organization Internationale de la Vigne et du Vin (OIV) regulates the enzymes used in wine processing in EU (Gómez-Plaza et al., 2010; Bruchmann & Fauveau, 2009; Aehle, 2007) and has recognized the important role of pectinases, hemicellulases, cellulose, β-glucanase, and glycosidase. The European Commission Regulation 1493/1999 authorises the use of pectinases from *Aspergillus niger*, β-glucanase from *Trichoderma harzanium* and urease from *Lactobacillus fermentum*. Pectinases are assigned a GRAS status by United States Food and Drug Administration (Bruchmann & Fauveau, 2009). In India, Food Safety and Standards Authority of India (FSSAI) regulates the use and safety of food enzymes. As per the regulation Food Safety and Standards (Food Products Standards and Food Additives) (Amendment) Regulations, 2015, the food regulator had permitted the use of processing aids in bread. The enzymes permitted for usage are glucose oxidase, lipase, and xylanase. They are obtained from various microbial sources.

In some EU countries, namely Denmark, France, and United Kingdom, approval is required for the use of any food enzymes (Aehle, 2007). However, in countries like Poland, China, Japan, Australia, New Zealand, Canada, Brazil, and Mexico, approval is needed for the use of enzymes produced traditionally, especially if they are new enzymes (Aehle, 2007). On the other hand, countries such as Thailand, Korea, and Taiwan require registration prior to their enzyme use, while USA requires a GRAS (generally regarded as safe) assessment, GRAS notice, or Food additive (Aehle, 2007). Even though countries have different set regulations, enzyme preparations must comply with specifications recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by Food Chemical Codex (FCC) for food enzymes (Bruchmann & Fauveau, 2009; Grassin & Coutel, 2009).

References

Abid, M., Jabbar, S., Hu, B., Hashim, M. M., Wu, T., Lei, S., Khan, M. A., & Zeng, X. (2014). Thermosonication as a potential quality enhancement technique of apple juice. *Ultrasonics Sonochemistry, 21*(3), 984–990. https://doi.org/10.1016/j.ultsonch.2013.12.003

Achir, N., Dhuique-Mayer, C., Hadjal, T., Madani, K., Pain, J. P., & Dornier, M. (2016). Pasteurization of citrus juices with ohmic heating to preserve the carotenoid profile. *Innovative Food Science and Emerging Technologies, 33*, 397–404.

Aehle, W. (Ed.). (2007). Enzymes in industry: Production and applications (3rd completely rev. ed.). Wiley-VCH.

Aguero, M. V., Ansorena, M. R., Roura, S. I., & Del Valle, C. E. (2008). Thermal inactivation of peroxidase during blanching of butternut squash. *Food Science and Technology, 41*, 401–407.

Aguilar-Rosas, S., Ballinas-Casarrubias, M., Elias-Ogaz, L., Martin-Belloso, O., & Ortega-Rivas, E. (2013). Enzyme activity and colour changes in apple juice pasteurised thermally and by pulsed electric fields. *Acta Alimentaria, 42*, 45–54.

Aguiló-Aguayo, I., Oms-Oliu, G., Soliva-Fortuny, R., & Martín-Belloso, O. (2009). Changes in quality attributes throughout storage of strawberry juice processed by high-intensity pulsed electric fields or heat treatments. *LWT—Food Science and Technology, 42*(4), 813–818. https://doi.org/10.1016/j.lwt.2008.11.008

Akacha, N. B., & Gargouri, M. (2009). Enzymatic synthesis of green notes with hydroperoxide-lyase from olive leaves and alcohol-dehydrogenase from yeast in liquid/gas reactor. *Process Biochemistry, 44*(10), 1122–1127.

Albersheim, P., Darvill, A. G., O'Neill, M. A., Schols, H. A., & Voragen, A. G. J. (1996). An hypothesis: The same polysaccharides are components of the primary cell walls of all higher plants. In J. Visser & A. G. J. Voragen (Eds.), *Pectin and pectinases* (pp. 47–55). Elsevier Science B.V.

Alkorta, I., Garbisu, C., Llama, M. J., & Serra, J. L. (1998). Industrial applications of pectic enzymes: A review. *Process Biochemistry, 33*(1), 21–28.

Anaya-Esparza, L. M., Velázquez-Estrada, R. M., Roig, A. X., García-Galindo, H. S., Sayago-Ayerdi, S. G., & Montalvo-González, E. (2017). Thermosonication: An alternative processing for fruit and vegetable juices. *Trends in Food Science & Technology, 61*, 26–37. https://doi.org/10.1016/j.tifs.2016.11.020

Andreou, V., Dimopoulos, G., Katsaros, G., & Taoukis, P. (2016). Comparison of the application of high pressure and pulsed electric fields technologies on the selective inactivation of endogenous enzymes in tomato products. *Innovative Food Science and Emerging Technologies, 38*, 349–355. https://doi.org/10.1016/j.ifset.2016.07.026

Arjmandi, M., Otón, M., Artés, F., Artés-Hernández, F., Gómez, P. A., & Aguayo, E. (2016). Semi-industrial microwave treatments positively affect the quality of orange-colored smoothies. *Journal of Food Science and Technology, 53*, 3695–3703.

Armada, L., & Falqué, E. (2007). Repercussion of the clarification treatment agents before the alcoholic fermentation on volatile composition of white wines. *European Food Research and Technology, 225*(3–4), 553–558. https://doi.org/10.1007/s00217-006-0453-3

Armada, L., Fernández, E., & Falqué, E. (2010). Influence of several enzymatic treatments on aromatic composition of white wines. *LWT—Food Science and Technology, 43*(10), 1517–1525.

Augusto, P. E. D., Tribst, A. A. L., & Cristianini, M. (2018). High hydrostatic pressure and high-pressure homogenization processing of fruit juices. In *Fruit juices* (pp. 393–421). Elsevier. https://doi.org/10.1016/B978-0-12-802230-6.00020-5

Baker, R. A., & Bruemmer, J. H. (1989). Quality and stability of enzymatically peeledand sectioned citrus fruit. In J. J. Jen (Ed.), *Quality factors of fruits and vegetables* (pp. 140–148). American Chemical Society.

Bansal, N., Tewari, R., Gupta, J. K., Soni, S. K., & Soni, R. (2011). A novel strain of *Aspergillus niger* producing a cocktail of industrial depolymerising enzymes for the production of second generation biofuels. *BioResources, 6*, 552–569.

Bartolome, L. G., & Hoff, J. E. (1972). Firming of potatoes. Biochemical effects of preheating. *Journal of Agricultural and Food Chemistry, 20*(2), 266–270. https://doi.org/10.1021/jf60180a028

Bautista-Ortín, A. B., Martinez-Cutillas, A., Ros-Garcia, J. M., Lopez-Roca, J. M., & Gomez-Plaza, E. (2005). Improving colour extraction and stability in red wines: The use of maceration enzymes and enological tannins. *International Journal of Food Science and Technology, 40*(8), 867–878. https://doi.org/10.1111/j.1365-2621.2005.01014.x

Baysal, A. H., & Icier, F. (2010). Inactivation kinetics of *Alicyclobacillus acidoterrestris* spores in orange juice by ohmic heating: Effects of voltage gradient and temperature on inactivation. *Journal of Food Protection, 73*, 299–304.

Beaulieu, J. C., & Baldwin, E. A. (2002). Flavor and aroma of fresh-cut fruits and vegetables. In O. Lamikanra (Ed.), *Fresh-cut fruits and vegetables: Science, technology, and market* (pp. 391–425). CRC Press.

Belluzzo, A. S. F., Fleuri, L. F., Macedo, J. A., & Macedo, G. A. (2009). Characterization of Biuti peach polyphenol oxidase. *Food Science and Biotechnology, 18*, 878–883.

Ben-Shalom, N., Levi, A., & Pinto, R. (1986). Pectolytic enzyme studies for peeling of grapefruit segment membrane. *Journal of Food Science, 51*(2), 421–423.

Berg, M., Roubos, J., & A.; & Parenicova, L. (2010). Enzymes in fruit and vegetable processing: Future trends in enzyme discovery, design, production and application. In A. Bayindirli (Ed.), *Enzymes in fruit and vegetable processing: Chemistry and engineering applications* (pp. 341–358). CRC Press.

Berry, R. E., Baker, R. A., & Bruemmer, J. H. (1988). Enzyme separated sections: A new lightly processed citrus product. In R. Goren & K. Hendel (Eds.), *Proceedings of the Sixth International Citrus Congress* (pp. 1711–1716). Balaban.

Bhowmik, P. K., & Dris, R. (2004). Enzymes and quality factors of fruits and vegetables. In R. Dris & S. M. Jain (Eds.), *Production practices and quality assessment of food crops* (pp. 1–25). Springer.

Bourke, P., Tiwari, B., O'Donnell, C., & Cullen, P. J. (2010). Effect of ultrasonic processing on food enzymes of industrial importance. *Trends in Food Science and Technology, 21*(7), 358.

Briones-Labarca, V., Plaza-Morales, M., Giovagnoli-Vicuña, C., & Jamett, F. (2015). High hydrostatic pressure and ultrasound extractions of antioxidant compounds, sulforaphane and fatty acids from Chilean papaya (Vasconcellea pubescens) seeds: Effects of extraction conditions and methods. *LWT—Food Science and Technology, 60*(1), 525–534. https://doi.org/10.1016/j.lwt.2014.07.057

Bruchmann, A., & Fauveau, C. (2009). Enzymes in potable alcohol and wine production. In R. J. Whitehurst & M. van Oort (Eds.), *Enzymes in food technology* (pp. 195–210). Wiley-Blackwell. https://doi.org/10.1002/9781444309935.ch9

Bruemmer, J. H., Griffin, A. W., & Onayami, O. (1978). Sectionizing grapefruit by enzyme digestion. *Proceedings of the Florida State Horticultural Society, 91*, 112–114.

Byarugaba-Bazirake, G. W. (2008). *The effect of enzymatic processing on banana juice and wine*. Dissertation, Institute of Wine Technology at Stellenbosch University.

Calligaris, S., Foschia, M., Bartolomeoli, I., Maifreni, M., & Manzocco, L. (2012). Study on the applicability of high-pressure homogenization for the production of banana juices. *LWT—Food Science and Technology, 45*(1), 117–121. https://doi.org/10.1016/j.lwt.2011.07.026

Carpita, N., & Gibeaut, D. (1993). Structural models of primary cell walls in flowering plants. *The Plant Journal: For Cell and Molecular Biology, 3*, 1–30.

Carrín, M. (2004). Characterization of starch in apple juice and its degradation by amylases. *Food Chemistry, 87*(2), 173–178. https://doi.org/10.1016/j.foodchem.2003.10.032

Castro, S. M., Saraiva, J. A., Lopes-da-Silva, J. A., Delgadillo, I., Loey, A. V., Smout, C., & Hendrickx, M. (2008). Effect of thermal blanching and of high pressure treatments on sweet

green and red bell pepper fruits (*Capsicum annuum* L.). *Food Chemistry, 107*(4), 1436–1449. https://doi.org/10.1016/j.foodchem.2007.09.074

Cautela, D., Castaldo, D., Servillo, L., & Giovan, A. (2010). Enzymes in citrus juice processing. In A. Bayindirli (Ed.), *Enzymatic processing in fruits and vegetables: Chemistry and engineering applications* (pp. 197–214). CRC Press, Taylor and Francis Group.

Ceci, L. N., & Lozano, J. E. (2010). Use of enzymes for non-citrus fruit juice production. In A. Bayindirli (Ed.), *Enzymatic processing in fruits and vegetables: Chemistry and engineering applications* (pp. 175–195). CRC Press, Taylor and Francis Group.

Chaisakdanugull, C., Theerakulkait, C., & Wrolstad, R. E. (2007). Pineapple juice and its fractions in enzymatic browning inhibition of banana (Musa [AAA Group] Gros Michel). *Journal of Agriculture and Food Chemistry, 55*, 4252–4257.

Chen, D., Xi, H., Guo, X., Qin, Z., Pang, X., Hu, X., Liao, X., & Wu, J. (2013). Comparative study of quality of cloudy pomegranate juice treated by high hydrostatic pressure and high temperature short time. *Innovative Food Science and Emerging Technologies, 19*, 85–94.

Chen, L., Bi, X., Guo, D., Xing, Y., & Che, Z. (2019). The effect of high-power ultrasound on the quality of carrot juice. *Food Science and Technology International, 25*(5), 394–403. https://doi.org/10.1177/1082013219825736

Claus, H., & Mojsov, K. (2018). Enzymes for wine fermentation: Current and perspective applications. *Fermentation, 4*(3), 52. https://doi.org/10.3390/fermentation4030052

Constabel, C. P., & Ryan, C. A. (1998). A survey of wound- and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. *Phytochemistry, 47*(4), 507–511.

Cruz, R. M. S., Vieira, M. C., & Silva, C. L. M. (2006). Effect of heat and thermosonication treatments on peroxidase inactivation kinetics in watercress (Nasturtium officinale). *Journal of Food Engineering, 72*(1), 8–15. https://doi.org/10.1016/j.jfoodeng.2004.11.007

Cunha, A. G., & Gandini, A. (2010). Turning polysaccharides into hydrophobic materials: A critical review. Part 2. Hemicelluloses, chitin/chitosan, starch, pectin and alginates. *Cellulose, 17*, 1045–1065.

de Carvalho, J. M., Maia, G. A., da Fonseca, A. V., de Sousa, P. H., & Rodrigues, S. (2015). Effect of processing on physicochemical composition, bioactive compounds and enzymatic activity of yellow mombin (*Spondias mombin* L.) tropical juice. *Journal of Food Science and Technology, 52*, 1182–1187.

de Castro Leite Júnior, B. R., Tribst, A. A. L., & Cristianini, M. (2017). Effect of high-pressure technologies on enzymes applied in food processing. In M. Senturk (Ed.), *Enzyme inhibitors and activators*. InTech. https://doi.org/10.5772/66629

Degraeve, P., Saurel, R., & Coutel, Y. (2003). Vacuum impregnation pretreatment with pectinmethylesterase to improve firmness of pasteurized fruits. *Journal of Food Science, 68*(2), 716–721. https://doi.org/10.1111/j.1365-2621.2003.tb05738.x

Dervilly, G., Leclercq, C., Zimmerman, D., Roue, C., Thibault, J. F., & Sauliner, L. (2002). Isolation and characterization of high molecular mass water-soluble arabinoxylans from barley malt. *Carbohydrate Polymers, 47*, 143–149.

Dhillon, G. S., Kaur, S., & Brar, S. K. (2013). Perspective of apple processing wastes as low-cost substrates for bioproduction of high value products: A review. *Renewable and Sustainable Energy Reviews, 27*, 789–805.

Dinu, D., Nechifor, M. T., Stoian, G., Costache, M., & Dinischiotu, A. (2007). Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. *Journal of Biotechnology, 131*, 128–137.

Eisenmenger, M. J., & Reyes-De-Corcuera, J. I. (2009). High pressure enhancement of enzymes: A review. *Enzyme and Microbial Technology, 45*, 331–347.

El Darra, N., Turk, M. F., Ducasse, M.-A., Grimi, N., Maroun, R. G., Louka, N., & Vorobiev, E. (2016). Changes in polyphenol profiles and color composition of freshly fermented model wine due to pulsed electric field, enzymes and thermovinification pretreatments. *Food Chemistry, 194*, 944–950. https://doi.org/10.1016/j.foodchem.2015.08.059

Espachs-Barroso, A., Van Loey, A., Hendrickx, M., & Martín-Belloso, O. (2006). Inactivation of plant pectin methylesterase by thermal or high intensity pulsed electric field treatments. *Innovative Food Science and Emerging Technologies, 7*(1–2), 40–48. https://doi.org/10.1016/j.ifset.2005.07.002

Espejo, F. (2020). Role of commercial enzymes in wine production: A critical review of recent research. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-020-04489-0

Fatibello-Filho, O., & Vieira, I. C. (2002). Uso analítico de tecidos e de extratos brutos vegetais como fonte enzimática. *Química Nova, 25*(3), 455–464.

Feng, H., Yang, W., & Hielscher, T. (2008). Power ultrasound. *Food Science and Technology International, 14*(5), 433–436. https://doi.org/10.1177/1082013208098814

Fleuri, L. F., Delgado, C. H. O., Novelli, P. K., Pivetta, M. R., do Prado, D. Z., & Simon, J. W. (2016). *Enzymes in food and beverage processing* (pp. 255–280). CRC Press, Taylor and Francis Group.

Freitas, A. A., Francelin, M. F., Hirata, G. F., Clemente, E., & Schmidt, F. L. (2008). Atividades das enzimas peroxidase (POD) e polifenoloxidase (PPO) nas uvas das cultivares benitaka e rubi e em seus sucos e geleias. *Ciência e Tecnologia de Alimentos, 28*, 172–177.

Funk, C., Weber, P., Thilker, J., Grabber, J. H., Steinhart, H., & Bunzel, M. (2006). Influence of lignification and feruloyation of maize cell walls on the adsorption of heterocyclic aromatic amines. *Journal of Agricultural and Food Chemistry, 54*, 1860–1867.

Gao, Y., Zietsman, A. J. J., Vivier, M. A., & Moore, J. P. (2019). Deconstructing wine grape cell walls with enzymes during winemaking: New insights from glycan microarray technology. *Molecules, 24*(1), 165. https://doi.org/10.3390/molecules24010165

Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., & Mahajan, R. (2016). Microbial pectinases: An ecofriendly tool of nature for industries. *Biotech, 6*(1), 47. https://doi.org/10.1007/s13205-016-0371-4

Goff, S. A., & Klee, H. J. (2006). Plant volatile compounds: Sensory cues for health and nutritional value? *Science, 311*(5762), 815–819.

Gómez-Plaza, E., Romero-Cascales, I., & Bautista-Ortín, A. B. (2010). Use of enzymes in wine production. In A. Bayindirli (Ed.), *Enzymatic processing in fruits and vegetables: Chemistry and engineering applications* (pp. 215–243). CRC Press, Taylor and Francis Group.

Gonçalves, E. M., Pinheiro, J., Abreu, M., Brandão, T. R. S., & Silva, C. L. M. (2007). Modelling the kinetics of peroxidase inactivation, colour and texture changes of pumpkin (Cucurbita maxima L.) during blanching. *Journal of Food Engineering, 81*, 693–701.

Gonzalez, M. E., & Barrett, D. M. (2010). Thermal, high pressure, and electric field processing effects on plant cell membrane integrity and relevance to fruit and vegetable quality. *Journal of Food Science, 75*, R121–R130.

Granada, G. L., Vendruscolo, J. L., & Treptow, R. O. (2001). Caracterização química e sensorial de sucos clarificados de amora-preta (Rubus spp. L.). *Revista Brasileira de Agrociencia, 7*, 143–147.

Grassin, C., & Coutel, Y. (2009). Enzymes in fruit and vegetable processing and juice extraction. In R. J. Whitehurst & M. van Oort (Eds.), *Enzymes in food technology* (pp. 236–263). Wiley-Blackwell. https://doi.org/10.1002/9781444309935.ch11

Grassino, A. N., Brnčić, M., Vikić-Topić, D., Roca, S., Dent, M., & Brnčić, S. R. (2016). Ultrasound assisted extraction and characterization of pectin from tomato waste. *Food Chemistry, 198*, 93–100.

Guerrero-Beltrán, J. A., Barbosa-Cánovas, G. V., & Swanson, B. G. (2005). High hydrostatic pressure processing of fruit and vegetable products. *Food Reviews International, 21*(4), 411–425. https://doi.org/10.1080/87559120500224827

Guillemin, A., Guillon, F., Degraeve, P., Rondeau, C., Devaux, M.-F., Huber, F., Badel, E., Saurel, R., & Lahaye, M. (2008). Firming of fruit tissues by vacuum-infusion of pectin methylesterase: Visualisation of enzyme action. *Food Chemistry, 109*(2), 368–378. https://doi.org/10.1016/j.foodchem.2007.12.050

Guo, X., Zhao, W., Pang, X., Liao, X., Hu, X., & Wu, J. (2014). Emulsion stabilizing properties of pectins extracted by high hydrostatic pressure, high speed shearing homogenization and traditional thermal methods: A comparative study. *Food Hydrocolloids, 35*, 217–225.

Hsu, C. P., Deshpande, S. N., & Desrosier, N. W. (1965). Role of pectin methylesterase in firmness of canned tomatoes. *Journal of Food Science, 30*(4), 583–588. https://doi.org/10.1111/j.1365-2621.1965.tb01806.x

Huang, W., Bi, X., Zhang, X., Liao, X., Hu, X., & Wu, J. (2013). Comparative study of enzymes, phenolics, carotenoids and colour of apricot nectars treated by high hydrostatic pressure and high temperature short time. *Innovative Food Science and Emerging Technologies, 18*, 74–82.

İyidoğan, N. F., & Bayındırlı, A. (2004). Effect of l-cysteine, kojic acid and 4-hexylresorcinol combination on inhibition of enzymatic browning in Amasya apple juice. *Journal of Food Engineering, 62*(3), 299–304. https://doi.org/10.1016/S0260-8774(03)00243-7

Jaiswal, A. K., & Sharma, S. (2016). Enzymes in synthesis of novel functional food ingredients. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 381–400). CRC Press, Taylor and Francis Group.

Javeri, H., Toledo, R., & Wicker, L. (1991). Vacuum infusion of citrus pectinmethylesterase and calcium effects on firmness of peaches. *Journal of Food Science, 56*(3), 739–742. https://doi.org/10.1111/j.1365-2621.1991.tb05371.x

Jecu, L. (2000). Solid state fermentation of agricultural wastes for endoglucanase production. *Industrial Crops and Products, 11*(1), 1–5. https://doi.org/10.1016/S0926-6690(99)00022-9

Jensen, M., Petersen, B. R., & Adler-Nissen, J. (2004). Enzymatic firming of processed red pepper by means of exogenous pectinesterase. *Food Biotechnology, 18*(2), 217–227. https://doi.org/10.1081/FBT-200025667

Jobling, S. (2004). Improved starch for food and industrial applications. *Current Opinion of Plant Biology, 7*, 210–218.

Juturu, V., & Wu, J. C. (2013). Insight into microbial hemicellulases other than xylanases: A review: Microbial hemicellulases other than xylanases. *Journal of Chemical Technology and Biotechnology, 88*(3), 353–363. https://doi.org/10.1002/jctb.3969

Kant, S., Vohra, A., & Gupta, R. (2013). Purification and physicochemical properties of polygalacturonase from *Aspergillus niger* MTCC 3323. *Protein Expression and Purification, 87*, 11–16.

Kavuthodi, B., & Sebastian, D. (2018). Review on bacterial production of alkaline pectinase with special emphasis on Bacillus species. *Bioscience Biotechnology Research Communications, 11*, 18–30.

Kc, S., Upadhyaya, J., Joshi, D. R., Lekhak, B., Chaudhary, D. K., Pant, B. R., & Raghavan, V. (2020). Production, characterisation and industrial applications pectinases enzyme isolated from fungal strains. *Fermentation, 6*(2), 59.

Keenan, D. F., Rößle, C., Gormley, R., Butler, F., & Brunton, N. P. (2012). Effect of high hydrostatic pressure and thermal processing on the nutritional quality and enzyme activity of fruit smoothies. *LWT—Food Science and Technology, 45*(1), 50–57. https://doi.org/10.1016/j.lwt.2011.07.006

Kelebek, H., Canbas, A., Cabaroglu, T., & Selli, S. (2007). Improvement of anthocyanin content in the cv. Öküzgözü wines by using pectolytic enzymes. *Food Chemistry, 105*(1), 334–339. https://doi.org/10.1016/j.foodchem.2006.11.068

Khairnar, Y., Krishna, V. K., Boraste, A., Gupta, N., Trivedi, S., Patil, P., Gupta, G., Gupta, M., Jhadhav, A., Mujapara, A., et al. (2009). Study of pectinase production in submerged fermentation using different strains of Aspergillus niger. *International Journal of Microbiology Research, 1*, 13–17.

Kim, J. Y., Seo, Y. S., Kim, J. E., Sung, S.-K., Song, K. J., An, G., & Kim, W. T. (2001). Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple. *Plant Science, 161*(6), 1145–1152. https://doi.org/10.1016/S0168-9452(01)00522-2

Kim, Y. J., & Uyama, H. (2005). Tyrosinase inhibitors from natural and synthetic sources: Structure, inhibition mechanism and perspective for the future. *Cellular and Molecular Life Sciences, 62*, 1707–1723.

Knorr, D., Zenker, M., Heinz, V., & Lee, D.-U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science and Technology, 15*(5), 261–266. https://doi.org/10.1016/j.tifs.2003.12.001

Kola, O., Kaya, C., Duran, H., & Altan, A. (2010). Removal of limonin bitterness by treatment of ion exchange and adsorbent resins. *Food Science and Biotechnology, 19*(2), 411–416.

Kollarigowda, R. H. (2015). Novel polysaccharide nanowires; synthesis from pectin-modified methacrylate. *RSC Advances, 5*, 102143–102146.

Kubra, K. T., Ali, S., Walait, M., & Sundus, H. (2018). Potential applications of pectinases in food, agricultural and environmental sectors. *Journal of Pharmaceutical, Chemical and Biological Sciences, 6*, 23–34.

Labuza, T. P., Lillemo, J. H., & Taoukis, P. S. (1992). Inhibition of polyphenol oxidase by proteolytic enzymes. *Fruit Processing, 2*(1), 9–13.

Leadlay, P. F. (1993). *An introduction to enzyme chemistry* (p. 82). The Royal Society of Chemistry.

Lee, J. Y., Kim, S. S., & Kang, D. H. (2015). Effect of pH for inactivation of *Escherichia coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* in orange juice by ohmic heating. *LWT—Food Science and Technology, 62*, 83–88.

Li, J.-Y., & Yeh, A.-I. (2001). Relationships between thermal, rheological characteristics and swelling power for various starches. *Journal of Food Engineering, 50*, 141–148.

Liu, F., Osman, A., Yusof, S., & Ghazali, H. M. (2005). Effects of enzyme-aided peeling on the quality of local mandarin (*Citrus reticulata b.*) segments: Enzyme-aided peeling of mandarins. *Journal of Food Processing and Preservation, 28*(5), 336–347. https://doi.org/10.1111/j.1745-4549.2004.21145.xC

Loizzo, M. R., Tundis, R., & Menichini, F. (2012). Natural and synthetic tyrosinase inhibitors as antibrowning agents: An update. *Comprehensive Reviews in Food Science and Food Safety, 11*(4), 378–398. https://doi.org/10.1111/j.1541-4337.2012.00191.x

Lopes, A. S., & Clemente, E. (2002). Minerais e enzimas oxidativas em brócolis (Brassica oleracea L. cv. Itálica) minimamente processado. *Acta Scientiarum, 24*(6), 1615–1618.

Lu, F.-J., Chu, L. H., & Gau, R.-J. (1998). Free radical—Scavenging properties of lignin. *Nutrition and Cancer, 30*, 31–38.

Markets & Markets. (2020). Retrieved June 20, 2020 from https://www.marketsandmarkets.com/Market-Reports/fruit-vegetable-processing-enzymes-market

Marshall, M. R., Kim, J., & Wei, C. (2000). *Enzymatic browning in fruits, vegetables and seafoods*. FAO.

Marszałek, K., Krzyżanowska, J., Woźniak, L., & Skąpska, S. (2017). Kinetic modelling of polyphenol oxidase, peroxidase, pectin esterase, polygalacturonase, degradation of the main pigments and polyphenols in beetroot juice during high pressure carbon dioxide treatment. *LWT—Food Science and Technology, 85*, 412–417.

Marszałek, K., Mitek, M., & Skąpska, S. (2015). The effect of thermal pasteurization and high pressure processing at cold and mild temperatures on the chemical composition, microbial and enzyme activity in strawberry purée. *Innovative Food Science and Emerging Technologies, 27*, 48–56. https://doi.org/10.1016/j.ifset.2014.10.009

Merino, M. T., Humanes, L., Lopez-Ruiz, A., Diez, J., & Roldan, J. M. (1997). High-performance liquid chromatography quantitation of limonin D-ring lactone hydrolase and limonoate dehydrogenase activities. *Journal of Chromatography A, 760*(2), 173–178.

Miller, F. A., & Silva, C. L. M. (2012). Thermal treatment effects in fruit juices. In S. Rodrigues & F. Fan (Eds.), *Advances in fruit processing technologies* (e-book ed., pp. 363–383). CRC Press. ISBN: 978-1-4398-5153-1.

Min, S., Evrendilek, G. A., & Zhang, H. Q. (2007). Pulsed electric fields: Processing system, microbial and enzyme inhibition, and shelf life extension of foods. *IEEE Transactions on Plasma Science, 35*(1), 59–73. https://doi.org/10.1109/TPS.2006.889290

Nandakumar, R., & Wakayama, M. (2015). Enzymes in flavours and food additives. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 321–340). CRC Press.

Noci, F., Riener, J., Walkling-Ribeiro, M., Cronin, D. A., Morgan, D. J., & Lyng, J. G. (2008). Ultraviolet irradiation and pulsed electric fields (PEF) in a hurdle strategy for the preservation of fresh apple juice. *Journal of Food Engineering, 85*(1), 141–146. https://doi.org/10.1016/j.jfoodeng.2007.07.011

O'Donnell, C. P., Tiwari, B. K., Bourke, P., & Cullen, P. J. (2010). Effect of ultrasonic processing on food enzymes of industrial importance. *Trends in Food Science and Technology, 21*(7), 358–367. https://doi.org/10.1016/j.tifs.2010.04.007

Oey, I. (2010). Effect of novel food processing on fruit and vegetable enzymes. In A. Bayindirli (Ed.), *Enzymatic processing in fruits and vegetables: Chemistry and engineering applications* (pp. 245–312). CRC Press, Taylor and Francis Group.

Oliveira, C. M., Ferreira, A. C. S., De Freitas, V., & Silva, A. M. S. (2011). Oxidation mechanisms occurring in wines. *Food Research International, 44*(5), 1115–1126. https://doi.org/10.1016/j.foodres.2011.03.050

Omaña-Molina, M., González-Robles, A., Iliana Salazar-Villatoro, L., Lorenzo-Morales, J., Cristóbal-Ramos, A. R., Hernández-Ramírez, V. I., et al. (2013). Reevaluating the role of Acanthamoeba proteases in tissue invasion: Observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells. *BioMed Research International, 2013*, 461329.

Oumer, O. J. (2017). Pectinase: Substrate, production and their biotechnological applications. *International Journal of Environment, Agriculture and Biotechnology, 2*, 1007–1014.

Oumer, O. J., & Abate, D. (2018). Screening and molecular identification of pectinase producing microbes from coffee pulp. *BioMed Research International*. https://doi.org/10.1155/2018/2961767

Pagán, A., Ibarz, A., & Pagán, J. (2005). Kinetics of the digestion products and effect of temperature on the enzymatic peeling process of oranges. *Journal of Food Engineering, 71*(4), 361–365. https://doi.org/10.1016/j.jfoodeng.2004.10.039

Palma-Fernandez, E. R. D., Gomes, E., & Silva, R. D. (2002). Purification and characterization of two β-Glucosidases from the thermophilic Fungus Thermoascus aurantiacus. *Folia Microbiologica, 47*(6), 685–690.

Pan, X., Tu, T., Wang, L., Luo, H., Ma, R., Shi, P., Meng, K., & Yao, B. (2014). A novel low-temperature-active pectin methylesterase from *Penicillium chrysogenum* F46 with high efficiency in fruit firming. *Food Chemistry, 162*, 229–234. https://doi.org/10.1016/j.foodchem.2014.04.069

Perera, N., Gamage, T. V., Wakeling, L., Gamlath, G. G. S., & Versteeg, C. (2010). Colour and texture of apples high pressure processed in pineapple juice. *Innovative Food Science and Emerging Technologies, 11*, 39–46.

Placido, D., Fernandes, C. G., Isidro, A., Carrondo, M. A., Henriques, A. O., & Archer, M. (2008). Auto-induction and purification of Bacillus subtilis transglutaminase (Tgl) and its preliminary crystallographic characterization. *Protein Expression and Purification, 59*, 1–8.

Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., & Amorim, D. S. (2005). Xylanases from fungi: Properties and industrial applications. *Applied Microbiology and Biotechnology, 67*(5), 577–591. https://doi.org/10.1007/s00253-005-1904-7

Poojary, M. M., Roohinejad, S., Koubaa, M., Barba, F. J., Passamonti, P., Režek Jambrak, A., Oey, I., & Greiner, R. (2017). Impact of pulsed electric fields on enzymes. In D. Miklavčič (Ed.), *Handbook of electroporation* (pp. 2369–2389). Springer International Publishing. https://doi.org/10.1007/978-3-319-32886-7_173

Prakash, S., Singhal, R., & Kulkarni, P. (2001). Enzymic peeling of Indian grapefruit (Citrus paradisi). *Journal of the Science of Food and Agriculture, 81*(15), 1440–1442. https://doi.org/10.1002/jsfa.969

Pretel, M. T., Botella, M. Á., Amorós, A., Serrano, M., Egea, I., & Romojaro, F. (2007a). Obtaining fruit segments from a traditional orange variety (*Citrus sinensis* (L.) Osbeck cv. Sangrina) by enzymatic peeling. *European Food Research and Technology, 225*(5–6), 783–788. https://doi.org/10.1007/s00217-006-0482-y

Pretel, M. T., Botella, M. A., Amorós, A., Zapata, P. J., & Serrano, M. (2007b). Optimization of vacuum infusion and incubation time for enzymatic peeling of 'Thomson' and 'Mollar' oranges. *LWT—Food Science and Technology, 40*(1), 12–20. https://doi.org/10.1016/j.lwt.2005.07.021

Pretel, M. T., Sánchez-Bel, P., Egea, I., & Romojaro, F. (2008). Enzymatic peeling of citrus fruits: Factors affecting degradation of the albedo. In T. da Silva & J. A. Islework (Eds.), *Tree and forestry science and biotechnology* (Vol. 2, pp. 52–59). Global Science Books.

Pretel, M. T., Sánchez-Bel, P., Egea, I., & Romojaro, F. (2010). Enzymatic peeling of citrus fruits. In A. Bayindirli (Ed.), *Enzymatic processing of fruits and vegetables: Chemistry and engineering applications* (pp. 145–174). CRC Press, Taylor and Francis Group.

Qudsieh, H. Y. M., Yusof, S., Osman, A., & Rahman, R. A. (2002). Effect of maturity on chlorophyll, tannin, color and polyphenol oxidase (PPO) activity of sugarcane juice (Saccharum officinarum var. Yellow cane). *Journal of Agricultural and Food Chemistry, 50*, 1615–1618.

Rawson, A., Patras, A., Tiwari, B. K., Noci, F., Koutchma, T., & Brunton, N. (2011). Effect of thermal and non thermal processing technologies on the bioactive content of exotic fruits and their products: Review of recent advances. *Food Research International, 44*(7), 1875–1887. https://doi.org/10.1016/j.foodres.2011.02.053

Revilla, I., & Gonzalez-San Jose, M. L. (2003). Addition of pectolytic enzymes: An enological practice which improves the chromaticity and stability of red wines. *International Journal of Food Science and Technology, 38*(1), 29–36. https://doi.org/10.1046/j.1365-2621.2003.00628.x

Ribeiro, D. S., Henrique, S. M. B., Oliveira, L. S., Macedo, G. A., & Fleuri, L. F. (2010). Enzymes in juice processing: A review. *International Journal of Food Science and Technology, 45*(4), 635–641. https://doi.org/10.1111/j.1365-2621.2010.02177.x

Rojas, M. L., Miano, A. C., & Augusto, P. E. D. (2017). Ultrasound processing of fruit and vegetable juices. In *Ultrasound: Advances for food processing and preservation* (pp. 181–199). Elsevier. https://doi.org/10.1016/B978-0-12-804581-7.00007-5

Rolff, M., Schottenheim, J., Decker, H., & Tuczek, F. (2011). Copper–O2 reactivity of tyrosinase models towards external monophenolic substrates: Molecular mechanism and comparison with the enzyme. *Chemical Society Reviews, 40*(7), 4077. https://doi.org/10.1039/c0cs00202j

Romero-Cascales, I., Fernández-Fernández, J. I., Ros-García, J. M., López-Roca, J. M., & Gómez-Plaza, E. (2008). Characterisation of the main enzymatic activities present in six commercial macerating enzymes and their effects on extracting colour during winemaking of Monastrell grapes. *International Journal of Food Science and Technology, 43*(7), 1295–1305. https://doi.org/10.1111/j.1365-2621.2007.01608.x

Rouhana, A., & Mannheim, C. H. (1994). Optimization of enzymatic peeling of grapefruit. *Lebensmittel Wissenschaft und Technology, 27*, 103–107.

Saeeduddin, M., Abid, M., Jabbar, S., Wu, T., Hashim, M. M., Awad, F. N., Hu, B., Lei, S., & Zeng, X. (2015). Quality assessment of pear juice under ultrasound and commercial pasteurization processing conditions. *LWT—Food Science and Technology, 64*(1), 452–458. https://doi.org/10.1016/j.lwt.2015.05.005

Schwab, W., Davidovich-Rikanati, R., & Lewinsohn, E. (2008). Biosynthesis of plant-derived flavor compounds. *The Plant Journal, 54*, 712–732.

Segovia, F. J., Luengo, E., Corral-Pérez, J. J., Raso, J., & Almajano, M. P. (2015). Improvements in the aqueous extraction of polyphenols from borage (Borago officinalis L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. *Industrial Crops and Products, 65*, 390–396. https://doi.org/10.1016/j.indcrop.2014.11.010

Sessa, D. J., & Anderson, R. L. (1981). Soybean peroxidases: Purification and some properties. *Journal of Agricultural and Food Chemistry, 29*, 960.

Sharma, H. P., Patel, H., & Sugandha. (2017). Enzymatic added extraction and clarification of fruit juices—A review. *Critical Reviews in Food Science and Nutrition, 57*(6), 1215–1227. https://doi.org/10.1080/10408398.2014.977434

Sheu, S. C., & Chen, A. O. (1991). Lipoxygenase as blanching index for frozen vegetable soybeans. *Journal of Food Science, 56*(2), 448–451.

Sila, D. N., Duvetter, T., De Roeck, A., Verlent, I., Smout, C., Moates, G. K., Hills, B. P., Waldron, K. K., Hendrickx, M., & Van Loey, A. (2008). Texture changes of processed fruits and vegetables: Potential use of high-pressure processing. *Trends in Food Science and Technology, 19*(6), 309–319. https://doi.org/10.1016/j.tifs.2007.12.007

Singh, N., Singh, J., Kaur, L., Sodhi, N. S., & Gill, B. S. (2003). Morphological, thermal and rheological properties of starches from different botanical sources. *Food Chemistry, 81*(2), 291–231.

Singhania, R. R., Saini, J. K., Saini, R., Adsul, M., Mathur, A., Gupta, R., & Tuli, D. K. (2010). Bioethanol production from wheat straw via enzymatic route employing *Penicillium janthinellum* cellulases. *Bioresource Technology, 169*, 490–495.

Siwach, R., & Kumar, M. (2012). Comparative study of thermosonication and thermal treatments on pectin methyl esterase inactivation in mosambi juice. *Journal of Dairying, Foods and Home Sciences, 31*, 290–296.

Somavat, R., Mohamed, H. M. H., & Sastry, S. K. (2013). Inactivation kinetics of *Bacillus coagulans* spores under ohmic and conventional heating. *LWT—Food Science and Technology, 54*, 194–198.

Sundarram, A., & Murthy, T. P. K. (2014). α-Amylase production and applications: A review. *Journal of Applied and Environmental Microbiology, 2*(4), 166–175.

Suutarinen, M., Mustranta, A., Autio, K., Salmenkallio-Marttila, M., Ahvenainen, R., & Buchert, J. (2003). The potential of enzymatic peeling of vegetables. *Journal of the Science of Food and Agriculture, 83*(15), 1556–1564. https://doi.org/10.1002/jsfa.1579

Swami Hulle, N. R., & Rao, P. S. (2016). Effect of high pressure and thermal processing on quality changes of Aloe vera-litchi mixed beverage (ALMB) during storage. *Journal of Food Science and Technology, 53*, 359–369.

Szymańska-Chargot, M., Chylińska, M., Gdula, K., Koziol, A., & Zdunel, A. (2017). Isolation and characterisation of cellulose from different fruit and vegetable pomaces. *Polymers, 9*(10), 495.

Terefe, N. S., Buckow, R., & Versteeg, C. (2014). Quality related enzymes in plant based products: Effects of novel food processing technologies, Part 1: High-pressure processing. *Critical Reviews in Food Science and Nutrition, 54*, 24–63.

Terefe, N. S., Tepper, P., Ullman, A., Knoerzer, K., & Juliano, P. (2016). High pressure thermal processing of pears: Effect on endogenous enzyme activity and related quality attributes. *Innovative Food Science and Emerging Technologies, 33*, 56–66. https://doi.org/10.1016/j.ifset.2015.12.001

Terrasan, C. R. F., Temer, B., Duarte, M. C. T., & Carmona, E. C. (2010). Production of xylanolytic enzymes by Penicillium janczewskii. *Bioresource Technology, 101*, 4139–4143.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin—A review. *Critical Reviews in Food Science and Nutrition, 37*(1), 47–73.

Tiwari, B. K., O'Donnell, C. P., & Cullen, P. J. (2009). Effect of non-thermal processing technologies on the anthocyanin content of fruit juices. *Trends in Food Science and Technology, 20*(3–4), 137–145. https://doi.org/10.1016/j.tifs.2009.01.058

Tochi, B. N., Wang, Z., Xu, S. Y., & Zhang, W. (2009). Effect of stem bromelain on the browning of apple juice. *American Journal of Food Technology, 4*(4), 146–153.

Toker, İ., & Bayındırlı, A. (2003). Enzymatic peeling of apricots, nectarines and peaches. *LWT—Food Science and Technology, 36*(2), 215–221. https://doi.org/10.1016/S0023-6438(02)00203-7

Tourino, M. C. C., Chitarra, A. B., & Gavilanes, M. C. (1993). Injúria mecânica em tecidos de frutos de pessegueiros (Prununs persica [L.] Batsch): Mecanismos de cura. *Boletim da Sociedade Brasileira de Ciência e Tecnologia de Alimentos, 27*(2), 69–78.

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: Applications of lignocellulolytic enzymes. *Journal of Food Science, 82*(3), 585–593. https://doi.org/10.1111/1750-3841.13636

Uneojo, M., & Pastore, G. M. (2007). Pectinases: Aplicações industriais e perspectivas. *Química Nova, 30*(20), 1–14.

Valderrama, P., Marangoni, F., & Clemente, E. (2001). Efeito do tratamento térmico sobre a atividade de peroxidase (POD) e polifenoloxidase (PPO) em maçã (Mallus comunis). *Ciência e Tecnologia de Alimentos, 21*, 321–325.

van der Maarel, M. J. E., van der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. *Journal of Biotechnology, 94*, 137–155.

Villena, M. A., Iranzo, J. F. Ú., & Pérez, A. I. B. (2007). β-Glucosidase activity in wine yeasts: Application in enology. *Enzyme and Microbial Technology, 40*(3), 420–425. https://doi.org/10.1016/j.enzmictec.2006.07.013

Vincken, J. P., Beldman, G., & Voragen, A. G. J. (1994). The effect of xyloglucans on the degradation of cell wall embdedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. *Plant Physiology, 104*(1), 99–107.

Wang, K., Jiang, X., Xu, F., Sun, R. C., & Baird, M. S. (2010). Influence of steam pressure on the physiochemical properties of degraded hemicelluloses obtained from steam-exploded Lespedeza stalks. *BioResources, 5*, 1717–1732.

Welti-Chanes, J., Ochoa-Velasco, C. E., & Guerrero-Beltrán, J. Á. (2009). High-pressure homogenization of orange juice to inactivate pectinmethylesterase. *Innovative Food Science and Emerging Technologies, 10*(4), 457–462. https://doi.org/10.1016/j.ifset.2009.05.012

Yang, Y., Zhu, N., Yang, J., Lin, Y., Liu, J., Wang, R., Wang, F., & Yuan, H. (2017). A novel bifunctional acetyl xylan esterase/arabinofuranosidase from *Penicillium chrysogenum* P33 enhances enzymatic hydrolysis of lignocellulose. *Microbial Cell Factories, 16*(1), 166. https://doi.org/10.1186/s12934-017-0777-7

Yoruk, R., & Marshall, M. R. (2003). Physiochemical properties and function of plant polyphenol oxidase: A review. *Journal of Food Biochemistry, 27*(5), 361–422. https://doi.org/10.1111/j.1745-4514.2003.tb00289.x

Zabetakis, I., Leclerc, D., & Kajda, P. (2000). The effects of high hydrostatic pressure on the strawberry anthocyanins. *Journal of Agriculture and Food Chemistry, 48*, 2749–2754.

Zanatta, C. L., Zotarelli, M. F., & Clemente, E. (2006). Peroxidase (POD) e Polifenoloxidase (PPO) em polpa de goiaba (Psidium guajava R.). *Ciência e Tecnologia de Alimentos, 26*(3), 705–708.

Zhang, L., Zhang, L., Yi, H., et al. (2012). Enzymatic characterization of transglutaminase from Streptomyces mobaraensis DSM 40587 in high salt and effect of enzymatic cross-linking yak milk proteins on functional properties of stirred yogurt. *Journal of Dairy Science, 95*, 3559–3568.

Zhu, F.-M., Du, B., & Li, J. (2014). Aroma enhancement and enzymolysis regulation of grape wine using β-glycosidase. *Food Science and Nutrition, 2*(2), 139–145. https://doi.org/10.1002/fsn3.84

Production of α, β, and γ-Cyclodextrin Gluconotransferase (CGTase) and Their Applications in Food Industry

4

Rizul Gautam and Shailendra Kumar Arya

Abstract

Cyclodextrins (CD) are cyclic oligosaccharides which can be produced by the enzymatic degradation of starch and have the ability to form inclusion complex (IC) with variety of molecules. Formation of inclusion complexes with cyclodextrins (CDs) is known to enhance guest solubility in aqueous medium. CD has α-(1–4) linkages forming a cone shaped structure with a hydrophilic outer surface which makes them water-soluble. The rate of hydrolysis increases in the order of α-CD < β-CD < γ-CD. Gamma cyclodextrins have been proved to retain the maximum retention properties than the three cyclodextrins. Cyclodextrins are also used to retain colours of food substances. Three common types of CDs are α-CD, β-CD, and γ-CD with 6, 7, and 8 sugar-membered ring molecules. This book chapter will discuss the preparation and properties of three types of CDs with their applications in food industry.

Keywords

α-Cyclodextrin · β-Cyclodextrin · γ-Cyclodextrin · Food industry · Applications

4.1 Introduction

Cyclodextrins (CDs) are a family of cyclic oligosaccharides that show the ability to form inclusion complex (IC) through variety of molecules (like Allyl isothiocyanate (AITC)) (Del Valle, 2004). These molecules are produced through the action of

R. Gautam · S. K. Arya (✉)
Department of Biotechnology, University Institute of Engineering Technology, Panjab University, Chandigarh, India
e-mail: skarya@pu.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_4

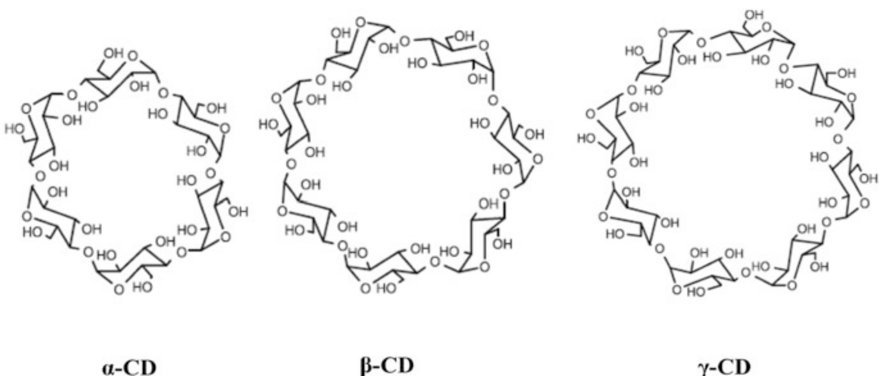


Fig. 4.1 Basic structure of alpha, beta, and gamma cyclodextrins (Radi & Eissa, 2010; Das et al., 2013; Astray et al., 2009)

cyclodextrin glycosyltransferase (CGTase) enzyme on starch or starch derivatives. This enzymatic activity sometimes results in changing the properties of many molecules like their stability, reactivity, bioavailability, and solubility (Larsen et al., 1998). On the basis of number of D-glucose units attached to CDs, they are classified as six-α-cyclodextrin, seven-β-cyclodextrin, and eight-γ-cyclodextrin (Blackwood & Bucke, 2000; Singh et al., 2002). Cyclodextrins cannot be formed if less than six glucopyranose units used because of steric hindrances (Qi & Romberger, 1988). Cyclodextrin possesses a hydrophobic central and hydrophilic outside cavity. The α-(1–4) linkages of CD help in the formation of a cone-shaped structure that results in its hydrophilicity. The central cavity of the cone is hydrophobic in nature because of the presence of carbon and oxygen atoms of the glucose residues. This arrangement of a hydrophilic exterior with a hydrophobic interior design of CDs helps cyclodextrins to make inclusion complexes or compounds with hydrophobic guest molecules. α-, β- and γ-CD are 6, 7, and 8 sugar-membered ring molecules, respectively, and are the most common CD types (Del Valle, 2004), as shown in Fig. 4.1 (Del Valle, 2004; Radi & Eissa, 2010; Das et al., 2013). Cyclodextrins have number of applications in food industry due to their ability of encapsulations of molecules and formation of inclusion complex with hydrophobic compounds (Gawande et al., 1998). Cyclodextrins are preferred due to the following reasons: they are made from renewable raw material such as starch or starch derivatives. They are relatively cheap. They are toxic-free and beneficial for human body. Applications of cyclodextrins in food industry are expressed in many different ways, like for encapsulation of flavours, used as food preservative, used as an eliminator of bitter tastes from food, used against oxidative degradations, heat- and light-induced changes, and for modification of taste and odours of foods (Astray et al., 2009).

Among all the three cyclodextrins, gamma cyclodextrin proves to be more suitable to be used in the field of pharmaceutical and food industries because of its properties like water solubility, size of internal cavity, etc. (Li et al., 2007). However,

the most marketed utilized CD is β-CD and to lesser extent α-CD. The production of gamma CD is less due to high cost and low production associated with it. The CGTases producing cyclodextrin of single type are of interest as it is difficult to isolate the CD of interest from a complex mixture of CDs. Cyclodextrins are hydrophobic molecules and their solubility increases with ethanol concentration. The spectroscopic studies imply that the confirmation of cyclodextrins in solution is similar to that in crystalline state; however, β-CD has a perfect regularity, whereas alpha and gamma are slightly distorted (Astray et al., 2009).

Cyclodextrins are useful because of following reasons: They are made from renewable material (starch). They are relatively cheap. Due to their biodegradability, they do not produce any harmful effect to the environment. They are nontoxic for human body (Duca & Boldescu, 2008). The stability of the cyclodextrins follows $\alpha > \beta > \gamma$. The cyclodextrins are as stable as sucrose in solid state and are resistant to degradation and can be stored for several years (Kurkovsergey & Loftssonthorsteinn, 2013). These are homogenous, crystalline, and non-hygroscopic substances (Stella & Rajewski, 1997). The alpha cyclodextrin has six glucopyranose rings, beta has seven, and gamma has eight glucopyranose units (Astray et al., 2009). The rate of hydrolysis accelerates in the order of α-CD < β-CD < γ-CD. Gamma cyclodextrins have been proved to retain the maximum retention properties than the three cyclodextrins. Cyclodextrins are also used to retain colours of food substances (Duca & Boldescu, 2008; Lopez-Nicolas et al., 2007a, b). The hydrophobic interior of the cavity possesses some lewis base characteristics due to the presence of high electron density.

4.2 History

First time cyclodextrin was discovered by Villiers in 1891 from starch using a strain of *Bacillus amylobacter* (Das et al., 2013; Szejtli, 1990). The following table represents the discoveries of cyclodextrins according to time passage and work done on cyclodextrins in different fields (Del Valle, 2004; Dardeer, 2014).

Year of working	Work done on cyclodextrin	References
1891	First time CDs produced from starch by digesting it with *Bacillus amylobacter* strain by scientist Villiers. Villiers also produces cyclodextrin from impure culture by using strain *Bacillus macerans*.	Singh et al. (2002)
1903	Schardinger produces two crystalline products and named it as dextrin A and dextrin B, but the strain from which they are produced are not maintained.	Eastburn and Tao (1994)
1904	Schardinger discovered a new organism that has capability of producing ethyl	Eastburn and Tao (1994)

(continued)

Year of working	Work done on cyclodextrin	References
	alcohol and acetone from sugar and starch-containing plant materials.	
1911	Schardinger produced large amount of cyclodextrin from strain *Bacillus macerans* about 25–30% and named it α-CD and β-CD.	Eastburn and Tao (1994)
1935	Schardinger discovered γ-CD. After that, Freudenberg and Cramer explained the existence of large cyclodextrin molecules.	Loftsson and Brewester (1996), Matsuda and Arima (1999), Mabuchi and Ngoa (2001)
1942	Schardinger demonstrated the structures of β-CD and α-CD using X-ray crystallography.	Buschmann and Schollmeyer (2002)
1948	γ-CD structure is determined by using X-ray crystallography. At that time, it is observed that cyclodextrins can form inclusion complexes.	Buschmann and Schollmeyer (2002)
1961	Some evidences show the existence of natural cyclodextrins like ξ, δ, ζ, and even η-cyclodextrin.	Qi and Romberger (1988), Hirose and Yamamoto (2001)
1965	A method is developed in this year for isolation of larger homologues structures of ζ-cyclodextrin and η-cyclodextrin, method named fractionation method.	Kainuma (1984)
1970	In 1970, several numbers of microorganisms are considered as a source for cyclodextrin glucanotransferases and glucanotransferase was purified and named during the exploration period. Applications of cyclodextrins in different industries started in this period.	Kainuma (1984)
1980	Development in biotechnology in the mid of 1980s, improved the purification and production of cyclodextrins and led to increases in their value for commercial uses.	Crini (2014)
1982	In 1982, first book on cyclodextrins was published, written by Szejtli.	Crini (2014)
1992	In this period, Hedges R.A. published a book on cyclodextrin under a title Cyclodextrins and Their Applications in Biotechnology.	Hedges (1992)
2003	In this period, Tomasik P. published a book on cyclodextrins, under a title Cyclodextrins Chemical and Functional Properties of Food Saccharides.	Szejtli (2004a)
2004	In this year, review articles were published on data related to cyclodextrin and its	Szejtli (2004b)

(continued)

Year of working	Work done on cyclodextrin	References
	future aspects and also on application of cyclodextrins.	
2006	Helena Dodziuk published a book on Cyclodextrins and Their complexes: Chemistry, Analytical Methods and Applications.	Kainuma (1984)
2007	Investigation of cyclodextrin glucanotransferase production using alginate-immobilized cells of alkalophilic *Bacillus* sp. in an airlift reactor.	Kunamneni et al. (2007)
2010	Biochemical characterization of α-cyclodextrin glycosyltransferase and its extracellular expression from *Paenibacillus macerans* have been published by Zhaofeng Li et al.	Zhaofeng et al. (2010)
2011	Encapsulation of ethylene gas into α-cyclodextrin and characterization of the inclusion complexes.	Ho et al. (2011)
2012	Effects of cyclodextrins on the antimicrobial activity of plant-derived essential oil compounds.	Liang et al. (2012)
2013	Optimization of reaction of γ-cyclodextrin glycosyltransferase and isoamylase for enhanced production of c-cyclodextrin.	Wang et al. (2013)
2014	Cyclodextrins in capillary electrophoresis: Recent developments and new trends.	Escuder-Gilabert et al. (2014)
2015	Experimental setup and kinetic study of ultrasonic-assisted transesterification of waste cooking oil over sulfonated carbon catalyst derived from cyclodextrin.	Maneechakr et al. (2015)
2016	Use of cyclodextrins to recover catechin and epicatechin from red grape pomace.	Lopez-Miranda et al. (2016)

4.3 Properties of Cyclodextrins

CD have hydrophobic cavity and the ability to form reversible host–guest complexes or termed as supramolecular complex via weak forces like, dipole–dipole interactions, Van der Waals interactions, and hydrogen bonding with molecules (Escuder-Gilabert et al., 2014). CDs have the ability to solubilize hydrophobic materials and thus can entrap volatile components by forming inclusion complexes with organic compounds, which results in enhancing their physical and chemical properties. They have the property of selective recognition of a chiral compound and enantio recognition of enantiomers of chiral compounds (Del Valle, 2004; Steinbrunn & Wenz, 1996). Low toxicity and immunogenicity of CDs allow for

their use as a transport system into the living systems. Also, CDs have the capacity to form pseudorotaxanes and rotaxanes through number of linear species. Rotaxanes are the compounds that consist linear and cyclic species and are linked together by non-covalent interaction (Isnin & Kaifer, 1993; Buston et al., 2001). As inclusion bodies, CDs help in increasing the stability, solubility, and bioavailability of lipophilic organic molecules, improve the stability, increase the permeability of water-insoluble compounds, and diminish the drug toxicity by manufacturing the compound effective at lesser doses (Del Valle, 2004; Kamimura et al., 2014).

4.3.1 CGTase Synthesis

Cyclodextrin glucanotransferases (EC 2.4.1.19, CGTases) using starch and its derivatives are used for the production of cyclic α-(1,4)-linked oligosaccharides commonly known as cyclodextrins (CDs) via the cyclization reaction (Tonkova, 1998). CGTase is isolated from *Bacillus* sp. and degrade the starch from raw substrate (like potato) to form cyclodextrin. It was reported that immobilization increases the activity of CGTase enzyme, as when it was immobilized in mixed gel beads, its activity increases. This fermentation process is carried out in a continuous stirred tank reactor (CSTR) and a packed bed reactor (PBR) for commercial production. Enzyme preparation is done according to production process required. Generally, CGTase is isolated from *Bacillus* sp. C26 and inoculated into medium and then incubated at 37 °C at 200 rpm for 48–50 h. Centrifugation is done for 15 min to remove the cells and insoluble material at 4000 × *g* and at 4 °C. Precipitation of enzyme is done by adding the cell-free supernatant with ammonium sulfate and keeping them for overnight in controlled conditions at 4 °C temperature and pH is maintained at 7. Product purification is done by dialysing the solution obtained after overnight incubation with molecular cut-off 8000 Da. Enzyme activity is monitored by using phenolphthalein colourimetric method, and to measure the enzyme protein concentration, folin ciocaltue method is used. It was reported that CGTases are an extracellular enzyme produced by number of microorganisms, for example, bacteria (mostly from *Bacillus* species) (Leemhuis et al., 2010).

B. circulans DF 9R was isolated from rotten potatoes (Ferrarotti et al., 1996). Culture was then transferred to medium containing cassava starch, $(NH_4)_2SO_4$, phosphate buffer, $MgSO_4$, and $FeSO_4$. Culture was grown aerobically until the absorbance at 620 nm becomes 0.3 and is then used as inoculum. The medium is then transferred to 1000 mL Erlenmeyer flask containing medium. Then the mixture is centrifuged and the supernatant obtained is used as a crude enzyme (Rosso et al., 2002; Kunamneni et al., 2007). The microorganism producing β-CGTase is alkalophilic *Bacillus* species (*Bacillus* sp. AK11) that has the capacity to grow in high alkaline condition when cultivated in air lift reactor. Alkalophilic bacilli are more trending and more commonly in use due to broad range specificities of pH and temperature (Kunamneni et al., 2007; Park et al., 1999; Vassileva et al., 2005). Microorganism is then transferred to growth medium containing starch, peptone, KH_2PO_4, and $MgSO_4$ and medium is adjusted to pH 8 using sodium carbonate and

maintained at a temperature of 37 °C. Cells were immobilized using sodium alginate method (Kunamneni et al., 2007; Higuti et al., 2004). The alginate beads were loaded into the reactor containing the production medium and allowed to stay for 24 h to activate cells. The broth obtained was drained and further replaced with production medium which contained different molar concentrations of the added ingredients. The production medium is then constantly added so that there is continuous mixing of the solution and overflow is regulated by peristaltic pump and finally its activity can then be measured spectrometrically. It was observed that with the increase in the air flow rate, the production of CGTase increased. The concentration of carbon and nitrogen needs to be maintained for effective CGTase production using immobilized beads (Kunamneni et al., 2007).

However, it was observed that the average volume productivity increased during immobilization as compared to free cells (Kunamneni et al., 2007). The concentration of α-CD production is measured by observing the decrease in absorbance at 507 nm. The decrease in absorbance is due to formation of methyl orange α-CD complex (Kunamneni et al., 2007; Higuti et al., 2004).

One unit of CGTase is defined as the amount of enzyme catalysing the production of 1 μmol of β-CD per minute under the reaction conditions. The cyclising activity of CGTase was tested by phenolphthalein method. In this test, the production of β-cyclodextrin is measured on the basis of its ability to form inclusion complex with this dye which is then measured by taking absorbance at 550 nm (Kunamneni et al., 2007; Goel & Nene, 1995). Phenolphthalein is diluted in ethanol and the pH is adjusted to 11 using Na_2CO_3. The activity assay consisted of substrate solution containing 1% (w/v) maltodextrin in tris-HCl buffer at 50 °C, taking water as a blank.

The concentration of γ-CD was measured at 630 nm due to formation of inclusion complex with bromcrescol green (Goel & Nene, 1995; Natalia et al., 2007) taking water as a blank. In this case, the reaction mixture consists of 1% (w/v) soluble starch in gly-NaOH buffer, pH 10, and enzyme solution diluted by buffer incubated at 40 °C for 10 min. Then 0.1 N HCl was used to stop the reaction. Finally, the citric acid–citrate sodium buffer (pH 4.2) were added to the reaction mixture, and then the absorbance is taken at 630 nm.

Methods for CD-complex formation: The forces that are responsible for interaction of these inclusion complexes include Van der Waals forces, hydrophobic interactions and hydrogen bonds (as shown in Fig. 4.2). These inclusion complexes change the chemical and physical properties of the molecule. The energy of Van der Waals is proportional to molecular polarizability and molecular refraction. The stability of the complex increases with the increase in the electron donor ability of the substituents (Astray et al., 2009).

Cyclodextrins can also form complexes with the gases such as CH_4, C_2H_6, C_3H_8, Cl_2, Xe, etc. Alpha cyclodextrin due to its smaller diameter (470–530 pm) can form stable complexes with guest molecules having five carbon atoms or less. However, ethylene gas is more suitable for formation of complexes with alpha CDs. The encapsulation of ethylene into alpha cyclodextrin was done by dissolving alpha cyclodextrin powder in water at 25 °C. After the powder is completely dissolved,

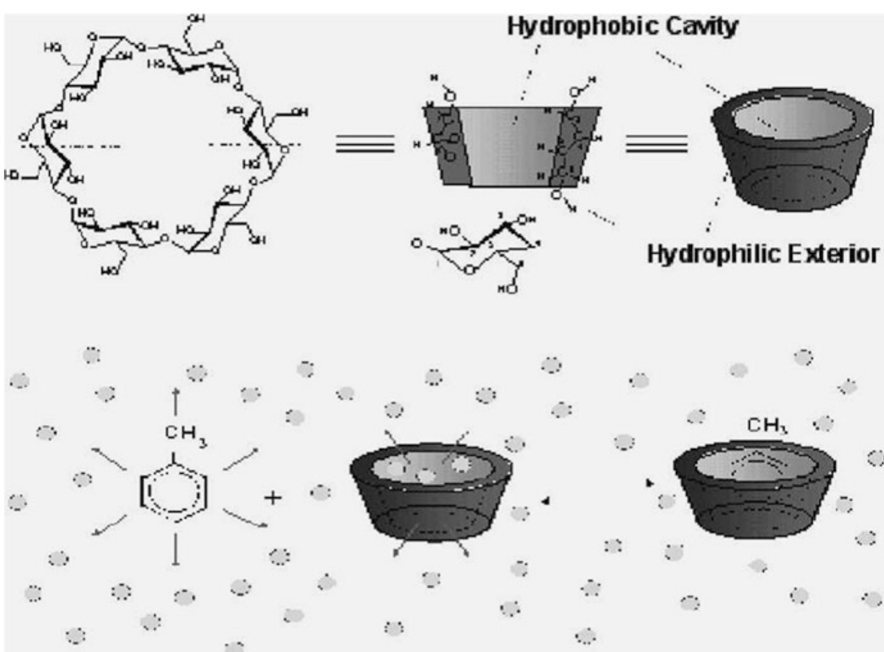


Fig. 4.2 Cyclodextrins structure and inclusion complex formation (Schmid, 1989; Gomes et al., 2014; Garnero et al., 2010)

solution is then stirred for complete 5 min. Saturated alpha cyclodextrin obtained is added to plastic container and ethylene gas was then pressurized at 0.2, 0.5, 1, and 1.5 Pa at 25 °C. Afterwards, crystal precipitates formed are collected by vacuum filtration and then analysed for ethylene gas concentrations and further characterized for their physiochemical properties (Ho et al., 2011).

CDs have hydrophobic cavity. CDs have the ability to form host–guest complexes via weak forces, like Van der waal interactions, dipole–dipole interactions, and hydrogen bonding with molecules (Szejtli, 1988). CDs can solubilize hydrophobic materials and thus entrap volatile components by forming inclusion complex with organic compounds, resulting in enhancing their chemical and physical properties. CGTase production at large scale by using *Bacillus* sp. depends upon the kinetic model of cyclodextrin-glucanotransferase (Burhan et al., 2005). Advantages of CD to form IC are: higher thermal stability, high solubility, bioavailability of hydrophobic guests, control volatility, masking off unpleasant odours, and controlling release of drugs and flavours.

4.3.2 CGTase Sources

This table represents the various sources of CGTase from different spices, their mode of production, and their optimum parameters like temperature and pH.

Sources of CGTase	Production	Optimum temperature and pH	References
Bacillus macerans IFO 3490	*Bacillus macerans* IFO 3490 mainly produces α-CGTase. Fermentation medium is soluble starch, corn steep liquor.	Optimum temperature is 55 °C and pH 5.2–5.7.	Fujiwara et al. (1974), Kitahata et al. (1974), Rimphanitchayakit et al. (2005)
Bacillus circulance E 192	*Bacillus circulance* E 192 produces mainly β-CGTase. Agar-immobilized cells are used for high productivity of CGTase. *B. circulance* requires alkaline pH for an efficient CGTase formation.	Optimum temperature 60 °C and pH 5.5–5.8.	Charoenlap et al. (2004), Vassileva et al. (2005)
Paenibacillus	Main product is β-CD.	Optimum temperature is 50 °C and pH is 6.0–8.0.	Larsen et al. (1998)
Klebsiella pneumonia	The CGTase from *Klebsiella pneumonia*. Main product is α-CD.	It can be preferred at pH 6–8, temperatures of 40–50 °C.	Gawande and Patkar (2001)
Bacillus stearothermophillus	CGTase from *Bacillus stearothermophillus* ET1 is a potential antistaling enzyme with cyclodextrin (CD)-producing activity. Main product is α- and β-CD.	Its optimum activity at 80 °C temperature.	Rahman et al. (2004)
Bacillus lehensis	*Bacillus lehensis* is isolated from cassava starch wastewater. Main product is β-CD. Its CGTase activity is 18.9 μm/L	Optimum temperature is 55 °C and pH 8.0.	Blanco et al. (2014)
Micrococcus	*Micrococcus* variant M 849 produces α and β-CD as main products.	Its optimum activity temperature is 55–65 °C and pH is 5.0–9.0.	Kim et al. (1997)
Thermoanaerobacter sp.	*Thermoanaerobacter* sp. ATCC 53627 mainly used for the	Its optimum activity temperature is 90–95 °C.	Starnes et al. (1992)

(continued)

Sources of CGTase	Production	Optimum temperature and pH	References
	production of β-CGTase.		
Alkalophilic *Bacillus* sp.	Alkalophilic *Bacillus* sp. 38-2 mainly produces β-CD.	Optimum temperature is 45 °C.	Sian et al. (2005)
Bacillus amyloliquefaciens	CGTase is synthesized by *B. amyloliquefaciens* by immobilized method in batch and continuous cultures.	Growth medium is maintained at pH 6.5 and microorganism is maintained at 4 °C on potato dextrose agar slants.	Andel-Naby et al. (2000)

4.3.3 Methods for Production of Inclusion Complexes

In this table, methods for CDs complexes or inclusion complexes formation have been explained and the manufacturing of CDs.

Methods for CD-complex formations	Process	References
Precipitation method	Precipitation method is most widely used for inclusion complex production. According to Sapkal et al., by using co-precipitation method, a poor aqueous solubility guest molecule is combined with β-cyclodextrin to form inclusion complex. In this method, drug and CD are dissolved in water and solvent (like acetone) is heated at 75 °C. Stirring is done for 1 h at 75 °C to obtain concentrated, viscous, and translucent liquid and then cooled at room temperature. Precipitates are filtered or separated than dried and stored to get solid inclusion complex.	Das et al. (2013), Sapkal et al. (2007)
Kneading method	Conventional kneaders like low and high shear mixers are used to prepare CD paste, paste is prepared with small amount of water in which guest molecule is added (without a solvent) in a small amount of ethanol, and then after grinding, solvent is evaporated and powdered form cyclodextrin-inclusion formed.	Das et al. (2013), Miller et al. (2007)
Spray drying	In this method, first cyclodextrin is dissolved in solution that is already alkalinized with 25% aqueous ammonia, then guest molecule is dissolved in 96% ethanol alcohol solution in 100 mL, both solutions are then mixed, and finally sonicated. The l solution obtained is then spray-dried to get inclusion complexes; this is done at	Das et al. (2013), Arias et al. (2000)

(continued)

Methods for CD-complex formations	Process	References
	equilibrium state which is attained by removing the solvent by spray drying.	
Neutralization method	The guest molecule is dissolved in basic or acidic aqueous cyclodextrin solution. Cyclodextrin and drug are dissolved separately in 0.1 N NaOH solution, mixed and stirred for half an hour; pH is maintained by 0.1 N HCl at 7.5; at this pH, complexes precipitate and then residues are separated and washed until free from chlorine and then dried at 250 °C for 24 h	Das et al. (2013), Choi et al. (2001)

4.3.4 Production Methods

(a) Batch process: Generally CDs are produced through batch process which is quite simple and easy to control process. But there are several limitations of this method, like long operation hours, high labour cost, and high enzyme concentration requirement that leads to switching to other production method (Sakinah et al., 2014; Lia et al., 2014).

(b) Enzymatic membrane reactor (EMR): EMR is basically a stirred tank reactor combined with a separation membrane that recirculates the reaction mixture through a membrane module placed outside the enzymatic reactor. As compared to batch process, this is a continuous process method and is more practical and economical. Also, reusability of the enzyme results in high yield productions of cyclodextrins as compared to batch process (Lia et al., 2014). In this process, product (CD) is separated from the enzyme and substrate through a semi-permeable membrane, which is composed of an ultra-thin separation layer widely used in enzyme separation. During the separation process, CDs penetrate through the membrane and the enzymes get retained by the membrane within the reaction reactor (Bodalo et al., 2001).

The working principle is such that the pressure difference across the membrane, also called the transmembrane pressure (TMP), impels the product through the membrane, while the unreacted substrate and free enzyme are recirculated to the enzymatic reactor (Sakinah et al., 2009). Advantages of enzymatic membrane reactor over batch reactor are its high production flow rates; reductions in cost, energy, and waste products by the recycling practice; easy reactor operation and control; and the high yields of pure material (Katzir, 1993). But the main limitation of this method is fouling of membrane as the substrate gets deposited onto the membrane surface and enzyme precipitated within the membrane pores (Hughes & Field, 2006; Balakrishnan & Dua, 2000; Yuan & Zydney, 1999; Bansal et al., 2006). However, this problem can be overcome by employing a high cross-flow velocity (CFV), but this condition requires large amount of

energy and sometimes reduces the enzyme activity (Sakinah et al., 2014; Gugliemi et al., 2007; Priyananda & Chen, 2006).

4.4 Alpha Cyclodextrin Production

α-Cyclodextrin containing six 1,4-linked D-glucose units makes it cyclic polysaccharide, which differentiates it from other two, β (seven) and γ (eight)-cyclodextrins. It is commercially produced from starch (used as a substrate) by an enzymatic production process. α-Cyclodextrin has variety of application in food industry due its small internal cavity and high resistance to its enzymatic hydrolysis. α-Cyclodextrin is less important than other CDs because of its low production yield and high cost. α-Cyclodextrin is generally used as a dietary fibre food. α-Cyclodextrins are used in food industry due to following reasons: α-CDs has both nutritional and technological properties (Lia et al., 2014). It acts as a carrier for natural flavour, colours, and vitamins, and as a stabilizer of oil in water emulsions. It is also used as a solubilizer of lipids.

A number of applications for α-cyclodextrins have been published in a variety of papers. Some of the important applications are: α-cyclodextrin have been used in bakery products: like biscuits, rolls, and cakes; snack food: like salty snacks, etc.; dairy desserts: such as yoghurt and dessert mixes; beverages and powders: like coffee whitener, vegetable and fruit juice, soy drinks, and soft drinks (diet); grain-based foods: like pasta, maggie, macaronis, noodles, and instant rice dishes; and so many other food items like chewing gum, sauces, fat, and oil products like mayonnaise and dressing. So these all are the proposed applications of cyclodextrins in food products by some users in particular papers (Lia et al., 2014).

4.4.1 Production

CDs' production can be divided into four stages as follows:

1. Production of CGTase in the reaction medium from the cultivation of the microorganism.
2. Extracting the enzyme through separation, concentration, and purification method.
3. Converting pre-hydrolysed starch into cyclic and acyclic dextrin with the action of enzyme.
4. Separation, purification, and crystallization of CDs (Katzir, 1993; Moriwaki et al., 2014; Astray et al., 2009).

Cyclodextrins are produced from starch or their derivatives because of enzymatic conversion that is catalysed by cyclodextrin glycosyltransferase (CGTase) (Rios et al., 2004). In simple words, cyclodextrins are produced by cyclodextrin glucosyltransferase (CGTase) through cyclization reaction of gelatinized starch

(Rakmai et al., 2015; Iyer et al., 2003). The result is a mixture of α-, β-, and γ-cyclodextrins, containing trace amounts of cyclodextrins (Sakinah et al., 2014).

1. α-Cyclodextrin: It has small internal cavity and high resistance to enzymatic hydrolysis, which make it suitable for application in the food industry. But because of its low production yield and high price, its market share is currently smaller than that of β-cyclodextrin. Thus, the production of α-cyclodextrin can be improved by modifying the properties of CGTases.

4.4.2 Properties of α-Cyclodextrin

α-CD has the lowest hydrogen bond strengths between the 3-OH and 2-OH groups around the wider rim. Diameter of internal cavity is 4.7–5.3 Å and the cavity volume is about 66% or 41% of that of β- or γ-cyclodextrins, respectively (Li et al., 2007). It typically forms inclusion complexes with benzene derivatives because of Van der Waals interactions. α-Cyclodextrin has modest solubility in water which means it is almost eight times greater than that of β-cyclodextrin, but 1.6 times lower than that of γ-cyclodextrin at 25 °C. Also, it is more resistant to hydrolysis in acid solutions. It was observed that even after 3 h of incubation period in extremely acidic conditions (pH = 2.4) at 100 °C, no breakdown of α-cyclodextrin is recognized (Jodai et al., 1984; Saha & Zeikus, 1992). But α-cyclodextrin can't be hydrolysed by human salivary and pancreatic amylases for its commercial production (Kondo et al., 1990). α-CGTases from *Bacillus macerans* or *Paenibacillus macerans* are most commonly used. It is also effective at solubilizing free fatty acids (Qi & Zimmermann, 2005).

4.4.3 Enzymatic Production of α-Cyclodextrin

There are two types of processes that result in the production of cyclodextrins:

Non-solvent process: In this process, cyclodextrin mixture is produced without the addition of any complexing agents and the mixture produced can be further separated by chromatographic procedures.

Solvent process: This process requires an organic complexing agent that helps in selecting one type of cyclodextrin and thus directs the enzymatic reaction to produce the cyclodextrin of interest. This method is commonly used for the production of α-cyclodextrin on industrial scale as shown below in the Fig. 4.3 (Flaschel et al., 1984; Rendleman, 1997).

4.4.4 Applications

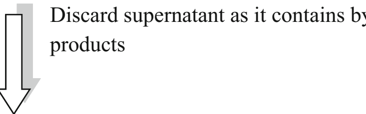
Cyclodextrins are used as browning inhibitors in different fruit juices. The oxidation of volatile precursors present in freshly squeezed pear juices can be prevented by

20-30% Gelatinized and liquefied starch (using α-amylase)

Add α-CGTase (under controlled conditions)
Add 1-Decanol (to form inclusion complex)

Formation of α-cyclodextrin/1-decanol complex by conversion of starch

Separation of complex by centrifugation

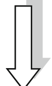


Re- suspension and re- precipitation of recovered complex in water

Centrifugation (to recover precipitate) + steam distillation (to remove 1-decanol)

Crystallization of α- cyclodextrin by concentration and cooling

Filtration and drying of crystals



White powder α-cyclodextrin with at least 98% purity

Fig. 4.3 Flow chart is showing the production of α-CD (Lia et al., 2014; Rendleman, 1997)

adding α-cyclodextrin (90 mM). It is one of the most suitable agents for encapsulating flavours that are encapsulated in powder form by spray drying with α-cyclodextrin (Shiga et al., 2004). α-Cyclodextrin is also a dietary fibre as it forms complex with triglyceride at a ratio 1:1 that is typical for dietary fibres and form a stable complex with dietary fat (Ferrari et al., 2013; Threapleton et al., 2013; Zhang et al., 2013a, b). It reduces the absorption and bioavailability of dietary fat, which makes it practical as a weight loss supplement (Suzuki & Sato, 1985). α-Cyclodextrin is effective in reducing and/or maintaining body weight with

increasing energy intake for obese patients with type 2 diabetes (Gallaher et al., 2007; Grunberger et al., 2007). It is effective in improving metabolic syndrome by reducing serum triglyceride and leptin levels and increasing insulin sensitivity and faecal fat excretion in rats. α-Cyclodextrin may also be useful as an ingredient for reducing the glycaemic impact of foods (Penninga et al., 1995).

4.4.5 Alternative Method for the Production of α-Cyclodextrin

Alpha CGTase from *Paenibacillus macerans* is most commonly used for the production of alpha cyclodextrin. However, the production of enzyme can be increased by expressing it in *E. coli* which can bring a benefit in reducing cost of alpha cyclodextrin. Alpha CGTases produced in *E. coli* are accumulated in the cytosol in the form of inactive inclusion bodies. In this case, alpha CGTase from *P. macerans* JFB05-01 is expressed extracellularly in *E. coli*. The recombinant enzyme is then purified and characterized with respect to cyclization activity, including optimum conditions effect of metal ions, etc.

4.4.6 Steps for the Production of Alpha Cyclodextrin

The plasmid pET20b is used for the production of alpha cyclodextrin. At 25 °C, very little recombinant alpha CGTase was secreted into the medium. But after 90 h of incubation period, the production rate increased rapidly and the cyclization activity of alpha CGTase also increased which was 42 times higher than the parent strain. A single colony of *E. coli* BL21cells harbouring plasmid CGT/pET20B was inoculated into LB medium containing ampicillin. For inducing the expression of α-CGTase, different amounts of isopropyl 1-thio-b-D-galactopyranoside (IPTG) were added. The recombinant alpha CGTase contains a histidine sequence on a C-terminal domain which can be separated by metal binding affinity. To overcome the heterogeneity, the recombinant enzyme is purified by chromatographic separation techniques which include Q-sepharose and phenyl-superose chromatography. The purified protein obtained is then separated by SDS page. From the SDS PAGE, it was found that alpha CGTase migrated as a single band with molecular mass of approximately 72 kDa. The optimum temperature of alpha CGTase recombinant enzyme was 45° which was below that of the native enzyme which is 50 °C. The recombinant and native CGTase both showed the best cyclization activity at pH 5.5 at a pH value; above or below this, the cyclization activity decreases. Enzyme is stable in Na_2HPO_4/NaH_2PO_4 at the same pH. The cyclization activity of alpha CGTase is inhibited completely by Hg^{2+} and slightly by Fe^{2+} or Co^{2+}. The addition of 2 mM Ca^{2+}, 2 mM Ba^{2+}, or 0.1 mM Zn^{2+} activated the cyclization activity considerably. It is also observed that the growth medium has a great impact on the secretion of proteins in *E. coli*. However, TB medium was considered the best for extracellular production of alpha CGTase as TB medium has beneficial effect on cell growth because of its relative high buffering capacity. Also, the low IPTG

concentration (0.01 mM) favoured the extracellular enzyme production. The extracellular productivity of the recombinant enzyme was 40 fold higher than the parent enzyme *P. macerans* JFB05-01. The metal chelator like EDTA doesn't show any effect on the activity of α-CGTase enzyme even at relatively high concentration (10 mM), which indicates that a metal cofactor was not required for the function of the enzyme. Furthermore, α-CGTase could be activated in the presence of Ca^{2+}, which was probably because of the binding of Ca^{2+} with its binding sites which are located at the active site of the protein molecule, resulting in stabilizing the substrate binding cleft of CGTase (Eastburn & Tao, 1994). Following table represents number of the sources of alpha cyclodextrin production, optimum temperature, optimum pH, and main products (Stella & Rajewski, 1997; Buschmann & Schollmeyer, 2002; Hirose & Yamamoto, 2001).

Source of α-CD production	Optimum pH	Optimum temperature (°C)	Main product	References
CGTase produced from *Klebsiella pneumoniae* M5	6–8	40–50	In this method, alpha-cyclodextrin is the main product, produced by enzymatic degradation of starch, starch used as a substrate. The yield of alpha-cyclodextrin is lower by the enzymatic method.	Flaschel et al. (1984)
CGTase produced from *Micrococcus* variant M 849	5.0–8.0	55–65	Main product is α-CDs, studied by purification and characterization of cyclodextrin glycosyltransferase from *Micrococcus* variant M 849.	Larsen et al. (1998), Yagi et al. (1980)
CGTase produced from *Klebsiella oxytoca* M5A1	5.0–7.5	–	By using this sp., CGTase produces α-CDs as a main product. Purification and characterization of CGTase are done in this article.	Larsen et al. (1998), Bender (1983)
CGTase produced from *Bacillus macerans* IFO 3490	5.2–5.7	55	In this paper, we study the production of α-CDs by using sp. *Bacillus macerans*.	Larsen et al. (1998), Fujiwara et al. (1974)

The concentration of α-CD production is measured by observing the decrease in absorbance at 507 nm due to formation of methyl orange α-CD complex.

4.5 Beta-Cyclodextrin

Beta-cyclodextrin (β-CD) has the ability to encapsulate molecules that are hydrophobic in nature which helps in improving their aqueous solubility and also reduces their volatility (Lin et al., 2016).

Production of β-CD can be achieved in both batch and continuous process. But continuous process is preferred over batch because of its advantages like greater process control, high productivity, and improvement of quality and yield. Continuous process includes the combination of both CSTR (continuous stirred tank reactor) and PFR (plug flow reactor) and is considered to be more suitable for long-term use. Also in case of industrial scale production, CSTR is preferred over batch process because of its properties like high stability and low by-product formation, but the disadvantage of using CSTR is the undesired thermal gradients in case of PBR, poor radial mixing, and low mass transfer rate (Santos et al., 2015; Rakmai & Cheirsilp, 2016; Galan et al., 2011; Kittikun et al., 2008).

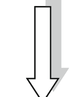
Gram-positive microorganisms *Bacillus firmus* strain and *Bacillus sphaericus* strain 41 were immobilized on a loofa sponge and are used as the direct source for the CGTase and CD production (Moriwak et al., 2014). The CGTase produced by *Bacillus* sp. C26 has been reported to have high activity for β-cyclodextrin production; immobilized CGTase is then applied for the production of β-cyclodextrin (Rakmai et al., 2015) (Fig. 4.4).

4.5.1 Alternative Method

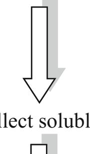
CGTase was produced by bacillus firmus isolated from brazillian soil of oat culture. The medium contained KH_2PO_4, $MgSO_4$, and Na_2CO_3 and the pH was maintained at 10.3. Then the medium is transferred to a 5 L medium and proceeded for cultivation for 5 days at 37 °C. The specific enzyme is isolated using affinity column chromatography and the protein concentration is estimated using Bradford method using bovine serum as standard, and using SDS-PAGE, molecular mass of protein is estimated. The CGTase activity is measured by replacing maltodextrin by cornstarch and potato starch. The maximum activity of enzyme is seen at a pH greater than 5 and less than 10 and at a temperature of 50 °C (Natalia et al., 2007).

Production of β-CD (10 whole till last): Immobilization of CGTases is more commonly used as it reduces the production costs and makes it feasible to be used in various food industries (Garcia-Galan et al., 2011). The enzyme immobilization has a lot of advantages as the enzyme can be reused, makes downstream processing easy, and increases thermo-stability of enzyme (Rodrigues et al., 2013; Natividade Schöffer et al., 2013). Chitosan derivative of chitin has required properties for enzyme immobilization (Muzzarelli, 1980). Chitosan has reactive amino and hydroxyl groups in its glucosamine chain (Chiou & Wu, 2004). Chitosan also exhibits mechanical stability, rigidity, and biocompatibility which permit its use in food industries (Krajewska, 2004). The following table represents the sources of β-cyclodextrins from various papers and its optimum pH, temperature, and fermentation methods.

Immobilized CGTase at 10 U/g-starch + 20 mL starch solution
(In a batch stirred-tank reactor)

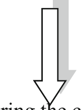


Agitation at 300 rpm using magnetic stirrer.



Collect soluble starch

Add phosphate buffer at varying concentrations of 1–8% (w/v) of pH 7.0



Incubation process (for measuring the concentration of β-cyclodextrin sample is collected after every 2 hour)

High yield of β-cyclodextrin production (8.2 g/L)

Fig. 4.4 Flow chart showing production of β-cyclodextrin using entrapped CGTase (Rakmai et al., 2015; Muria et al., 2011)

Source of β-CD production	Optimum pH	Optimum temperature	Main product and fermentation medium	References
CGTase produced from *Bacillus circulans* (ATCC 21783)	9.8–10	40 °C	In this article, cyclodextrin glucanotransferase is produced by immobilized and free cells of *Bacillus circulans* ATCC 21783 in various types of bioreactors. Main product is β-cyclodextrin.	Vassileva et al. (2007)
CGTase produced from *Bacillus* sp. (Alkalophilic bacteria)	6–7	40–90 °C	*Bacillus* sp. G1 is isolated from soil. Inoculum for *Bacillus* sp. G1 contains 1% (w/v) tapioca starch,	Mora et al. (2012)

(continued)

Source of β-CD production	Optimum pH	Optimum temperature	Main product and fermentation medium	References
			0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4$, and 1% (w/v) Na_2CO_3. Its main product is β-cyclodextrin.	
CGTase produced from *Bacillus firmus* strain 37	8.0	Different temperatures 37, 50, 60, and 70 °C at different substrate concentrates.	*B. firmus* strain 37, isolated from the soil. Composition of culture medium (%, w/v) is such that it contains 1.0 soluble starch, 0.5 polypeptone, 0.5 yeast extract, 0.1 K_2HPO_4, 0.02 $MgSO_4{\cdot}7H_2O$, 0.01 Congo red dye, 1.0 Na_2CO_3, and 1.5 agar. The plates were incubated at 37 °C for 48 h. Main product is β-CD.	Mazzer et al. (2008)
CGTase partially purified from *Bacillus circulans* (TISTR 907) by ammonium sulfate precipitation.	4.5–5.0	55–60 °C	Composition of medium: 10 g/L soluble starch, 5 g/L yeast extract, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 5 g/L peptone, 1 g/L K_2HPO_4, and 10 g/L NaCl at pH 10.0 and incubation temperature is 37 °C for complete 24 h with shaking at 200 rpm. Main product is β-CD.	Charoenlap et al. (2004)
CGTases produced by *Bacillus* sp. C26	5–8.5	60–65 °C	Composition of medium 1% w/v soluble starch, 0.5% w/v yeast extract, 0.5% w/v peptone, 0.02% w/v K_2HPO_4, 0.02% w/v $MgSO_4{\cdot}7H_2O$, and 1% w/v Na_2CO_3. Its main product is β-cyclodextrin.	Cheirsilp et al. (2010)

4.5.2 Applications of β-Cyclodextrin

β-Cyclodextrin is used in inhibiting the browning of potato crude extract and different fruit juices: β-Cyclodextrin acts as an anti-browning agent in potato extract, and in case of fruit juices, it helps in stabilizing the flavour, improves mouth feel and also a mild antimicrobial effect, antioxidant properties in grape juice, improves retention of volatiles in pear juice, and helps in elimination of off-flavours in lemon oil (Santos et al., 2015).

4.6 Gamma Cyclodextrin

Gamma cyclodextrin is produced from microbial strain *B. thuringiensis* GU-2. The optimum pH and temperature for γ-CD production are pH 8.5 and 37 °C, respectively, in the presence of 1.0% starch substrate which results in 95% production of product with high yield (Goo et al., 2014). γ-CD has the premier solubility and largest interior cavity. Bioconversion of gamma cyclodextrin (γ-CD) from starch in the presence of cyclodextrin glycosyltransferase is using a technique aqueous two-phase system (ATPS). This is a low pollution technique, simple, economic, and is used for purification and production process in single step (Lin et al., 2016).

γ-CD production was to a great extent enhanced with the use of cyclododecanone, 5-cyclohexadecen-1-one, and glycyrrhizic acid. Optimum pH for reaction conditions is between 7 and 10 using 15% potato starch. All these parameters resulted in the corresponding increase in the yield of γ-CD from 21.2% to 41.6%. Optimum temperature range for the reaction is between 40° and 65°. Higher enzyme dosages had a detrimental effect on the CD yield, probably because excess CGTase might have hydrolysed CDs to produce glucose and high quantities of malto-oligosaccharides, which might have facilitated coupling reaction with the CDs, thus severely degrading them. A CD was not detected. When soluble starch or potato starch as a source was used, it becomes very difficult to detect the amount of CD. Soluble starch and potato starch are responsible for the production of highest percentage of γ-CD among the total CDs (nearly 88%), when compared with the other two starches (Loftsson & Brewester, 1996).

Source of γ-CGTase	Optimum pH	Optimum temperature (°C)	Main product	References
Bacillus subtilis strain 313	8.0	65	γ-CD	Li et al. (2007), Kato and Horikoshi (1986)
Bacillus sp. strain AL-6	7.5–10.5	55	γ-CD	Li et al. (2007), Fugita et al. (1990)
Bacillus firmus strain 290-3	6–8	60	γ-CD	Li et al. (2007), Veen et al. (2000)
Bacillus cereus	8.0	37	γ-CD	Li et al. (2007), Lin et al. (2016), Ng et al. (2011)

4.6.1 Applications

Cyclodextrins are used in food due to the following reasons:

1. *Cyclodextrins are used to preserve the natural flavours, vitamins, and natural colours of foods*
   Manufacturing, packaging, and other factors modify the flavours by reducing aroma. There are wide techniques and processes used for retaining the properties and flavour of the food like spray drying, freeze-drying, fluidized bed coating, and crystallization. However, those involving the formation of flavour/CD molecular-inclusion complexes offer great potential for the protection of volatile substances throughout many rigorous food-processing methods such as freezing, thawing, and microwaving. The addition of β-CD has proved to be most effective in retaining the flavour against heat, evaporation, and also against thermal degradation (Jouquand et al., 2004). The flavour enhancing complexes consists of many components, thus it is important that there is proper incorporation of these components in the complex without causing any change in its composition (Astray et al., 2009). Natural and synthetic coffee flavours have also been retained by the use of beta cyclodextrins. When these complex bound flavours come in contact with water molecules, they get released immediately and thus responsible for the taste or flavour (Astray et al., 2009; Szente & Szejtli, 1986). In addition to coffee, β-CD is also used for tea aromatization (jasmine, peppermint, and cinnamon) and also results in stability as well as efficient storage.
2. *For the removal of undesired taste, flavour, microbial contamination, and other undesired products*
   For the rejection of various food items, bitter taste is responsible. It has been found that there are two classes of chemical compounds like flavonoids and limonoids that are responsible for bitterness. Fresh citrus juice turns bitter in case of storage which is dependent on pH and temperature. It is highly desirable to remove the bitter components without the addition of any other substance to the fruit juice. Therefore, cyclodextrins are used for masking the undesired components from food. When the cyclodextrins are added to the fruit juice which has a peculiar odour and an undesirable taste, it forms complexes and retains the flavour of the components. For example, this process is used for deodourizing soybean milk and soy protein which removes the peculiar fish odours. A mixture of cyclodextrins alpha, beta, and gamma is ordinarily used. Rice which gives an unpleasant odour when stored for a long time can be made odour-free by cooking the rice in the presence of 0.01–0.4% CD (Sakakibara et al., 1985). The taste of cooked ice can be improved by addition of maltosyl beta CD. When CDs are complexed with sweetening agents such as aspartame; it helps in stabilizing and improving the taste of product. This complexation process also helps in eliminating the bitter aftertaste of other sweeteners like stevioside, glycyrrhizin, and rubusoside. CD itself is a promising new sweetener (Singh

et al., 2002). When these cyclodextrins or cyclodextrin-complexed antimicrobial agents are incorporated into food packaging plastic films, they help in effectively reducing the loss of the aroma substances and also improve the microbiological preservation during storage. Various CD complexes can be utilized in food as an antiseptic or conserving agents; 0.1% iodine-β-CD inhibits putrefying for 2 months at 20 °C in fish paste or in frozen seafood products (Hara & Hashimoto, 2002). Incorporating fungicide CD complexes into films-for example, packaging of hard cheeses-significantly elongates the shelf life of the product by inhibiting the rapid development of mould colonies on surface of the packaged cheese. The characteristic colour of muttons and fish and bad odour of bone powder can be removed by the use of cyclodextrins. The bitter taste of grape juice also reduced considerably when 0.3% of β cyclodextrin was added prior to heat treatment of canned juice. Empty beta cyclodextrin has also been used in food industries to remove undesirable compounds such as bitter taste from tea and coffee (Shiga et al., 2004) and fruit juices (Ferrari et al., 2013; Threapleton et al., 2013).

3. *To protect against oxidative degradation, heat-induced changes, and light-induced decomposition*

   The lipophilic components present in food which are sensitive to light- or heat-induced degradation can be protected because of the formation of inclusion complexes with CDs. The formation of inclusion complexes with cyclodextrins removes the polyphenol oxidase which converts the colourless polyphenols to coloured compounds which then leads to browning reaction in case of preparation of fruit juices. Cyclodextrins remove the polyphenol oxidase by forming complexes (Del Valle, 2004).

   In case of apple and pear juice, the maltosyl beta cyclodextrin can enhance the ability of ascorbic acid to prevent enzymatic browning due to protective effect of maltosyl beta cyclodextrin against ascorbic acid oxidation. Hence, maltosyl beta cyclodextrin is considered as secondary antioxidant reducing apple and pear juice browning reaction. During industrial food processing when these inclusion complexes are heated, they exhibit high thermal stability, are stable, and last for a longer period of time. This can be supported by taking an example such as in case of aromatizing substances which are sensitive to some kind of radiation, like citral (responsible for fresh citrus odour) cyclizes under exposure to UV irradiation. This can be prevented by complexation with beta cyclodextrins. It has been found that complexation is more effective (protective) in solid state than in aqueous solution. Another application of cyclodextrins is that since they can form inclusion complexes with the substances, they can be used to retain or scavenge substances such as odour, lactose, and cholesterol, thus retaining the aroma, colours which could enhance shelf life, and also the quality of the packaged product (Zhang et al., 2013b; Artiss et al., 2006; Suzuki & Sato, 1985).

4. *Food preservation*

   This is the most important application of cyclodextrins in food. Cyclodextrins help in reducing the volatile organic contaminants present in packaging materials

and also improve the barrier properties (diffusion rate and transmission rate), thus maintaining the food quality (Suzuki & Sato, 1985). Cyclodextrins can release antimicrobials and antioxidant compounds using CDs as carriers. As the humidity levels in freshly cut vegetables or fruits increase, they can lead to spoilage; this can be reduced by using cyclodextrins. At high relative humidity, high water–CD interaction weakens the host guest interaction and consequently there is a release of antimicrobial molecule which should protect the molecule against microbial growth (Astray et al., 2009; Ayala-Zavala et al., 2008).

5. *Cyclodextrins as stabilizers*
   Cyclodextrins are used for the preparation of stable water in oil emulsion such as mayonnaise and salad dressing due to their hydrophobic cavity and hydrophilic outer surface. In tomato ketchup, natural food colouring components can be stabilized by the addition of beta cyclodextrins. This ketchup when prepared by heating at 100° for 2 h did no discolouration, while the control did. Also, the addition of cyclodextrins to emulsified cheese increased the water retention and shelf life. However, the control discoloured when heated to this temperature (Szente & Szejtli, 2004). Cyclodextrins also help in processed meat products in improving water retention and texture (Ota & Takeda, 1981).
6. The browning or caking formation in the solid compositions containing sugars and amino acids can be prevented by addition of oligosaccharides such as cyclodextrin
   By the addition of 20% of β-CD to powdered juice containing anhydrous glucose, aspartic acid, d-alanine, citric acid, etc., the shelf life and also stability of the product are enhanced. Even after 30–35 days at 40 °C, no change in colour was observed (Fujimoto, 1981). The control started to discolour even on the second day and turned brown on fourth day (Szente & Szejtli, 2004). Starch gelatinization is the process in which heat treatment is given to starch and water, resulting in swelling of starch granules. However, addition of β-CD considerably improves the gelatinization of wheat flour. Addition of 1.5% beta cyclodextrin improves the solubility and swelling properties of starch granules. It has been observed that the adding up of 1.5% of beta cyclodextrin augmented the viscosity of starch paste to 4%. Milk casein hydrolysate is a readily digestible protein and its bitter protein can be removed by addition of 10% of beta cyclodextrin (Specht et al., 1981). Similarly, the bitter taste of ginseng extract and propylene glycol can also be eliminated by the use of beta cyclodextrin (Akiyama & Miyao, 1979).
7. *Extraction efficiency is increased with the use of beta cyclodextrin*
   When the roasted coffee beans are extracted using beta cyclodextrin, the extraction becomes more effective and the complex containing beta cyclodextrin and coffee can be used for effectual preservation of volatile components and also provide better aroma of the product (Wagner et al., 1988). Low cholesterol butter is also made by the addition of beta cyclodextrin. Molten butter is blended with crystalline beta cyclodextrin, which forms stable complexes with triglycerides/cholesterol and the cholesterol/β-CD is easily removed from molten butter.

Ninety percent of cholesterol in fat milk can also be separated by the addition of beta cyclodextrin; the butter obtained after this process does not retain any of the cyclodextrin (Szente & Szejtli, 2004). Cyclodextrins are widely used in order to modify the natural properties of materials, for example simply eggs have been produced in USA which means a low cholesterol egg which is produced by the addition of cyclodextrins to reduce cholesterol.

8. β-Cyclodextrin is more extensively used in food applications due to its availability at low cost, chemically stable structure, and non-hygroscopic, non-toxic, and edible nature. Ethyl vinyl alcohol copolymers are widely used in food industry due to their properties like gas barrier to oxygen and organic compounds and high transparency (Sanchez-Chaves et al., 2007; Lopez-de-Dicastilloa et al., 2010). On comparing all the three forms of cyclodextrins, γ-cyclodextrins show the highest flavour retention capacity among the other three.

4.6.2 Properties

S. no.	Properties	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin	References
1.	Form complexes with	Benzene derivatives	Aromatic or heterocycles	Macrocycles or steroids	Threapleton et al. (2013)
2.	Water solubility	Modest solubility (14.5)	Lowest solubility (1.85)	Highest solubility (23.2)	Threapleton et al. (2013), Arya et al. (2006)
3.	Hydrolysis by human salivary and pancreatic amylases	Resistant to hydrolysis	Resistant to hydrolysis	Completely digested by amylase	Threapleton et al. (2013)
4.	Production from halophilic α-CGTase	1 (produced in much higher amount)	0.6	0.3	Threapleton et al. (2013)
5.	No. of glucopyranose units	6	7	8	Qi and Romberger (1988), Radi and Eissa (2010), Arya et al. (2006)
6.	Molar mass (g/mol)	972	1135	1297	Arya et al. (2006)
7.	Inner cavity diameter (Å)	4.7–5.2	6.0–6.4	7.5–8.3	Arya et al. (2006)
8.	Outer cavity diameter (Å)	14.6	15.4	17.5	Arya et al. (2006)
1. 9.	Cost per production			Highest	Threapleton et al. (2013)

(continued)

S. no.	Properties	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin	References
10.	$\Delta H°$ (ionization), kcal/mol $\Delta H°$ (solution), kcal/mol	8.36 7.66	9.98 8.31	11.22 7.73	Arya et al. (2006)
11.	$\Delta S°$ (solution), kcal/mol $\Delta S°$ (ionization), cal/mol/K	7.67 −28.3	8.31 −22.4	7.73 −17.6	Arya et al. (2006)

References

Arya S. K., & Srivastava S. K. (2006, July 3). *Enzyme and microbial technology, 39* (3), 507–510.

Akiyama, Y., & Miyao, K. (1979). *Microencapsulation of royal jelly*. Japanese Kokai JP 77 80 463.

Andel-Naby, M. A., Reyad, R. M., & Abdel-Fattah, A. F. (2000). Biosynthesis of cyclodextrin glucosyltransferase by immobilized Bacillus amyloliquefaciens in batch and continuous cultures. *Biochemical Engineering Journal, 5*, 1–9.

Arias, M. J., Moyano, J. R., Munoz, P., Gines, J. M., Justo, A., & Giordano, F. (2000). Study of omeprazole-gamma-cyclodextrin complexation in the solid state. *Drug Development and Industrial Pharmacy, 26*, 253–259.

Artiss, J. D., Brogan, K., Brucal, M., Moghaddam, M., & Jen, K. L. (2006). The effects of a new soluble dietary fiber on weight gain and selected blood parameters in rats. *Metabolism, 55*, 195–202.

Astray, G., Barreiro, C. G., Mejuto, J. C., Otero, R. R., & Gándara, J. S. (2009). A review on the use of cyclodextrins in foods. *Food Hydrocolloids, 23*(7), 1631–1640.

Ayala-Zavala, J. F., Del-Toro-Sánchez, L., Lvarez-Parrilla, A. E., & Gonzalez-Aguilar, G. A. (2008). High relative humidity in-package of fresh-cut fruits and vegetables: Advantage or disadvantage considering microbiological problems and antimicrobial delivering systems. *Journal of Food Science, 73*(4), R41–R47.

Balakrishnan, M., Dua, M., & Bhagat, J. J. (2000). Effect of operating parameters on sugarcane juice ultrafiltration: Results of a field experience. *Separation and Purification Technology, 19*, 209–220.

Bansal, B., Ali, R. A., Prieto, R. M., & Chen, X. D. (2006). Rinsing and cleaning of alactalbumin fouled MF membranes. *Separation and Purification Technology, 48*, 202–207.

Bender, H. (1983). An improved method for the preparation of cyclooctaamylose, using starches and the cyclodextrin glycosyltransferase of Klebsiella pneumoniae M5 al. *Carbohydrate Research, 124*, 225–233.

Blackwood, A. D., & Bucke, C. (2000). Addition of polar organic solvents can improve the product selectivity of cyclodextrin glycosyltransferase solvent effects on CGTase. *Enzyme and Microbial Technology, 27*, 704–708.

Blanco, K. C., Moraes, F. F. D., Bernardi, N. S., Vettori, M. H. P. B., Monti, R., & Contiero, J. (2014). Cyclodextrin production by *Bacillus lehensis* isolated from cassava starch: Characterisation of a novel enzyme. *Czech Journal of Food Sciences, 32*(2014), 48–53.

Bodalo, A., Gomez, J. L., Gomez, E., Bastida, J., Maximo, M. F., & Montiel, M. C. (2001). Ultrafiltration membrane reactors for enzymatic resolution of amino acids: Design model and optimization. *Enzyme and Microbial Technology, 28*, 355–361.

Burhan, N., Sapundzhiev, T. S., & Beschkov, V. (2005). Mathematical modeling of cyclodextrin-glucanotransferase production by batch cultivation. *Biochemical Engineering Journal, 24*, 73–77.

Buschmann, H. J., & Schollmeyer, E. (2002). Applications of cyclodextrins in cosmetic products: A review. *Journal of Cosmetic Science, 53*, 575–592.

Buston, J. E. H., Marken, F., & Anderson, H. L. (2001). Enhanced chemical reversibility of redox processes in cyanine dye rotaxanes. *Chemical Communications, 11*, 1046–1047.

Charoenlap, N., Dharmsthiti, S., Sirisansaneeyakul, S., & Lertsiri, S. (2004). Optimization of cyclodextrin production from sago starch. *Bioresource Technology, 92*, 49–54.

Cheirsilp, B., Kitcha, S., & Maneerat, S. (2010). Kinetic characteristics of β-cyclodextrin production by cyclodextrin glycosyltransferase from newly isolated *Bacillus* sp. C26. *Electronic Journal of Biotechnology, 13*, 717–3458.

Chiou, S. H., & Wu, W. T. (2004). Immobilization of Candida rugosa lipase on chitosan with activation of the hydroxyl groups. *Biomaterials, 25*(2), 197–204.

Choi, H. G., Lee, B. J., Han, J. H., Lee, M. K., Park, K. M., Yong, C. S., Rhee, J. D., Kim, Y. B., & Kim, C. K. (2001). Terfenadine-β-cyclodextrin inclusion complex with antihistaminic activity enhancement. *Drug Development and Industrial Pharmacy, 27*(8), 857–862.

Crini, G. (2014). Review: A history of cyclodextrins. *Chemical Reviews, 114*, 10940–10975.

Dardeer, H. M. (2014). Importance of cyclodextrins into inclusion complexes. *International Journal of Advances Research, 2*(4), 414–428.

Das, S. K., Rajabalaya, R., David, S., Gani, N., Khanam, J., & Nanda, A. (2013). Cyclodextrins: The molecular container. *Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4*(2), 1694.

Del Valle, E. M. M. (2004). Cyclodextrin and their uses: A review. *Process Biochemistry, 39*, 1033–1046.

Duca, G., & Boldescu, V. (2008). Cyclodextrin: Fields of application. *Chemistry Journal of Moldova: General, Industrial and Ecological Chemistry, 3*, 30–37.

Eastburn, S. D., & Tao, B. Y. (1994). Applications of modified cyclodextrins. *Journal of Biotechnology Advances, 12*, 325–339.

Escuder-Gilabert, L., Martín-Biosca, Y., Medina-Hernández, M. J., & Sagrado, S. (2014). Cyclodextrins in capillary electrophoresis: Recent developments and new trends. *Journal of Chromatography A, 1357*, 2–23.

Ferrari, P., Rinaldi, S., Jenab, M., Lukanova, A., Olsen, A., Tjonneland, A., et al. (2013). Dietary fiber intake and risk of hormonal receptor-defined breast cancer in the European Prospective Investigation into Cancer and Nutrition study. *American Journal of Clinical Nutrition, 97*, 344–353.

Ferrarotti, S., Rosso, A., Marechal, M., Krymkiewicz, N., & Marechal, L. (1996). Isolation of two strains (S–R type) of Bacillus circulans and purification of a cyclomaltodextrin-glucanotransferase. *Cellular and Molecular Biology, 42*(5), 653–657.

Flaschel, E., Landert, J. P., Spiesser, D., & Renken, A. (1984). The production of alpha-cyclodextrin by enzymatic degradation of starch. *Annals of the New York Academy of Sciences, 434*, 70–77.

Fugita, Y., Tsubouchi, H., Inagi, Y., Tomita, K., Ozaki, A., & Nakanishi, K. (1990). Purification and properties of cyclodextrin glycosyltransferase from Bacillus sp. AL-6. *Journal of Fermentation and Bioengineering, 70*, 150–154.

Fujimoto, S. (1981). *Prevention of quality deterioration in rice*. Japanese Kokai JP 8196670.

Fujiwara, S., Kakihara, H., Woo, K. B., Lejeune, A., Kanemoto, M., Sakaguchi, K., & Imanaka, T. (1974). Cyclization characteristics of cyclodextrin glucanotransferase are conferred by the NH2-terminal region of the enzyme. *Applied and Environmental Microbiology, 58*, 4016–4025.

Galan, C. G., Murcia, A. B., Lafuente, R. F., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis and Catalysis, 353*(16), 2885–2904.

Gallaher, D. D., Gallaher, C. M., & Plank, D. W. (2007). Alphacyclodextrin selectively increases fecal excretion of saturated fats. *FASEB Journal, 21*, A730.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis & Catalysis, 353*(16), 2885–2904.

Garnero, C., Zoppi, A., Genovese, D., & Longhi, M. (2010). Studies on tri-methoprim: Hydroxypropyl-β-cyclodextrin: Aggregate and complex formation. *Carbohydrate Research, 345*, 2550–2556.

Gawande, B., & Patkar, A. (2001). Alpha-cyclodextrin production using cyclodextrin glycosyltransferase from Klebsiella pneumoniae AS-22. *Starch-Starke, 53*(2), 75–83.

Gawande, B. N., Singh, R. K., Chauhan, A. K., Goel, A., & Patkar, A. Y. (1998). Optimization of cyclomaltodextrin glucanotransferase production from Bacillus firmus. *Enzyme and Microbial Technology, 22*, 288–291.

Goel, A., & Nene, S. (1995). Modifications in the phenolphthalein method for spectro-photometric estimation of beta cyclodextrin. *Starch, 47*, 399–400.

Gomes, L. M. M., Petito, N., Costa, V. G., & Deborah, Q. F. (2014). Inclusion complexes of red bell pepper pigments with b-cyclodextrin preparation, characterisation and application as natural colorant in yogurt. *Food Chemistry, 148*, 428–436.

Goo, B. G., Hwang, Y. J., & Park, J. K. (2014). Bacillus thuringiensis: A specific gamma-cyclodextrin producer strain. *Carbohydrate Research, 386*, 12–17.

Grunberger, G., Jen, K. L., & Artiss, J. D. (2007). The benefits of early intervention in obese diabetic patients with FBC—A new dietary fibre. *Diabetes/Metabolism Research and Reviews, 23*, 56–62.

Gugliemi, G., Chiarani, D., Judd, S. J., & Andreottola, G. (2007). Flux criticality and sustainability in a hollow fibre submerged membrane bioreactor for municipal wastewater treatment. *Journal of Membrane Science, 289*, 241–248.

Hara, H., & Hashimoto, H. (2002). *Antimicrobial and insect-repellent cyclodextrin films*. Japanese Kokai JP 2002029901.

Hedges, R. A. (1992). Cyclodextrins and their applications in biotechnology. In *Minutes of the Sixth International Symposium on Cyclodextrins* (pp. 380–389). Editions de Sante.

Higuti, I. H., da Silva, P. A., Papp, J., de Eiro, M., Okiyama, V., Alves de Andrade, E., Abreu Marcondes, A., & do Nascimento, A. J. (2004). Colorimetric determination of α & β-cyclodextrins and studies on optimization of CGTase production from B. firmus using factorial designs. *Brazilian Archives of Biology and Technology, 47*(6), 837–841.

Hirose, T., & Yamamoto, Y. (2001). *Hinokitol containing cyclodextrin polymer compositions and their molding with excellent antimicrobial and gas barrier properties*. Journal of Japanese Patent 55480.

Ho, B. T., Joyce, D. C., & Bhandari, B. R. (2011). Encapsulation of ethylene gas into α-cyclodextrin and characterisation of the inclusion complexes. *Food Chemistry, 127*, 572–580.

Hughes, D., & Field, R. W. (2006). Cross-flow filtration of washed and unwashed yeast suspensions at constant shear under nominally sub-critical conditions. *Journal of Membrane Science, 280*, 89–98.

Isnin, R., & Kaifer, A. E. (1993). A new approach to cyclodextrin-based rotaxanes. *Pure and Applied Chemistry, 65*, 495–498.

Iyer, J. L., Shetty, P., & Pai, J. S. (2003). Immobilization of cyclodextrin glucanotransferase from B. circulans ATCC21783 on purified seasand. *Journal of Industrial Microbiology & Biotechnology, 30*, 47–51.

Jodai, I., Kandra, L., Harangi, J., Nanasi, P., Debrecen, & Szejtli, J. (1984). Hydrolysis of cyclodextrin by Aspergillus oryzae alphaamylase. *Starch-Starke, 36*, 140–143.

Jouquand, C., Ducruet, V., & Giampaoli, P. (2004). Partition coefficients of aroma compounds in polysaccharide solutions by the phase ratio variation method. *Food Chemistry, 85*, 467–474.

Kainuma, K. (1984). Chapter V: Starch oligosaccharides: Linear, branched, and cyclic. In R. L. Whistler, J. N. Bemiller, & E. F. Paschall (Eds.), *Starch: Chemistry and technology* (2nd ed., p. 125). Academic Press Ltd.

Kamimura, J. A., Santos, E. H., Hill, L. E., & Gomes, C. L. (2014). Antimicrobial and antioxidant activities of carvacrol microencapsulated in hydroxypropyl-beta-cyclodextrin. *LWT—Food Science and Technology, 57*, 701–709.

Kato, T., & Horikoshi, K. A. (1986). New γ-cyclodextrin forming enzymeprodiced by Bacillus subtilis no. 313. *Journal of the Japanese Society of Starch Science, 34*, 137–143.

Katzir, E. K. (1993). Immobilized enzymes: Learning from past successes and failures. *Trends in Biotechnology, 11*, 471–478.

Kim, Y. H., Bae, K. H., Kim, T. J., Park, K. H., Lee, H. S., & Byun, S. M. (1997). Effect on product specificity of cyclodextrin glycosyltransferase by site-directed mutagenesis. *IUBMB Life Journal, 41*(2), 227–234.

Kitahata, S., Tsuyama, N., & Okada, S. (1974). Purification and some properties of cyclodextrin glycosyltransferase from a strain of Bacillus species. *Agricultural and Biological Chemistry, 38*, 387–393.

Kittikun, A. H., Kaewthong, W., & Cheirsilp, B. (2008). Continuous production of monoacylglycerols from palm olein in packed-bed reactor with immobilized lipase PS. *Biochemical Engineering Journal, 40*, 116–120.

Kondo, H., Nakatani, H., & Hiromi, K. (1990). In vitro action of human and porcine alpha-amylases on cyclomaltooligosaccharides. *Carbohydrate Research, 204*, 207–213.

Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. *Enzyme and Microbial Technology, 35*(2–3), 126–139.

Kunamneni, A., Prabhakar, T., Jyothi, B., & Ellaiah, P. (2007). Investigation of continuous cyclodextrin glucanotransferase production by the alginate-immobilized cells of alkalophilic *Bacillus* sp. in an airlift reactor. *Enzyme and Microbial Technology, 40*, 1538–1542.

Kurkovsergey, V., & Loftssonthorsteinn. (2013). Review: Cyclodextrins. *International Journal of Pharmaceutics, 453*, 167–180.

Larsen, K. L., Duedahl-Olesen, L., Christensen, H. J. S., Mathiesen, F., Pedersen, L. H., & Zimmermann, W. (1998). Purification and characterisation of cyclodextrin glycosyltransferase from Paenibacillus sp. F8. *Carbohydrate Research, 310*, 211–219.

Leemhuis, H., Kelly, R. M., & Dijkhuizen, L. (2010). Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. *Applied Microbiology and Biotechnology, 85*, 823–835.

Li, Z., Wang, F., Wang, M., Gu, Z., Du, G., Wu, J., & Chen, J. (2007). γ-Cyclodextrin: A review on enzymatic production and applications. *Journal of Applied Microbiology and Biotechnology, 77*, 245.

Lia, Z., Chena, S., Gua, Z., Chena, J., & Wua, J. (2014). Alpha-cyclodextrin: Enzymatic production and food applications. *Trends in Food Science and Technology, 35*, 151–160.

Liang, H., Yuan, Q., Vriesekoop, F., & Lv, F. (2012). Effect of cyclodextrin on the antimicrobial activity of plant-derived essential oil compounds. *Food Chemistry, 135*(3), 1020–1027.

Lin, Y. K., Show, P. L., Yap, Y. J., Ariff, A. B., Annuar, M. S. M., Lai, O. M., Tang, T. K., Juan, J. C., & Ling, T. C. (2016). Production of g-cyclodextrin by Bacillus cereus cyclodextrin glycosyltransferase using extractive bioconversion in polymer-salt aqueous two-phase system. *Journal of Bioscience and Bioengineering, 121*, 692–696.

Loftsson, T., & Brewester, M. J. (1996). Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *Pharmaceutical Sciences, 85*(10), 1017–1025.

Lopez-de-Dicastilloa, C., Gallurb, M., Cataláa, R., Gavaraa, R., & Hernandez-Munoz, P. (2010). Immobilization of β-cyclodextrin in ethylene-vinyl alcohol copolymer for active food packaging applications. *Journal of Membrane Science, 353*, 184–191.

Lopez-Miranda, S., Serrano-Martinez, A., Hernandez-Sanchez, P., Guardiola, L., Perez-Sanchez, H., Fortea, I., Gabaldon, J. A., & Nunez-Delicado, E. (2016). Use of cyclodextrins to recover catechin and epicatechin from red grape pomace. *Food Chemistry, 203*, 379–385.

Lopez-Nicolas, J. M., Andreu-Sevilla, A. J., Garcia-Carmona, F. J., & Carbonell-Barrachina, A. (2007a). Effects of addition of α-cyclodextrin on the sensory quality, volatile compounds, and color parameters of fresh pear juice. *Journal of Agricultural and Food Chemistry, 57*(20), 9668–9675.

Lopez-Nicolas, J. M., Perez-Lopez, A. J., Carbonell-Barrachina, A., & Garcia-Carmona, F. (2007b). Use of natural and modified cyclodextrins as inhibiting agents of peach juice enzymatic browning. *Journal of Agricultural and Food Chemistry, 55*(13), 5312–5319.

Mabuchi, N., & Ngoa, M. (2001). *Controlled release powdered flavour preparations and confectioneries containing preparations*. Journal of Japanese Patent 128,638.

Maneechakr, P., Samerjit, J., Uppakarnrod, S., & Karnjanakom, S. (2015). Experimental design and kinetic study of ultrasonic assisted transesterification of waste cooking oil over sulfonated carbon catalyst derived from cyclodextrin. *Journal of Industrial and Engineering Chemistry, 32*, 128–136.

Matsuda, H., & Arima, H. (1999). Cyclodextrins in transdermal and rectal delivery. *Journal of Advances in Drug Delivery Reviews, 36*, 81–99.

Mazzer, C., Ferreira, L. R., Rodella, J. R. T., Moriwaki, C., & Matioli, G. (2008). Cyclodextrin production by Bacillus firmus strain 37 immobilized on inorganic matrices and alginate gel. *Biochemical Engineering Journal, 41*, 79–86.

Miller, L. A., Carrier, R. L., & Ahmed, I. (2007). Practical considerations in development of solid dosage forms that contain cyclodextrin. *Journal of Pharmaceutical Sciences, 96*(7), 1691–1707.

Mora, M. M. M., Sánchezl, K. H., Santana, R. V., Rojas, A. P., Ramírez, H. L., & Torres-Labandeira, J. J. (2012). Partial purification and properties of cyclodextrin glycosiltransferase (CGTase) from alkalophilic Bacillus species. *Springer Plus, 1*, 61.

Moriwak, C., Mangolim, C. S., Ruiza, G. B., Morais, G. R. D., Baesso, M. L., & Matioli, G. (2014). Biosynthesis of CGTase by immobilized alkalophilic bacilli and crystallization of beta-cyclodextrin: Effective techniques to investigate cell immobilization and the production of cyclodextrins. *Biochemical Engineering Journal, 83*, 22–32.

Muria, S. R., Cheirsilp, B., & Kitcha, S. (2011). Effect of substrate concentration and temperature on the kinetics and thermal stability of cyclodextrin glycosyl transferase for the production of β-cyclodextrin: Experimental results vs mathematical model. *Process Biochemistry, 46*, 1399–1404.

Muzzarelli, R. A. A. (1980). Immobilization of enzymes on chitin and chitosan. *Enzyme and Microbial Technology, 2*(3), 177–184.

Natalia, S., Ignacio, S., & Mabel, R. A. (2007). Cyclodextrin production by cyclodextrin glycosyltransferase from Bacillus circulans DF 9R. *Bioresource Technology, 98*, 2886–2891.

Natividade Schöffer, J. D., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). Continuous production of β-cyclodextrin from starch by highly stable cyclodextrin glycosyltransferase immobilized on chitosan. *Carbohydrate Polymers, 98*, 1311–1316.

Ng, H. S., Tan, C. P., Chen, S. K., Mokhtar, M. N., Ariff, A., & Ling, T. C. (2011). Primary capture of cyclodextrin glycosyltransferase derived from Bacillus cereus by aqueous two phase system. *Separation and Purification Technology, 81*(2011), 318–324.

Ota, T., & Takeda, F. (1981). *Cyclodextrins as cheese additives*. Japanese Kokai JP 8175060.

Park, T.-H., Shin, H.-D., & Lee, Y.-H. (1999). Characterization of the beta cyclodextrin glucanotransferase gene of *Bacillus firmus* var. alkalophilus and its expression in *E. coli*. *Journal of Microbiology and Biotechnology, 9*, 811–819.

Penninga, D., Strokopytov, B., Rozeboom, H. J., Lawson, C. L., Dijkstra, B. W., Bergsma, J., et al. (1995). Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from Bacillus circulans strain 251 affect activity and product specificity. *Biochemistry, 34*, 3368–3376.

Priyananda, P., & Chen, V. (2006). Separation of residual fatty acids from aqueous solutions using an agitated solution of protein and membrane filtration. *Separation and Purification Technology, 48*, 113–120.

Qi, Q., & Zimmermann, W. (2005). Cyclodextrin glucanotransferase: From gene to applications. *Applied Microbiology and Biotechnology, 66*, 475–485.

Qi, Z., & Romberger, M. L. (1988). Cyclodextrin. In R. H. Walter (Ed.), *Polysaccharide association structures in food* (pp. 207–226). Marcel Dekker.

Radi, A. E., & Eissa, S. (2010). Electrochemistry of cyclodextrin inclusion complexes of pharmaceutical compounds. *Journal of the Open Chemical and Biomedical Methods, 3*, 74–85.

Rahman, R. A., Illias, R. M., Nawawi, M. G. M., Ismail, A. F., Hassan, Q., & Kamaruddin, K. (2004). Optimisation of growth medium for the production of cyclodextrin glucanotransferase from Bacillus stearothermophillus HR1 using response surface methodology. *Process Biochemistry, 39*, 2053–2060.

Rakmai, J., & Cheirsilp, B. (2016). Continuous production of β-cyclodextrin by cyclodextrin glycosyltransferase immobilized in mixed gel beads: Comparative study in continuous stirred tank reactor and packed bed reactor. *Biochemical Engineering Journal, 105*, 107–113.

Rakmai, J., Cheirsilp, B., & Prasertsan, P. (2015). Enhanced thermal stability of cyclodextrin glycosyl transferase in alginate–gelatine mixed gel beads and the application for β-cyclodextrin production. *Biocatalysis and Agricultural Biotechnology, 4*, 717–726.

Rendleman, J. A. J. (1997). Enhancement of cyclodextrin production through use of debranching enzymes. *Applied Biochemistry and Biotechnology, 26*, 51–61.

Rimphanitchayakit, V., Tonozuka, T., & Sakano, Y. (2005). Construction of chimeric cyclodextrin glucanotransferases from Bacillus circulans A11 and Paenibacillus macerans IAM1243and analysis of their product specificity. *Carbohydrate Research, 340*, 2279–2229.

Rios, G. M., Belleville, M. P., Paolucci, D., & Sanchez, J. (2004). Progress in enzymatic membrane reactors—A review. *Journal of Membrane Science, 242*, 189–196.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., & Fernandez-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews, 42*, 6290–6307.

Rosso, A., Ferrarotti, S., Krymkiewicz, N., & Nudel, C. (2002). Optimisation of batch culture conditions for cyclodextrin glucanotransferase production from Bacillus circulans DF 9R. *Microbial Cell Factories, 1*(3), 1–10.

Saha, B. C., & Zeikus, J. G. (1992). Cyclodextrin degrading enzymes. *Starch-Starke, 44*, 312–315.

Sakakibara, S., Sugisawa, K., Matsui, F., & Sengoku, K. (1985). *Cyclodextrins: New versatile food additive*. Indian. Japanese Patent JP 851 248 075.

Sakinah, A. M. M., Ismail, A. F., Hassan, O., Zularisam, A. W., & Illias, R. M. (2009). Influence of starch pretreatment on yield of cyclodextrins and performance of ultrafiltration membranes. *Desalination, 239*, 317–333.

Sakinah, A. M. M., Ismail, A. F., Illias, R. M., Zularisam, A. W., Hassan, O., Matsuura, T., & T. (2014). Effect of substrate and enzyme concentration on cyclodextrin production in a hollow fibre membrane reactor system. *Separation and Purification Technology, 124*, 61–67.

Sanchez-Chaves, M., Fernandez-García, M., & Cerrada, M. L. (2007). Ethylene-vinyl alcohol copolymers partially modified with benzoate groups: Study of their polymorphic behaviour. *Journal of Polymer Science Part B: Polymer Physics, 45*, 1026–1036.

Santos, E. H., Kamimura, J. A., Hill, L. E., & Gomes, C. L. (2015). Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications. *LWT—Food Science and Technology, 60*, 583–592.

Sapkal, N. P., Kilor, V. A., Bhusari, K. P., & Daud, A. S. (2007). Evaluation of some methods for preparing gliclazide-β-cyclodextrin inclusion complexes. *Tropical Journal of Pharmaceutical Research, 6*(4), 833–840.

Schmid, G. (1989). Cyclodextrin glucanotransferase production: Yield enhancement by over expression of cloned genes. *Trends in Biotechnology, 7*, 244–248.

Shiga, H., Yoshii, H., Ohe, H., Yasuda, M., Furuta, T., Kuwahara, H., et al. (2004). Encapsulation of shiitake (Lenthinus edodes) flavours by spray drying. *Bioscience, Biotechnology and Biochemistry, 68*, 66–71.

Sian, H. K., Said, M., Hassan, O., Kamaruddin, K., Ismail, A. F., Rahman, R. A., Mahmood, N. A. N., & Illias, R. M. (2005). Purification and characterization of cyclodextrin glucanotransferase from alkalophilic Bacillus sp. G1. *Process Biochemistry, 40*, 1101–1111.

Singh, M., Sharma, R., & Banerjee, U. C. (2002). Biotechnological applications of cyclodextrins. *Biotechnology Advances, 20*, 341–359.

Specht, M. Rothe, M., Szente, L., & Szejtli, J. (1981). *Removal of phenylalanine from protein hydrolysates*. Ger. Offen. 147 615.

Starnes, R. L., Amemiya, K., Jorgensen, P. L., & Jorgensen, S. (1992). Cloning and expression of the Thermoanaerobacter sp. cyclodextrin glycosyl transferase gene in E. coli and B. subtilis. In A. R. Hedges (Ed.), *Minutes of the Sixth International Symposium on Cyclodextrins* (pp. 46–52). Editions de Sante.

Steinbrunn, M. B., & Wenz, G. (1996). Synthesis of water-soluble inclusion compounds from polyamides and cyclodextrins by solid-state polycondensation. *Angewandte Chemie International Edition in English, 35*, 2139–2141.

Stella, V. J., & Rajewski, R. A. (1997). Cyclodextrins: Their future in drug formulation and delivery. *Pharmaceutical Research, 14*, 556–5567.

Suzuki, M., & Sato, A. (1985). Nutritional significance of cyclodextrins: Indigestibility and hypolipemic effect of alpha-cyclodextrin. *Journal of Nutritional Science and Vitaminology (Tokyo), 31*, 209–223.

Szejtli, J. (1988). *Cyclodextrin technology* (pp. 1–78). Kluwer Academic Publishers.

Szejtli, J. (1990). The cyclodextrin and their applications in biotechnology. *Journal of Carbohydrate Polymers, 12*, 375–392.

Szejtli, J. (2004a). Chapter 17: Cyclodextrins. In P. Tomasik (Ed.), *Chemical and functional properties of food saccharides* (pp. 271–290). CRC Press.

Szejtli, J. (2004b). Past, present and future of cyclodextrin research. *Pure and Applied Chemistry, 76*, 1825–1845.

Szente, L., & Szejtli, J. (1986). Molecular encapsulation of natural and synthetic coffee flavor with β-cyclodextrin. *Journal of Food Science, 51*(4), 1024–1027.

Szente, L., & Szejtli, J. (2004). Cyclodextrins as food ingredients. *Trends in Food Science and Technology, 15*, 137–142.

Threapleton, D. E., Greenwood, D. C., Evans, C. E., Cleghorn, C. L., Nykjaer, C., Woodhead, C., et al. (2013). Dietary fiber intake and risk of first stroke: A systematic review and meta-analysis. *Stroke, 44*, 1360–1368.

Tonkova, A. (1998). Bacterial cyclodextrin glucanotransferase. *Enzyme and Microbial Technology, 22*, 678–686.

Vassileva, A., Atanasova, N., Ivanova, V., Dhulster, P., & Tonkova, A. (2007). Characterisation of cyclodextrin glucanotransferase from *Bacillus circulans* ATCC 21783 in terms of cyclodextrin production. *Annals of Microbiology, 57*(4), 609–615.

Vassileva, A., Beschko, V., Ivanova, V., & Tonkova, A. (2005). Continuous cyclodextrin glucanotransferase production by free and immobilized cells of *Bacillus circulans* ATCC 21783 in bioreactors. *Process Biochemistry, 40*, 3290–3295.

Veen, B. A. V., Uitdehaag, J. C. M., Dijkstra, B. W., & Dijkhuizen, L. (2000). The role of arginine 47 in the cyclization and coupling reactions of cyclodextrin glycosyltransferase from Bacillus circulans strain 251. *The FEBS Journal, 267*(12), 3432–3441.

Wagner, C. W., Wilson, C. W., & Shaw, P. E. (1988). Reduction of grape fruit bitter components by cyclodextrin polymers. *Journal of Food Science, 53*, 516.

Wang, L., Wu, D., Chen, J., & Wu, J. (2013). Enhanced production of γ-cyclodextrin by optimization of reaction of γ-cyclodextrin glycosyltransferase as well as synchronous use of isoamylase. *Food Chemistry, 141*, 3072–3076.

Yagi, Y., Kouno, K., & Inui, T. (1980). *Purification and characterization of cyclodextrin; glycosyltransferase from Paenibacillus sp. F8*. US Patent 4 317 881.

Yuan, W., & Zydney, A. L. (1999). Humic acid fouling during microfiltration. *Journal of Membrane Science, 157*, 1–12.

Zhang, Z., Xu, G., Liu, D., Zhu, W., Fan, X., & Liu, X. (2013a). Dietary fiber consumption and risk of stroke. *European Journal of Epidemiology, 28*, 119–130.

Zhang, Z., Xu, G., Ma, M., Yang, J., & Liu, X. (2013b). Dietary fiber intake reduces risk for gastric cancer: A meta-analysis. *Gastroenterology, 145*(1), 113–120.

Zhaofeng, L., Bin, L., Zhengbiao, G., Dua, G., Jing, W., & Chen, J. (2010). Extracellular expression and biochemical characterization of alpha-cyclodextrin glycosyltransferase from Paenibacillus macerans. *Journal of Carbohydrate Research, 345*, 886–892.

Check for updates

5 Enzyme in Milk and Milk Products: Role and Application

Aparna Agarwal, Naman Kaur, Nidhi Jaiswal,
Memthoi Devi Heirangkhongjam, and Kanika Agarwal

Abstract

Enzymes are biocatalysts that catalyse a desired chemical reaction. Enzymes are specific in their action and yield into products. The enzymes that are utilized in the dairy industry for processing milk and milk products, like yoghurt, cheese, and fermented milks, are commonly known as dairy enzymes. These enzymes mostly aid in coagulation, cheese production, and enhancing shelf life of various dairy products. The most used dairy enzymes include lactase, amylase, lipases, transglutaminase, protease, catalase, and rennet. The functions of enzymes vary with the kind of the product to be processed. Both endogeneous and exogeneous enzymes are important for dairy application to provide functionality and safety to the product along with shelf life extension.

Keywords

Enzymes · Dairy products · Cheese · Fermented products

5.1 Introduction

Enzymes, also known as biocatalysts, are proteins that immobilize chemical reactions (Godfrey & Reichelt, 1983). Almost all the processes that exist in nature require enzymes in order to occur at significant rates. During an enzymatic reaction, the substrate molecules present on the onset are converted by the enzymes into different molecules, called products (Whitehurst & Van Oort, 2009).

The nomenclature of enzymes depends upon the reaction carried out by them or the substrate that they act upon as enzymes are highly specific for their substrates,

A. Agarwal (✉) · N. Kaur · N. Jaiswal · M. D. Heirangkhongjam · K. Agarwal
Department of Food and Nutrition, Lady Irwin College, University of Delhi, New Delhi, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_5

and hence, of various reactions; they catalyse only some of the many possibilities. Generally, the suffix 'ase' is added to the substrate name (e.g. glucose-oxidase, an enzyme which oxidizes glucose) or the type of reaction (e.g. a polymerase for a polymerization). However, pepsin, rennin, and trypsin are enzymes that were studied originally, and hence, are the exceptions to this rule. Enzymes can be categorized based upon the reactions they catalyse. These include, EC 1 oxidoreductases, enzymes that immobilize oxidation or reduction reactions (e.g. oxidases or dehydrogenases); EC 2 transferases enzymes (e.g. transphosphorylases and phosphomutases, example of a phosphate group), transfer a functional group such as a methyl or phosphate group; EC 3 hydrolases (e.g. hydrolases, including carbohydrases, deaminases, and proteases; hydrases such as fumarase, enolase, and carbonic anhydrase) are enzymes which catalyse the process of hydrolysis of different bonds (involving mostly, addition or removal of water); EC 4 lyase enzymes split bonds by methods other than hydrolysis and oxidation or form a C=C bond; EC 5 isomerases stimulate isomerization modifications within a molecule (e.g. glucose-isomerase); and EC 6 ligases combine two molecules using covalent bonds (Whitehurst & Van Oort, 2009).

All enzymes reduce the energy required by a reaction to take place. Different enzymes have different mechanisms to catalyse reactions, which involve creating conditions in which the transition state is stabilized in order to reduce the activation energy. This can be accomplished by providing a substitute way which involves bonding, and consequently, stabilizing the transition-state conformation of the substrate. For example, temporary reaction with the substrate in order to form an intermediate enzyme–substrate complex, where an enzyme plays an indispensable role. Enzymes possess an interesting property which is their specificity. For example, certain enzymes display absolute specificity which implies that they immobilize only one specific reaction or are selective for a certain kind of chemical bond or functional group. Generally, there exist four types of specificity which are: Absolute specificity; here, highly specific enzymes stimulate only one reaction; Group specificity; here, enzymes that are group-specific act only on molecules with certain functional groups, such as phosphate, amino, or methyl groups; Linkage specificity, where enzymes act on chemical bonds of specific nature, regardless of the molecular structure; and, Stereochemical specificity, where enzymes act only on a certain steric or optical isomer and not on their isomeric counterparts (Whitehurst & Van Oort, 2009). These biocatalysts are highly potent, as even in a very small quantity, they are capable of augmenting the rate of reactions to several folds. For example, orotidine-5′-phosphate-decarboxylase is an enzyme that facilitates a reaction to occur in milliseconds which would generally take millions of years (Callahan & Miller, 2007; Radzicka & Wolfenden, 1995). As a result, enzymes have advanced as a vital component for the sustenance of life. Evidently, over 5000 types of biochemical reactions have been found to be catalysed by enzymes (Schomburg et al., 2013).

Enzymes take part in several metabolic functions of living organisms. For example, they (kinases and phosphatases) are crucial for processes like signal transduction and cell regulation. They are also responsible for inducing movement, with adenosine triphosphate (ATP) which hydrolyses myosin in order to produce

contraction in the muscles and to also mobilize cargo around the cell as part of the cytoskeleton (Macrae, 1985). They also serve a vital role in the 'digestive systems' of various mammals and animals. For example, amylases cleave starch molecules and proteases cleave protein molecules (Whitehurst & Van Oort, 2009). Besides, enzymes have also been utilized at an industrial scale for antibiotic production. Moreover, there are certain enzymes which are used in household products like detergents that help in cleaving several stains on clothes. Additionally, as a result of the ability of enzymes to alter and enhance the nutritional, functional, and organoleptic properties of various ingredients and products, they have also found a widespread application in the food industry. They are employed in the food industries to manufacture bread, alcoholic drinks, and milk-based products like cheese and yoghurt, and to improve the flavour and texture of dairy-based products (Qureshi et al., 2015).

The enzymes that are utilized in the dairy industry for processing milk and milk products like yoghurt and cheese are commonly known as dairy enzymes. These enzymes mostly aid in coagulation, cheese production, and enhancing shelf life of various dairy products. The most used dairy enzymes include lactase, amylase, lipases, transglutaminase, protease, catalase, and rennet. Lactase enzyme is mostly utilized for hydrolysing lactose molecules to glucose and galactose sugars and augment the solubility and sweet flavour in various dairy products. Amylases are biocatalysts which break down starch by immobilizing the process of hydrolysis of internal α-1,4-glycosidic bonds of polysaccharide sugars like maltose, glucose, dextrin, and maltotriose, while preserving the α-anomeric configuration in the products (Takata et al., 1992). Lipase is another commonly used enzyme which is employed mostly to mature cheese and improve flavours. Another dairy enzyme is transglutaminase, commonly known as TGases, which plays a pivotal role in the enhancement of protein functionality in dairy products. Similar to lipases are different types of protease enzymes which are also utilized in manufacturing of cheese to increase the rate of cheese aging, as a functional property, and modifying milk protein to lower the allergic effects in cow milk-based infant food products (Fox, 2002). Another frequently used dairy enzyme in cheese production is catalase. It is employed in producing some cheeses in lieu of pasteurization (e.g. Swiss cheese) to save regular milk proteins that are responsible for providing certain benefits to finished product and flavour enhancement of the cheese (Fox, 2002). Rennet, which is also known as rennin, is a combination of pepsin and chymosin extracted from animals as well as microbiological sources. It is also employed in the cheese manufacturing industry to curdle milk (Merheb-Dini et al., 2010).

This chapter aims to emphasize on various endogenous and exogenous dairy enzymes like lactase, amylase, lipases, transglutaminase, protease, catalase, and rennet which are used widely, not only in the production of milk and milk-based products, but also lactose-free products. It briefly delineates the structure, sources, and applications of these enzymes in the dairy industry. Additionally, the chapter also meticulously explains the use of these dairy enzymes in the production of cheese, lactose-free milk, fermented milk as well as in packaging.

5.2 Endogenous Enzymes

All biochemical processes and metabolic pathways are propelled and modulated by a group of enzymes mobilizing a series of reactions. These enzymes catalyse reactions and improve the product yield up to several folds. However, in their absence, the rate of reaction decelerates, and eventually reaches impasse. Enzymes, on the basis of their source, can be broadly classified into two categories—exogenous enzymes and endogenous enzymes (EHEs). Enzymes secreted by animals are known as exogenous enzymes. These are used to enhance the process of digestion in animals. Optimizing the conditions required for exogenous enzymes is a tiresome job, which include optimum temperature, pH, and enzyme-substrate concentration. Additionally, these enzymes are expensive, and hence, increase the ultimate cost of the by-product. Contrariwise, EHEs are enzymes that are added to the animal feed. The efficacy, ease of use, unique properties, and profitability of these enzymes make them an appealing candidate for industries for the production of numerous by-products (Ramawat & Mérillon, 2015).

5.2.1 Lactase

β-Galactosidase, also known as lactase, is an endogenous enzyme which is accountable for hydrolysing lactose, a disaccharide composed of subunits of galactose and glucose (Carrara & Rubiolo, 1994; Felicilda-Reynaldo & Kenneally, 2016; Vandenplas, 2015; Lukito et al., 2015). This enzyme is located in the brush border of the small intestine of humans and other mammals. It plays a crucial role in the absorption of lactose. Lactase cleaves lactose by breaking the bond that links the two monosaccharide sugars—galactose and glucose and eventually resulting in glycolysis.

This enzyme also results in transgalactosylation of lactose to allolactose, which is then split into monosaccharides. The binding of allolactose to lacZ repressor modulates the amount of lactase in the cell by generating positive feedback (Pivarnik & Senecal, 1995).

5.2.1.1 Structure

Lactase or β-Galactosidase is a tetramer, that is four identical polypeptide chains. Each chain comprises 1023 amino acids that amalgamate to create five distinct structural domains. Of the five domains, one is a jelly roll barrel, whereas the rest comprise β-sandwich, fibronectin, and a central domain. The central domain with TIM-type barrel is made of tetramer subunits and acts as the active site (Huber et al., 1976). However, cleavage of tetramer to dimers causes the active site to inactivate. The α-peptide present at the n-terminal of lactase is involved in α-complementation and contributes in subunit interface (Corral et al., 2006).

5.2.1.2 Sources

Lactase can be found in fungi, bacteria as well as in yeasts. It can be mostly found in plants, like apples, apricots, peaches, and almonds. However, for industrial and commercial scale, lactase is usually acquired from fungi like *Aspergillus* and *Kluyveromyces* (Zhou & Chen, 2001).

Bacterial Sources

Lactase derived from bacterial sources possesses high activity, stability, and ease of fermentation, and hence, it is utilized to hydrolyse lactose. Studies have reported *Lactobacillus* and *Bifidobacterium* species to be an efficacious probiotic. As a result, they are commonly used to obtain lactase. It has been reported that lactase derived from bacterial strains like *Bifidobacterium longum* strain CCRC 15708, *Bifidobacterium infantis* strain CCRC 14633, and *Bifidobacterium longum* CCRC 15708 possess high enzyme activity (Hsu et al., 2007).

Yeast Sources

Lactase is extracted from yeast to produce lactose-free products. *Kluyveromyces lactis*, a variety of yeast found in dairy environment, is one of the most commercially used sources of lactase (Pivarnik & Senecal, 1995). The optimal pH of lactase obtained from a yeast lies between 6.0 and 7.0. Studies have reported that *Kluyveromyces marxianus*, another variety of yeast, can be utilized to produce homologous enzymes of lactase and other heterologous proteins that have the ability to thrive on a variety of substrates with lactose as their only source of energy. It has been found that a cold-active acidic lactase extracted from *Guehomyce spullulans*, a strain of psychrophilic yeast species, can be utilized for whey and milk hydrolysis in food industries (Saqib et al., 2017).

Fungal Sources

Fungi have been known to yield enzymes that are highly stable. Lactases obtained from fungal sources have an optimal acidic pH range of 2.5–5.4. This makes them highly potent for hydrolysing lactose of an acidic material such as whey. The most commonly used fungal sources to produce lactases that have been accepted as "generally recognized as safe" (GRAS) by FDA are *Kluyveromyces lactis*, *Kluyveromyces fragilis* (*Saccharomyces fragilis*), and certain *Aspergillus* species. Lactase extracted from *Aspergillus oryzae* is used on commercial scale and has been reported to be an effective source for whey utilization. Lactase generated from another fungus, that is, *Aspergillus niger*, is used to remove galactose residues from oligosaccharides and polysaccharides that are obtained from plant (Kazemi et al., 2016).

Plants

Lactase can also be obtained from a variety of plant sources like apple, tomato, mango muskmelon, avocado, coffee, kiwifruit, and Japanese pear plants (Seddigh & Darabi, 2014). These enzymes contribute to plant growth, ripening of fruits, and hydrolysis of lactose. It has been reported that this enzyme reduces galactosyl

content of cell wall of persimmons, causes cell wall hydrolysis in papaya, and facilitates the process of fruit ripening (Saqib et al., 2017).

5.2.1.3 Applications in Dairy Industry

Lactase has multiple industrial applications. They are used to produce lactose-free food products for patients suffering from lactase deficiency (Karasova et al., 2002). As a result of the hygroscopic nature of lactase, which leads to crystallization in foods, it is utilized for hydrolysing lactose to avert crystallization of lactose in desserts that are frozen and concentrated. This helps in reducing the lactose content of milk to be consumed by individuals with lactase deficiency (Champluvier et al., 1988). Additionally, on a commercial scale, this enzyme is also used to dispose whey. Whey disposal is a grave environmental concern as it is disposed into water streams, which leads to water pollution (Brandão et al., 1987). Hence, lactase is utilized to convert whey into useful products that have a range of applications in food industry such as ethanol and sweet syrup (Zhou & Chen, 2001).

Production of Hydrolysed Milk Products

A significant percentage of the world's population has been known to develop lactase deficiency which causes lactose intolerance (Sitanggang et al., 2016). The burden of lactose intolerance among the Asian population is nearly 90%. Individuals suffering from lactose intolerance can consume food products with either no or least amount of lactose in them. A solution to this problem is to treat milk or milk-based products with lactase. Lactase helps in pre-hydrolysing lactose in these products, thus reducing the concentration of lactose in them (Ramana Rao & Dutta, 1978).

Besides, lactase is also preferred to decrease the crystallization in frozen and condensed milk and milk products which occurs as a result of high concentration of lactose. Lactase also helps in improving the texture of milk and milk-based foods and in making them even more digestible. The by-products obtained from lactose hydrolysis (i.e. glucose and galactose) have been reported to be fermented easily. This helps in minimizing the total time required to attain the optimal pH in various milk-based foods like cottage cheese and yogurts. Additionally, lactase has been reported in reducing the calorie content of the end product by reducing the requirement of adding extra sweeteners (Domingues et al., 2005).

Whey Utilization

Cheese industry has been known to yield a massive amount of whey as a derivative. The chief constituents of whey include lactose, proteins, and minerals. The lactose in whey is associated with chemical oxygen demand and biochemical oxygen demand. Therefore, despite the fact that in developing countries a substantial amount of whey is used to produce permeates and protein concentrates, whey, when disposed in water streams, leads to severe water pollution. Nevertheless, whey, when converted into beneficial products such as ethanol as well as lactase, can be utilized for the production of lactose-free food products (Kokkiligadda et al., 2016). Additionally, treating whey with lactase can help in converting it into a substrate that is readily available for cell cultivation (Parashar et al., 2016). Another application of lactase

includes recovering of highly functional whey proteins like lactalbumin through ultrafiltration and subsequently hydrolysing them to manufacture numerous pharmaceutical intermediates (Domingues et al., 2005).

Production of Galacto-Oligosaccharides

Another application of lactase enzyme includes the production of galacto-oligosaccharides (GOS). GOS are nondigestible prebiotics and hence play a crucial role in improving human health by modifying intestinal microflora of human and promoting the growth of useful bacteria in the intestine such as *Lactobacillus* and *Bifidobacterium* species. GOS are produced from the transglycosylation activity of lactase during lactose hydrolysis. It can be stored at room temperature for longer period of time due to its acidic conditions. This characteristic of GOS makes it capable of being used for the production of different products without any decomposition. The oligosaccharide content in GOS ranges from 1% to 45% and depends further on the source of enzyme and the total amount of saccharides (Pazur, 1953).

GOS is also present in human milk and is known to help in increasing the natural microflora of small intestine of a breast-fed infant, that is *bifidobacteria*. This bifidogenic activity of GOS plays a vital role in reducing the number of pathogenic bacteria. In view of this characteristic, companies dealing with infant foods are now producing milk- and cereal-based food products containing GOS (Matto & Husain, 2006). Additionally, GOS also functions as soluble fibres and gets fermented by the natural microflora present in large intestine. This fermentation produces end products that are short-chain fatty acids that help in reducing the pH of fecal matter. The GOS is indigestible by the bacteria present in mouth; due to this reason they do not contribute to the development of cariogenicity (Matioli et al., 2003).

5.2.2 Amylase

Amylases are enzymes that degrade starch by catalysing the process of hydrolysis of internal α-1,4-glycosidic bonds of polysaccharides like maltose, glucose, dextrin, and maltotriose, while retaining the α-anomeric configuration in the products (Takata et al., 1992). They can be found in a variety of organisms. These enzymes are known to have multiple applications, and hence, hold a major share in market for the sales of enzymes (Vaidya et al., 2015). For centuries together, amylases derived from microbial and plant sources have been utilized in the brewing industry. Amylases extracted from fungal sources have been employed in the Asian cuisine (Hernández et al., 2006). The growing research interest in amylases obtained from bacterial, fungal, and viral sources in comparison to amylases that are derived from animals and plants sources can be ascribed to the ease in its large-scale production and industrial application (Mukherjee et al., 2009).

Amylases (EC 3.2.1.0) are classified into three subtypes that are α-amylase, ß-amylase, and γ-amylase. The two types of amylases—endoamylases and exoamylases, are categorized on the basis of the method by which the glycosidic bond is attacked (Vaidya et al., 2015). α-Amylases (α-1,4-glucan-glucanohydrolase,

EC 3.2.1.1) are extracellular enzyme (Cherry et al., 2004) that is responsible for degrading α-1,4-glucosidic linkage of starch and associated products and producing oligosaccharides (Baysal et al., 2008). β-Amylase (1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase, EC 3.2.1.2), another form of amylase, works from its non-reducing end and mobilizes the process of hydrolysis of the second α-1,4-glycosidic bond by cleaving two maltose units at a time. At the time of fruit ripening, this enzyme helps in cleaving starch present in the fruit and results in the sweet flavour of ripe fruit. γ-Amylase (EC 3.2.1.3) (Alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase), the third sub-type of amylases, is known to cleave α-(1–6)-glycosidic linkage along with the cleavage of the last α-(1–4)-glycosidic linkages at the non-reducing end of amylose and amylopectin to yield glucose. This sub-type has an optimum pH of 3, and hence, is efficacious in acidic conditions (Saini et al., 2017).

5.2.2.1 Structure

Amylase possesses a three-dimensional structure that enables it to bind to substrates. The high activity of specific catalytic groups helps in the cleavage of the glycoside links (Iulek et al., 2000). The human α-amylase is made of 512 amino acids in a single oligosaccharide chain. It comprises calcium and has a molecular weight of 57.6 kDa (Whitcomb & Lowe, 2007). Amylase possesses three domains: A, B, and C. The A domain which is the largest exhibits a classical barrel-shaped $(\beta/\alpha)_8$ super structure. The B domain is cemented to A domain by disulphide bond and is positioned between the A and C domains. The C domain possesses a sheet-like organization and is attached to the A domain by a simple polypeptide chain. The substrate binding site or the active site of the enzyme is positioned in a long cleft which is situated between the carboxyl end of the A and B domains. The calcium ion (Ca^{2+}) is positioned amid the A and B domains. This ion may serve in the process of stabilizing the three-dimensional structure of the enzyme and act as an allosteric activator. The active site of the enzyme comprises 5 sub-sites with the catalytic site located at sub-site 3. Substrate can attach to the first glucose residue present in sub-site 1 or 2, thereby permitting the division to take place between the first and second or second and third glucose residues (Whitcomb & Lowe, 2007).

5.2.2.2 Sources

Amylases are a group of enzymes produced in nature by plants, animals, and microorganisms.

Alpha Amylase

Alpha amylase can be isolated from a variety of sources like plants, animals, and microbes (Pandey et al., 2000). Although alpha amylase has been extracted from a variety of bacteria, fungi, actinomycetes, and yeasts, the enzymes obtained from fungi and bacteria have been found to have major industrial applications. This can be ascribed to their consistency, cost-effectiveness, less time and space requirement for their production, and ease of process optimization and modification (Ellaiah et al.,

2002). The microbial enzyme derived from the microbial sources is capable of meeting the industrial demand and a huge amount of them are commercially available. For centuries, amylases derived from microbes and plants have been used as food additives (Hernández et al., 2006). The bacterial amylases are mostly derived from *Bacillus* sp. like *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens*. Most of the fungal sources of amylases belong to terrestrial isolates like *Aspergillus* species (Jamal et al., 2011). The amylases derived from fungi are extensively utilized to prepare oriental foods (Popovic et al., 2009). The alpha amylases have also been isolated from filamentous fungi, which are high-yielding producers of extracellular proteins (Coradin et al., 2011).

5.2.2.3 Applications in Dairy Industry

Effect of Exogenous Amylase on Lactation Performance of Dairy Cows
Many researches have evidently exhibited the resistance of certain exogenous enzymes to the degradation in the rumen, which has the potential to augment the digestibility of feeds and eventually enhance animal performance (Hristov et al., 1998). Various studies have been focussed on the addition of fibrolytic enzymes to ruminant diets as fiber digestion is generally never amplified in the rumen (Beauchemin et al., 2003). Certain in vitro and in vivo evidences have reported that even though starch digestion in the rumen is not usually considered to be limiting, amylase enzymes contribute in enhancing the animal performance (Andreazzi et al., 2018). Intensive lactation augments the intake of feed and passage throughout the rumen. Although this increases the volume of starch passing the rumen without degradation, the capacity of small intestine to digest starch is limited. Researches have reported that supplementing the feed with α-amylase may enhance ruminal starch digestion without an intensive production of volatile fatty acids and increased risk for acidosis (Nozière et al., 2014).

5.2.3 Transglutaminase

All transglutaminases (EC 2.3.2.13), also known as protein-glutamine γ-glutamyltransferase, fall under the class of transferases (Marx et al., 2007; Trespalacios & Pla, 2007). Transglutaminases (TGases) can be found naturally in a variety of living organisms. They are known to participate in several biological functions, which include clotting of the blood and healing of wounds (Jaros & Rohm, 2016). They mobilize the formation of an iso-peptide bond between the group of γ-carboxamides of glutamine residues (donor) and the first order ε-amine groups of different compounds, for example, proteins (acceptors of an acyl residue). Additionally, in the absence of free amine groups, TGase catalyses deamination, where water serves as an acyl acceptor. TGase mobilizes the reactions that bring about substantial modifications in the physical and chemical properties of proteins, which include viscosity, elasticity, thermal stability, and resilience.

For the first time, TGases were derived from tissues of mammals and body fluids to investigate their applications in industry. The first commercially available TGase, extracted from the liver of guinea pig, was not found to be appealing candidate for industrial use ascribing to the limited supply and requirement of Ca2þ for activation which resulted in the high production cost of the enzyme. Likewise, another TGase known as Factor XIII, which was derived from blood plasma, exhibited a major drawback of red pigmentation and the requirement of thrombin for activation. In 1980s, during the screening of microorganisms for TGase activity, microbial TGase, which is independent of Ca2þ and possesses low substrate specificity, was identified from *Streptomyces mobaraensis.* Production of mTGase results from conventional fermentation of the wild-type *S. mobaraensis* strain (GRAS status) with consequent extraction of the protein that is secreted. Thus far, mTGase is the only commercially available TGase in the food industry (Jaros & Rohm, 2016).

5.2.3.1 Structure

mTGase, a monomeric protein with 331 amino acids, has an isoelectric point of 8.9. Its secondary structure comprises 8 β-strands adjoined with 11 α-helices. The enzyme acquires a disk-like shape that has a deep cleft at the edge. At this cleft is situated a single cysteine residue (Cys64). Molar mass of mTGase as evaluated by the technique of mass spectrometry and amino acid sequencing is 37.86 kDa. Usually, mTGase exhibits activity at pH range of 4 and 9. However, the optimum pH of the enzyme lies in the range 6–7. The optimum temperature for mTGase is 50 °C. Although it is immobilized at 70 °C within minutes, it maintains some residual activity at near-freezing temperatures. Another characteristic of mTGase is its stability under high hydrostatic pressure. This characteristic provides the prospects of alteration of proteins, even in situations where they are not available for mTGase under atmospheric pressure (Jaros & Rohm, 2016).

5.2.3.2 Sources

TGases are widely spread in nature (Kashiwagi et al., 2002). They can be derived from various invertebrates and microbial cells (Yu et al., 2008; Griffin et al., 2002) as well as mammalian tissues (Yasueda et al., 1994). TGases can also be found in tissues of various plants such as orchard apple, soy, fodder beet, and topinambour (Falcone et al., 1993). These enzymes have been extracted from microbes belonging to *Streptoverticillium* sp. and *Physarum polycephalum*. TGases are biosynthesized as an extracellular enzyme by *Streptoverticillium* sp., *Streptoverticillium cinnamoneum* subsp. *cinnamoneum* (Duran et al., 1998), *Streptomyces netropsis* (Yu et al., 2008), *Streptoverticillium griseocarneum* (Gerber et al., 1994), *Streptoverticillium ladakanum* (Ho et al., 2000), and *Streptomyces lydicus* (Færgemand & Qvist, 1997).

5.2.3.3 Applications in Dairy Industry

Various researches have reported the commercial availability of TGases and their crucial role in enhancing protein functionality in dairy products. It has been found that these enzymes avert syneresis in yoghurts and result in firmer yet soft texture

(Kieliszek & Misiewicz, 2014). A study reported that TGases at the level of 0.02% improve melting, firmness, flexibility, and organoleptic properties of high fat Mozzarella cheese. However, in the case of Mozzarella with low fat content, 0.05% of TGase provides adequate meltability, flexibility, free oil formation, and organoleptically to the cheese. The cross-linking property of TGase helps in augmenting soft cheese production, reducing syneresis, extending shelf life, and decreasing nutrient bioavailability for deteriorative microorganisms (Mahmood, 2009). It has also been reported that this enzyme can be utilized to partially replace fat in ice-creams, and it is also responsible for increasing overrun, partial amalgamation of fat globules, consistency, melting resistance, firmness, and viscosity in ice cream (Tafes, 2019).

A study reported that up to 0.5% of TGase was more efficacious to enhance the functional properties of yogurt that was made from goat milk. The cross-linking property of TGase was found to be accountable for enhancing the gel consistency and reducing the whey separation substantially in yoghurt (Farnsworth et al., 2006). Another study reported that when stirred yogurt was developed through covalent cross-linking by the combined action of inactivated mTGase and glutathione, no negative effects on the fermentation of yogurt were observed. However, a substantial rise in apparent viscosity as well as protein polymerization was observed in yogurt that was only developed by TGase (Bönisch et al., 2007). The use of TGase in high concentration was found to reduce syneresis and increase the viscosity of yogurt. However, a minor bottleneck was observed with regard to the growth of Lactic Acid Bacteria. This led to reduced production of acid and acetaldehyde in comparison to those reported for the control. Nevertheless, TGase concentration up to 0.3 g/L was reported to be optimum and was recognized as an acceptable substitute to stabilizers to be added in the production of fat-free yogurt (Ozer et al., 2007). Studies identified TGase as an efficacious candidate to enhance the physical properties of yogurt when its constitution was modified by the addition of whey (Şanli et al., 2011).

5.3 Application in Dairy Industry

5.3.1 Lactose-Free Milk

Low levels of lactase production a human beings has been known to be a leading cause of "lactose intolerance", which is an intestinal symptomatic condition. A lactose-intolerant individual may experience clinical symptoms like nausea, abdominal pain, flatulence, diarrhea, and bloating after consuming food that contains lactose. It has been estimated that about 70% of the world population, including all the age groups, is suffering from lactase deficiency (Xavier et al., 2018). Lactose-intolerant individuals can consume dairy-fermented products that comprise very little or no lactose in them (Saqib et al., 2017). Currently, there is an increase in demand for lactose-free dairy among the lactose-intolerant populations. This can be ascribed to the presence of little or no lactose in these products and their ability to provide vital macro- and micro-nutrients present in milk to individuals suffering from lactose intolerance (Dekker et al., 2019).

The dairy industry employs different processing techniques to reduce the lactose content in the dairy products to produce lactose-free products. Cheese production involves certain techniques which help in reducing the lactose content. For example, Gouda cheese production involves a curd washing step to reduce the lactose content. Although the lactose content in most of the cheeses is already relatively low without ripening, in several other types of cheeses, the lactose content is reduced during the process of ripening with the help of lactic acid bacteria. Generally, aged and hard cheeses like Parmesan, Swiss, or Cheddar cheeses have a very low concentration of lactose. Contrariwise, young and fresh cheeses may consist enough lactose that may be capable of inducing a reaction among individuals with lactose intolerance, depending on the quantity consumed. Butter is another dairy product with low lactose content. The majority of water-soluble constituents of milk inclusive of lactose are eliminated, during the production of butter resulting in the reduction of lactose concentration in butter (Dekker et al., 2019).

Besides reducing the lactose content in dairy products, another solution to produce lactose-free dairy products includes hydrolysing lactose into glucose and galactose using the lactase enzyme. The monosaccharides produced get easily adsorbed in the small intestine and avert the incidence of symptoms associated with lactose intolerance. Currently, neutral lactases and acid lactases are the two kinds of commercially available lactases (Dekker & Daamen, 2011). The former is chiefly employed on an industrial scale to produce lactose-free dairy products, while the latter is provided as a dietary supplement to the consumers for its consumption along with regular dairy products to induce cleavage of lactose in the stomach (Dekker et al., 2019).

5.3.2 Production of Lactose-Free Milk

Researches have reported that lactose dosage of less than 12 g per meal may result in mild symptoms among lactose-intolerant individuals; however, the dairy industry aims to maintain the lactose content as low as possible (Suchy et al., 2010). Currently, there does not exist global agreement on the regulatory requirements for lactose-free claims. In the past, lactose reduction to 0.5% or 0.1% was considered enough by the dairy producers, but, in some countries, lactose content less than 0.01% is considered to be the current requirement to consider milk to be lactose-free. To achieve such low lactose concentration in milk production entails special attention to the milk processing, to the dosage and efficiency of the enzyme used in this process, and to sensitive analytical methods to determine such low amounts of lactose (van Scheppingen et al., 2017).

Presently, there exist two processes that are used in the production of lactose-free milk (Troise et al., 2016; Harju, 2004). These include batch process and aseptic process and both of them use soluble lactase enzyme. Although various studies have suggested the processes that rely on an immobilized enzyme, these processes have not been employed in industrial practice to produce lactose-free milk either in the

past or in present due to issues related to the microbial stability of the end product (Finocchiaro et al., 1980).

5.3.2.1 Batch Process (Pre-Hydrolysis)

The batch process involves the addition of a neutral sample of lactase to a tank containing raw milk and subsequent incubation for approximately 24 h accompanied with continuous slow stirring in order to prevent creaming. In order to prevent microbial growth in the milk, which is not yet sterile, cool conditions with temperature ranging between 4–8 °C are maintained to perform this process. Once the process of incubation is completed, the milk is then pasteurized, homogenized, and packaged.

It is important for the enzyme dosage to be adequate to reach the requisite limit for removing lactose at low temperature of the incubation and during the set time period. It is because of this reason that the enzyme dosage for this process is kept fairly high and enzymes are selected based on their high activity at neutral temperature. The batch incubation is discontinuous as it involves a tank in the factory, delaying the pasteurization of the milk by a day. This may prove to be a setback for certain factories during high productivity. In conditions like these, lactase enzyme having higher specific activity may be helpful in reducing the production time, thereby increasing the total yield of the factory. Additionally, to prevent microbial spoilage of milk that may result due to delay in pasteurization of the milk, it is important for the milk quality to be impeccable.

Generally, the lactose-free milk produced with the batch process is comparatively unresponsive to the side activities of the enzyme. This can be ascribed to the short storage period of the milk at refrigerated conditions and to the process of pasteurization or sterilization after incubation of the enzyme, which is responsible for the inactivation of most of the enzymatic activities. In the past, certain lactase preparations displayed proteolytic side activities; however, these issues seem to be resolved with only rare complaints occurring for the lactose-free milk produced with the batch process (Mittal et al., 1991).

Techniques like chromatography and ultra- and nano-filtration have been employed in combination with lactose hydrolysis to avoid the doubling of the milk sweetness (Harju et al., 2012; Jelen & Tossavainen, 2003). This results in the production of lactose-free milk of outstanding quality, with its taste nearly alike the regular milk.

5.3.2.2 Aseptic Process (Post-Hydrolysis)

The aseptic process involves the sterilization of the milk using the UHT technique and the subsequent injection of the prepared sterile lactase into the milk immediately before packaging (Dahlqvist et al., 1977). This packaged UHT milk is then kept quarantined for almost 3 days at optimum temperature, which provides adequate time for lactose hydrolysis to occur before the milk is distributed to the retailer. The aseptic process is not employed for pasteurized milk for the production of lactose-free milk, due to the absence of quarantine period (Dekker et al., 2019).

Fundamentally, there exist two techniques to obtain sterile lactase enzyme. The first technique involves pre-sterilization of lactase by the enzyme manufacturer and usage of distinct sterile dosing equipment that is required for the sterile injection. The second technique involves the filter-sterilization of the unsterile enzyme immediately before incorporating it to the sterilized milk at the dairy industry (Dekker et al., 2019).

Considering the high incubation time and temperature required in this process, the enzyme dosage can be kept much lower. Process control in this process is not present as the enzyme is vigorous only in the final milk package. The dairy manufacturer should also consider the aspects such as deviation in the storage temperature in un-thermostated warehouses, resulting from the seasonal transitions such as summer to winter, when dosing the enzyme (Dekker et al., 2019).

During the aseptic process, special equipment and consumable costs are essential requirements specifically for the in-factory filtration. Highly skilled operators are employed during the process of lactose injection in order to prevent microbial contamination in the milk. When organized appropriately, the process can function as a completely continuous operation, thus making the aseptic process a desired technique in industries requiring a high yield (Dekker et al., 2019). The aseptic process for the production of lactose-free UHT milk can only be considered to be a fully developed technique after the crucial refinement of the lactase enzyme quality. Researches have reported that the arylsulfatase side activity in the preparation of lactose may result in serious medicinal off-flavours during storage. This has been ascribed to formation of *p*-cresol from sulphonated-cresol, which occurs naturally in the milk (De Swaaf et al., 2006). Currently, arylsulfatase-free lactase enzymes are available commercially. It is important for a lactose-free UHT milk producer to consider utilizing only the supreme quality lactase enzymes for this process in order to avert shelf life-related complication (Dekker et al., 2019).

Lactose hydrolysis in milk has been known to augment the monosaccharides' content, resulting in an efficient Maillard reaction, which may be amplified due to the inadequate proteolysis by proteases. These proteases may be present naturally in the milk or they may originate from the lactase preparation. The amplified reactions lead to the increase in the formation of off-flavours and greater browning of lactose-free milk in comparison to regular milk. When stored at higher temperature, it also results in a reduced nutritional value of the product (Troise et al., 2016; Jansson et al., 2014; Evangelisti et al., 1999). The enhanced maillard reaction may be considered as the critical determinant of the decreased shelf life of lactose-free UHT milk in comparison to regular UHT milk. In the past, various researches have suggested that the batch process for the production of lactose-free UHT milk may result in comparatively higher browning than the milk produced using the aseptic process (Mendoza et al., 2005). However, recent studies have reported the relevance of the storage conditions (temperature) and lactase quality for determining shelf life (Troise et al., 2016). Excellent shelf life was found for lactose-free UHT milk produced with the batch process, and milk browning during storage is, therefore, largely independent of the production process that is used (Dekker et al., 2019).

5.3.3 Other Dairy Products

Besides lactose-free milk, various other lactose-free dairy products are manufactured with the use of enzymatic treatment. Flavoured milk is one such product that is produced using a similar process (McCain et al., 2018). The comparatively stronger production of colour and flavour by the flavoured lactose-free milk has been reported to reduce browning and production of off-flavours due to the maillard reaction. Unlike regular lactose-free milk, the flavoured lactose-free milk requires less addition of extra sugar, due to the lactose treatment; however, in certain flavoured milks like highly sugared chocolate milk, the process of lactose hydrolysis may not be adequate for complete replacement of sugar addition (Li et al., 2015), and thus there may be a requirement to add additional sweeteners. Another lactose-free dairy product is dairy powder, which can be developed from either milk or whey, which is made lactose-free with the batch process. A major setback reported during the production of lactose-free dairy powder is the high monosaccharide content in the treated milk, which causes a reduction in the glass-transition temperature. This phenomenon further results in the contamination of the spray dryer when the drying conditions are not adjusted (Torres et al., 2017). Additionally, packaging of the lactose-free dairy powder should be done cautiously as the high hygroscopic nature of the powder results in its caking during storage. It is because of these challenges that the lactose-free dairy powders are not a part of big markets like regular milk powders. Cheese is yet another dairy product that can be made lactose-free. This is done using lactase enzyme to incubate the cheese milk before renneting. This method is used majorly for cheeses that are young and fresh, as they comprise a considerable quantity of lactose. In the case of ripened cheeses, lactase incubation is not required as lactose gets completely consumed by the lactic acid bacteria. The studies conducted in the past have reported that unlike yoghurt, lactase treatment of cheese milk stimulates the process of acidification during cheese manufacturing. Furthermore, the addition of lactase during the process of ripening enhances the flavour of cheese. It is unclear if these effects are a result of the stimulation of the cheese microbial flora caused either by lactose hydrolysis or residual proteolytic activity occurring during lactase preparations that were available commercially in the past (Marschke et al., 1980). Lactose-free ice cream can also be produced using either lactose-free milk or powders in the ice cream mix (Abbasi & Saeedabadian, 2015). The production of lactose-free ice cream involves the addition of lactase during the ageing period before freezing, once the process of pasteurization and incubation is complete. The lactose hydrolysis increases the monosaccharide concentration which reduces the freezing point of the ice cream. This results in the production of ice cream with a soft texture at the same temperature. Although this may be a desirable attribute in several frozen desserts, it may also cause faster melting. Additionally, the sweetness resulting due to the lactose hydrolysis may help in reducing the addition of extra sugar in the ice creams, and consequently, raising the melting temperature again. In order to avert crystallization of lactose in the ice creams which causes a sensory defect known as "sandiness", lactase treatment is employed to convert lactose into two monosaccharides—glucose and

galactose, which are highly soluble at high temperature. This treatment is essential when ice cream is produced using whey powder or WPC as the high lactose content may result in the formation of crystals during freezing (Abbasi & Saeedabadian, 2015).

5.4 Lipase

Lipase, considered as triacylglycerol acylhydrolase, EC 3.1.1.3, is a ubiquitous enzyme belonging to the family of hydrolases which reacts with carboxylic ester bonds and is present everywhere, i.e. in plants, animals, and microorganisms. These enzymes are obtained from plant and animal tissues or through the cultivation of microbes. Also, the commercial production of lipase is majorly done with the help of microorganisms such as by genetic engineering through recombinant bacteria and yeasts (Houde et al., 2004; Schmid & Verger, 1998). Naturally, lipase enzymes are useful in hydrolysing fats and oils. They lead to the production of free fatty acids, glycerol, diacylglycerols, and monoacylglycerols from the hydrolysis of triacylglycerols by acting as a catalyst in the reaction. Also, this hydrolysis reaction is reversible in order to allow the enzymes to catalyse the hydrolysis of acylglycerols through free fatty acid and glycerol (Balcao & Malcata, 1998; Macrae & Hammond, 1985) (Fig. 5.1).

Nowadays, due to high catalytic performance, lipase is gaining wide attention in industries such as for flavor development in food industry, in paper manufacturing industry, and more important role in dairy industry. It can help in providing enhanced digestibility, structure, physical properties, and reduced calorie in dairy products (Balcao & Malcata, 1998; Bjorkling et al., 1991).

5.4.1 Characteristics of Lipase

Lipase enzyme is considered as a monomeric protein with molecular weight of 19–60 kDa. Its physical properties are dependent upon chain length of fatty acid,

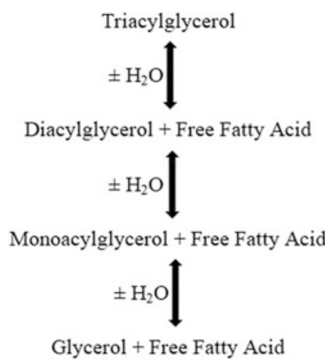


Fig. 5.1 Reaction process of lipase enzyme (Macrae & Hammond, 1985)

position of fatty acid in glycerol backbone, and degree of unsaturation. Also, its activity depends upon the pH of the solution such as lipase is stable at neutral pH and pH 4.0–8.0. For the catalytic activity of enzyme lipase, greater mild conditions and large surface area are required (Aravindan et al., 2007). Among the different lipases produced from plants, animals, and microorganisms, the most vital are microbial lipases. Microbial lipases are gaining greater attention due to their non-toxic nature, convenience, high catalytic activity, greater yield, rapid growth, and usage due to absence of seasonal interference as well as availability of cheaper media for growth (Ray, 2012). Microbial lipases are utilized in different areas such as in cosmetics, detergents, pharmaceuticals, formation of aliphatic acids, leather industry, and in treatment of waste excreted from different industries (Seitz, 1974).

Lipases obtained from microorganisms are broadly classified into two major categories on the basis of their specificity, namely, non-specific and 1,3-specific lipases. Non-specific lipases are those lipases which catalyse the reactions independent of the position esterified in glyceride molecules such as lipases obtained from *Staphylococcus aureus*, *Pseudomonas* spp., *Candida cylindracea*, and *Chromobacterium viscosum*. On the other hand, 1,3-specific lipases are those which react and catalyse only outer, i.e. *sn*-1 and *sn*-3, positions of the glycerol backbone such as lipases obtained from *Rhizopus oryzae*, *Rhizopus delemar*, *Rhizopus niveus*, *Aspergillus niger*, *Mucor javanicus*, *Candida lipolytica*, and *Humicola lanuginose* (Balcao & Malcata, 1998).

Lipases are used as biocatalysts, especially microbial lipases, in various industries such as food industry due to their various functions and properties such as regioselectivity, stereospecificity, able to function in different pH and temperature, substrate specificity, and potential to act upon heterogeneous reactions at the interface between water-soluble and water-insoluble systems (Verma & Kanwar, 2008). On the other hand, bacterial lipases are stable at a temperature range of 30–60 °C and under neutral or alkaline pH except the lipase produced from *P. fluorescens* SIK W1, which is stable under acidic pH of 4.8. Bacterial lipases are also stable in organic solvents (Verma et al., 2012).

5.4.2 Applications of Lipase in Dairy Industry

Lipase has a wide range of applications in dairy industry such as in improving the flavor and modifying the fatty acid chain of dairy products including cheese. The major role of lipases in dairy industry involves the hydrolysis of milk fat. Tissues from animals such as pancreatic glands, i.e. bovine and porcine, and pre-gastric tissues of young ruminants, i.e. lamb and calf for lipase production, are widely used in traditional cheese flavor enhancement process (Ray, 2012).

Recent applications of lipase in dairy industry involve the production of cheese, its flavor enhancement, and lipolysis of cream and butterfat. Lipases are utilized in the manufacturing of enzyme-modified cheese for providing concentrated flavor to cheese. In this process, cheese is incubated with enzymes at high temperature and thus leads to the formation of enzyme-modified cheese which can be used as an

ingredient in various products such as sauces, dressings, dips, snacks, and soups. Addition of lipases in the production of dairy products leads to the release of short chain fatty acids, namely C4 and C6, which provide sharp and tangy flavor to the products along with the release of medium chain fatty acids, namely C12 and C14, which provide soapy taste to the finished products (Hasan et al., 2006). Exogenous lipase enzyme helps in accelerating the cheese ripening process. On the other hand, addition of free lipases results in greater lipolysis which deteriorates the texture as well as flavor of cheese, but encapsulating the lipase helps in maintaining and stabilizing the enzyme-substrate ratio, thus eliminating the problem of excessive lipolysis (Houde et al., 2004). It is also used in the manufacturing of chocolates by production of cocoa butter-equivalent from palm oil with the technique using interesterification. There are many commercial lipase enzymes which are used for the production of different milkfat flavor profiles for milk-based products, such as Snow plum blossom and Palatase 20,000 L (Sarmah et al., 2017).

5.4.3 Other Applications of Lipase

In addition to the dairy industry, lipase also plays a vital role and possesses various applications in other industries such as cosmetics, pharmaceutical, leather, paper, food industry, etc. In food industry, lipase is widely used in the preparation of infant formulas which can provide an alternative of breast milk. The enzyme is also used for the modification of lipid characteristics of oils rich in omega-6 fatty acids such as coconut, sunflower, olive, rice bran, corn oil, and oils rich in omega-3 fatty acids such as linseed and fish oil. In pharmaceutical industries, lipase is used as an ingredient in the formation of products and also utilized in the interesterification of vegetable oil. It helps in enhancement of colour and texture of the dough and used as an emulsifier in many bakery industries. Lipase is also essential in the formation of cosmetic component, i.e. isopropyl myristate, for manufacturing of cosmetic items (Sarmah et al., 2017; Houde et al., 2004).

Lipases are also utilized in detergent industry as an additive in household and laundry detergents in order to vanish the oil stains from the fabrics as these enzymes can withstand harsh pH (10–11) and temperature (30–60 °C) required for washing. It also plays a vital role in paper manufacturing industry by removing the pitch, which is a hydrophobic portion of wood, i.e. waxes and triglycerides, from the pulp required for manufacturing paper (Sharma et al., 2001). In addition to this, microbial lipases such as *Candida rugosa*, *Mucor miehei*, *Pseudomonas fluorescens*, *Rhizopus oryzae*, and *Aspergillus niger* are used for the production of biodiesel. Presently, lipase produced from the microbe *Streptomyces* sp. was considered as most effective for biodiesel production (Chandra et al., 2020).

5.4.4 Introduction of Rennet

Rennet is obtained from the combination of two proteolytic enzymes, namely chymosin and pepsin, which are secreted from the fourth stomach of ruminants including calves, lambs, and kids. It is used in the cheese production from historical times, i.e. 6000 BC. Rennet is majorly available in powder, liquid, and paste forms. The powder and liquid rennet is produced on industrial scale and is obtained from calf abomasa, whereas rennet in paste form is obtained from lamb or kid abomasa. Powder and liquid rennet is most widely utilized in cheese production. Along with rennet, other enzymes produced from different origin are also utilized in manufacturing of cheese (Moschopoulou, 2011). Among the two proteolytic enzymes, chymosin is the most essential protease present in rennet. Chymosin is an aspartic protease which has the properties like pepsin and it cleaves *k*-casein in para-*k*-casein along with a glycomacropeptide (Fazouane-Naimi et al., 2010).

Clotting of milk with the help of rennet takes place in two step reaction; first includes the enzymic hydrolysis reaction and second step involves enzyme-independent protein aggregation reaction. As the concentration of enzyme increases, clotting time of milk decreases because of the greater amount of proteolysis of *k*-casein (Najera et al., 2003).

5.4.5 Different Types of Rennet

Different types of rennet are used in cheese manufacturing which are classified on the basis of their source such as animal rennet, plant rennet, and fungal rennet. Under the classification of animal rennet, calf rennet is widely utilized for cheese production, the reason being its high amount of chymosin. Presently, adult bovine rennet is used as an alternative towards calf rennet due to high pepsin content in adult bovine rennet that results in a product with high pH sensitivity and greater proteolytic activity (Harboe et al., 2010).

Many proteolytic enzymes are derived from plants such as bromelain from pineapple, ficin from *Ficus* spp., and papain from papaya. Other plants include fig, pumpkin, soybean, ash gourd, and milkweed. The enzymes are embedded in the buds, leaves, flowers, seeds, latex, and roots of the plants. But the proteolytic capability of enzymes from vegetable sources is low which makes them unsuitable for cheese production. And if excessive proteolysis occurs, it will lead to bitterness in the finished products. Although for cottage cheese and soft cheese production, extract from berries of *Withania coagulans* was used, it is also not suitable for cheddar cheese manufacturing (Garg & Johri, 1994).

Rennet clotting enzymes are also produced from fungi such as rennet from *Mucor miehei* and *Endothia parasitica*. Enzyme obtained from *Mucro miehei* results in an excellent quality of cheddar cheese, even with high ripening process due to greater stability between 4.0 and 6.0 pH with no loss of its activity and also due to its high heat stability. However, rennet obtained from *Endothia parasitica* is also useful in

cheddar cheese production, but its high proteolytic activity leads to 1.2% yield loss of the cheese (Brown & Ernstrom, 1988).

References

Abbasi, S., & Saeedabadian, A. (2015). Influences of lactose hydrolysis of milk and sugar reduction on some physical properties of ice cream. *Journal of Food Science and Technology, 52*(1), 367–374. https://doi.org/10.1007/s13197-013-1011-1

Andreazzi, A. S. R., Pereira, M. N., Reis, R. B., Pereira, R. A. N., Morais Júnior, N. N., Acedo, T. S., Hermes, R. G., & Cortinhas, C. S. (2018). Effect of exogenous amylase on lactation performance of dairy cows fed a high-starch diet. *Journal of Dairy Science, 101*(8), 7199–7207. https://doi.org/10.3168/jds.2017-14331

Aravindan, R., Anbumathi, P., & Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology, 6*, 141–158.

Balcao, V. M., & Malcata, F. X. (1998). Lipase catalyzed modification of milkfat. *Biotechnology Advances, 16*, 1–33.

Baysal, Z., Uyar, F., Doğru, M., & Alkan, H. (2008). Production of extracellular alkaline α-amylase by solid state fermentation with a newly isolated Bacillus sp. *Preparative Biochemistry and Biotechnology, 38*(2), 184–190. https://doi.org/10.1080/10826060701885167

Beauchemin, K., Colombatto, D., Morgavi, D., & Yang, W. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science, 81*(14_Suppl_2), E37–E47. https://doi.org/10.2527/2003.8114_suppl_2E37x

Bjorkling, F., Godtfredsen, S. E., & Kirk, O. (1991). The future impact of industrial lipases. *Trends in Biotechnology, 9*(1), 360–363. https://doi.org/10.1016/0167-7799(91)90119-3

Bönisch, M. P., Huss, M., Weitl, K., & Kulozik, U. (2007). Transglutaminase cross-linking of milk proteins and impact on yoghurt gel properties. *International Dairy Journal, 17*(11), 1360–1371. https://doi.org/10.1016/j.idairyj.2007.01.019

Brandão, R. L., Nicoli, J. R., & de Souza Figueiredo, A. F. (1987). Purification and characterization of a β-galactosidase from Fusarium oxysporum var. lini. *Journal of Dairy Science, 70*(7), 1331–1337. https://doi.org/10.3168/jds.S0022-0302(87)80152-2

Brown, R. J., & Ernstrom, C. A. (1988). Chapter 12: Milk-clotting enzymes and cheese chemistry. In *Fundamentals of dairy chemistry* (pp. 609–654). Van Nostrand Reinhold. https://doi.org/10.1007/978-1-4615-7050-9_12

Callahan, B. P., & Miller, B. G. (2007). OMP decarboxylase—An enigma persists. *Bioorganic Chemistry, 35*(6), 465–469. https://doi.org/10.1016/j.bioorg.2007.07.004

Carrara, C. R., & Rubiolo, A. C. (1994). Immobilization of β-galactosidase on chitosan. *Biotechnology Progress, 10*(2), 220–224. https://doi.org/10.1021/bp00026a012

Champluvier, B., Kamp, B., & Rouxhet, P. G. (1988). Immobilization of β-galactosidase retained in yeast: Adhesion of the cells on a support. *Applied Microbiology and Biotechnology, 27*(5–6), 464–469. https://doi.org/10.1007/BF00451614

Chandra, P., Enespa, Singh, R., & Arora, P. K. (2020). Microbial lipases and their industrial applications: A comprehensive review. *Microbial Cell Factories, 19*(1), 2–42. https://doi.org/10.1186/s12934-020-01428-8

Cherry, H. M., Hossain, M. T., & Anwar, M. N. (2004). Extracellular glucoamylase from the isolate Aspergillus fumigatus. *Pakistan Journal of Biological Sciences, 7*(11), 1988–1992. https://doi.org/10.3923/pjbs.2004.1988.1992

Coradin, J. H., Braun, A., Viccini, G., de Lima Luz, L. F., Krieger, N., & Mitchell, D. A. (2011). A three-dimensional discrete lattice-based system for modeling the growth of aerial hyphae of filamentous fungi on solid surfaces: A tool for investigating micro-scale phenomena in solid-state fermentation. *Biochemical Engineering Journal, 54*(3), 164–171. https://doi.org/10.1016/j.bej.2011.02.012

Corral, J. M., Bañuelos, O., Adrio, J. L., & Velasco, J. (2006). Cloning and characterization of a β-galactosidase encoding region in Lactobacillus coryniformis CECT 5711. *Applied Microbiology and Biotechnology, 73*(3), 640–646. https://doi.org/10.1007/s00253-006-0510-7

Dahlqvist, A., Asp, N. G., Burvall, A., & Rausing, H. (1977). Hydrolysis of lactose in milk and whey with minute amounts of lactase. *Journal of Dairy Research, 44*(3), 541–548. https://doi.org/10.1017/S0022029900020495

De Swaaf, M. P. M., van Dijk, A. A., Edens, L., & Dekker, P. J. T. (2006). Enzyme preparation yielding a clean taste. EP1954808 B1, November 28, 2006.

Dekker, P. J. T., & Daamen, C. B. G. (2011). Enzymes exogenous to milk in dairy technology: β-d-galactosidase. In *Encyclopedia of dairy sciences* (2nd ed., pp. 276–283). Academic Press. https://doi.org/10.1016/B978-0-12-374407-4.00152-7

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-free dairy products: Market developments, production, nutrition and health benefits. *Nutrients, 11*(3), 1–14. https://doi.org/10.3390/nu11030551

Domingues, L., Lima, N., & Teixeira, J. A. (2005). Aspergillus niger β-galactosidase production by yeast in a continuous high cell density reactor. *Process Biochemistry, 40*(3–4), 1151–1154. https://doi.org/10.1016/j.procbio.2004.04.016

Duran, R., Junqua, M., Schmitter, J. M., Gancet, C., & Goulas, P. (1998). Purification, characterisation, and gene cloning of transglutaminase from Streptoverticillium cinnamoneum CBS 683.68. *Biochimie, 80*(4), 313–319. https://doi.org/10.1016/S0300-9084(98)80073-4

Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., & Srinivasulu, B. (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated Aspergillus species. *Process Biochemistry, 38*(4), 615–620. https://doi.org/10.1016/S0032-9592(02)00188-7

Evangelisti, F., Calcagno, C., Nardi, S., & Zunin, P. (1999). Deterioration of protein fraction by Maillard reaction in dietetic milks. *Journal of Dairy Research, 66*(2), 237–243. https://doi.org/10.1017/S0022029999003453

Færgemand, M., & Qvist, K. B. (1997). Transglutaminase: Effect on rheological properties, microstructure and permeability of set style acid skim milk gel. *Food Hydrocolloids, 11*(3), 287–292. https://doi.org/10.1016/s0268-005x(97)80058-6

Falcone, P., Serafini-Fracassini, D., & Del Duca, S. (1993). Comparative studies of transglutaminase activity and substrates in different organs of Helianthus tuberosus. *Journal of Plant Physiology, 142*(3), 265–273. https://doi.org/10.1016/S0176-1617(11)80421-9

Farnsworth, J. P., Li, J., Hendricks, G. M., & Guo, M. R. (2006). Effects of transglutaminase treatment on functional properties and probiotic culture survivability of goat milk yogurt. *Small Ruminant Research, 65*(1–2), 113–121. https://doi.org/10.1016/j.smallrumres.2005.05.036

Fazouane-Naimi, F., Mechakra, A., Abdellaoui, R., Nouani, A., Daga, S. M., Alzouma, A. M., Gais, S., & Penninckx, M. J. (2010). Characterization and cheese-making properties of rennet-like enzyme produced by a local Algerian isolate of *Aspergillus niger*. *Food Biotechnology, 24*(3), 258–269. https://doi.org/10.1080/08905436.2010.507149

Felicilda-Reynaldo, R. F. D., & Kenneally, M. (2016, March 2–3). *Therapy: Pancreatic enzymes*.

Finocchiaro, T., Olson, N. F., & Richardson, T. (1980). Use of immobilized lactase in milk systems. *Advances in Biochemical Engineering, 15*, 71–88. https://doi.org/10.1007/3540096868_3

Fox, P. F. (2002). Significance of indigenous enzymes in milk and dairy products. In *Handbook of food enzymology* (pp. 270–293). CRC Press.

Garg, S. K., & Johri, B. N. (1994). Rennet: Current trends and future research. *Food Reviews International, 10*(3), 313–355. https://doi.org/10.1080/87559129409541005

Gerber, U., Jucknischke, U., Putzien, S., & Fuchsbauer, H. L. (1994). A rapid and simple method for the purification of transglutaminase from Streptoverticillium mobaraense. *Biochemical Journal, 299*(3), 825–829. https://doi.org/10.1042/bj2990825

Godfrey, T., & Reichelt, J. (1983). *Industrial enzymology: The application of enzymes in industry* (1st ed.). Macmillan Publishers Ltd.

Griffin, M., Casadio, R., & Bergamini, C. M. (2002). Transglutaminases: Nature's biological glues. *Biochemical Journal, 368*(2), 377–396. https://doi.org/10.1042/BJ20021234

Harboe, M., Broe, M. L., & Qvist, K. B. (2010). Chapter 3: The production, action and application of rennet and coagulants. In *Technology of cheesemaking* (2nd ed., pp. 98–125). John Wiley & Sons, Ltd.

Harju, M. (2004). Chromatographic and enzymatic removal of lactose from milk. *Bulletin of the International Dairy Federation, 389*, 4–8.

Harju, M., Kallioinen, H., & Tossavainen, O. (2012). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal, 22*(2), 104–109. https://doi.org/10.1016/j.idairyj.2011.09.011

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology, 39*, 235–251. https://doi.org/10.1016/j.enzmictec.2005.10.016

Hernández, M. S., Rodríguez, M. R., Guerra, N. P., & Rosés, R. P. (2006). Amylase production by Aspergillus niger in submerged cultivation on two wastes from food industries. *Journal of Food Engineering, 73*(1), 93–100. https://doi.org/10.1016/j.jfoodeng.2005.01.009

Ho, M. L., Leu, S. Z., Hsieh, J. F., & Jiang, S. T. (2000). Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from Streptoverticillium ladakanum. *Journal of Food Science, 65*(1), 76–80. https://doi.org/10.1111/j.1365-2621.2000.tb15959.x

Houde, A., Kademi, A., & Leblanc, D. (2004). Lipases and their industrial applications. *Applied Biochemistry and Biotechnology, 118*, 155–170.

Hristov, A. N., McAllister, T. A., & Cheng, K. J. (1998). Stability of exogenous polysaccharide-degrading enzymes in the rumen. *Animal Feed Science and Technology, 76*(1–2), 161–168. https://doi.org/10.1016/S0377-8401(98)00217-X

Hsu, C. A., Lee, S. L., & Chou, C. C. (2007). Enzymatic production of galactooligosaccharides by β-galactosidase from Bifidobacterium longum BCRC 15708. *Journal of Agricultural and Food Chemistry, 55*, 4–9. Retrieved from http://pubs.acs.org/doi/full/10.1021/jf063126+%0Apapers3://publication/doi/10.1021/jf063126

Huber, R. E., Kurz, G., & Wallenfels, K. (1976). A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (E. coli) on lactose. *Biochemistry, 15*(9), 1994–2001. https://doi.org/10.1021/bi00654a029

Iulek, J., Franco, O. L., Silva, M., Slivinski, C. T., Bloch, C., Rigden, D. J., & Grossi De Sá, M. F. (2000). Purification, biochemical characterisation and partial primary structure of a new α-amylase inhibitor from Secale cereale (rye). *International Journal of Biochemistry and Cell Biology, 32*(11–12), 1195–1204. https://doi.org/10.1016/S1357-2725(00)00053-4

Jamal, P., Idris, Z. M., & Alam, M. Z. (2011). Effects of physicochemical parameters on the production of phenolic acids from palm oil mill effluent under liquid-state fermentation by Aspergillus niger IBS-103ZA. *Food Chemistry, 124*(4), 1595–1602. https://doi.org/10.1016/j.foodchem.2010.08.022

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., Ray, C., Andersen, H. J., & Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry, 62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jaros, D., & Rohm, H. (2016). Enzymes exogenous to milk in dairy technology: Transglutaminase. In *Reference module in food science* (Issue July). Elsevier. https://doi.org/10.1016/b978-0-08-100596-5.21158-x

Jelen, P., & Tossavainen, O. (2003). Low lactose and lactose-free dairy products—Prospects, technologies and applications. *Australian Journal of Dairy Technology, 58*(2), 161–165.

Karasova, P., Spiwok, V., Mala, S., & Kralova, B. (2002). Beta-galactosidase activity in psychrotrophic microorganisms and their potential use in food industry. *Czech Journal of Food, 20*(2), 43–47. Retrieved from http://81.0.228.28/publicFiles/50865.pdf

Kashiwagi, T., Yokoyama, K.-I., Ishikawa, K., Ono, K., Ejima, D., Matsui, H., & Suzuki, E.-I. (2002). Crystal structure of microbial transglutaminase from Streptoverticillium mobaraense.

Journal of Biological Chemistry, 277(46), 44252–44260. https://doi.org/10.1074/jbc.M203933200

Kazemi, S., Khayati, G., & Faezi-Ghasemi, M. (2016). β-Galactosidase production by Aspergillus niger ATCC 9142 using inexpensive substrates in solid-state fermentation: Optimization by orthogonal arrays design. *Iranian Biomedical Journal, 20*(5), 287–294. https://doi.org/10.22045/ibj.2016.06

Kieliszek, M., & Misiewicz, A. (2014). Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiologica, 59*(3), 241–250. https://doi.org/10.1007/s12223-013-0287-x

Kokkiligadda, A., Beniwal, A., Saini, P., & Vij, S. (2016). Utilization of cheese whey using synergistic immobilization of β-galactosidase and saccharomyces cerevisiae cells in dual matrices. *Applied Biochemistry and Biotechnology, 179*(8), 1469–1484. https://doi.org/10.1007/s12010-016-2078-8

Li, X. E., Lopetcharat, K., Qiu, Y., & Drake, M. A. (2015). Sugar reduction of skim chocolate milk and viability of alternative sweetening through lactose hydrolysis. *Journal of Dairy Science, 98*(3), 1455–1466. https://doi.org/10.3168/jds.2014-8490

Lukito, W., Malik, S. G., Surono, I. S., & Wahlqvist, M. L. (2015). From "lactose intolerance" to "lactose nutrition". *Asia Pacific Journal of Clinical Nutrition, 24*(December), S1–S8. https://doi.org/10.6133/apjcn.2015.24.s1.01

Macrae, A. R. (1985). Microbial lipases as catalysts for the interesterification of oils and fats. In C. Ratledge, P. Dawson, & J. Rattray (Eds.), *Biotechnology for the oils and fats industry* (pp. 189–198). AOCS Press.

Macrae, A. R., & Hammond, R. C. (1985). Present and future applications of lipases. *Biotechnology and Genetic Engineering Reviews, 3*, 193–217.

Mahmood, W. (2009). Effect of microbial transglutaminase treatment on soft cheese properties. *Mesopotamia Journal of Agriculture, 37*(4), 19–27. https://doi.org/10.33899/magrj.2009.27525

Marschke, R. J., Nickerson, D. E. J., Jarrett, W. D., & Dulley, J. R. (1980). A cause of increased proteolysis in cheddar cheese manufactured from milk containing added Maxilact. *Australian Journal of Dairy Technology, 35*, 84–88.

Marx, C. K., Hertel, T. C., & Pietzsch, M. (2007). Soluble expression of a pro-transglutaminase from Streptomyces mobaraensis in Escherichia coli. *Enzyme and Microbial Technology, 40*(6), 1543–1550. https://doi.org/10.1016/j.enzmictec.2006.10.036

Matioli, G., De Moraes, F. F., & Zanin, G. M. (2003). Operational stability and kinetics of lactose hydrolysis by β-galactosidase from Kluyveromyces fragilis. *Acta Scientiarum—Health Sciences, 25*(1), 7–12.

Matto, M., & Husain, Q. (2006). Entrapment of porous and stable concanavalin A-peroxidase complex into hybrid calcium alginate pectin gel. *Journal of Chemical Technology and Biotechnology, 81*, 1316–1323. https://doi.org/10.1002/jctb.1540

McCain, H. R., Kaliappan, S., & Drake, M. A. (2018). Invited review: Sugar reduction in dairy products. *Journal of Dairy Science, 101*(10), 8619–8640. https://doi.org/10.3168/jds.2017-14347

Mendoza, M. R., Olano, A., & Villamiel, M. (2005). Chemical indicators of heat treatment in fortified and special milks. *Journal of Agricultural and Food Chemistry, 53*(8), 2995–2999. https://doi.org/10.1021/jf040406l

Merheb-Dini, C., Gomes, E., Boscolo, M., & da Silva, R. (2010). Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated Thermomucor indicae-seudaticae N31. (Milk-clotting protease from the newly isolated Thermomucor indicae-seudaticae N31). *Food Chemistry, 120*(1), 87–93. https://doi.org/10.1016/j.foodchem.2009.09.075

Mittal, S. B., Newell, G., Hourigan, J. A., & Zadow, J. G. (1991). The effect of protease contamination in lactase on the flavour of lactose-hydrolyzed milks. *Australian Journal of Dairy Technology, 46*(1), 46–47.

Moschopoulou, E. (2011). Characteristics of rennet and other enzymes from small ruminants used in cheese production. *Small Ruminant Research, 101*, 188–195. https://doi.org/10.1016/j.smallrumres.2011.09.039

Mukherjee, A. K., Borah, M., & Rai, S. K. (2009). To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by Bacillus subtilis DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. *Biochemical Engineering Journal, 43*(2), 149–156. https://doi.org/10.1016/j.bej.2008.09.011

Najera, A. I., Renobales, M., & Barron, L. J. R. (2003). Effects of pH, temperature, $CaCl_2$ and enzyme concentrations on the rennet-clotting properties of milk: A multifactorial study. *Food Chemistry, 80*, 345–352.

Nozière, P., Steinberg, W., Silberberg, M., & Morgavi, D. P. (2014). Amylase addition increases starch ruminal digestion in first-lactation cows fed high and low starch diets. *Journal of Dairy Science, 97*(4), 2319–2328. https://doi.org/10.3168/jds.2013-7095

Ozer, B., Avni Kirmaci, H., Oztekin, S., Hayaloglu, A., & Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal, 17*(3), 199–207. https://doi.org/10.1016/j.idairyj.2006.02.007

Pandey, A., Soccol, C. R., & Mitchell, D. (2000). New developments in solid state fermentation: I—Bioprocesses and products. *Process Biochemistry, 35*(10), 1153–1169. https://doi.org/10.1016/S0032-9592(00)00152-7

Parashar, A., Jin, Y., Mason, B., Chae, M., & Bressler, D. C. (2016). Incorporation of whey permeate, a dairy effluent, in ethanol fermentation to provide a zero waste solution for the dairy industry. *Journal of Dairy Science, 99*(3), 1859–1867. https://doi.org/10.3168/jds.2015-10059

Pazur, J. H. (1953). The enzymatic conversion of lactose into galactosyl oligosaccharides. *Science, 117*(3040), 355–356. https://doi.org/10.1126/science.117.3040.355

Pivarnik, L. F., & Senecal, A. G. (1995). Hydrolytic and transgalactosylic activities of commercial beta-galactosidase (lactase) in food processing. *Advances in Food and Nutrition Research, 38*, 1–102. https://doi.org/10.1016/s1043-4526(08)60083-2

Popovic, M. K., Jamrath, T., & Linder, C. (2009). Production of thermostable alpha amylase using food industry wastes. *New Biotechnology, 5*(1), 206–210. https://doi.org/10.1016/j.nbt.2009.06.147

Qureshi, M. A., Khare, A. K., & Pervez, A. (2015). Enzymes used in dairy industries. *International Journal of Applied Research, 1*(110), 523–527. www.allresearchjournal.com

Radzicka, A., & Wolfenden, R. (1995). A proficient enzyme. *Science, 267*, 90–931.

Ramana Rao, M. V., & Dutta, S. M. (1978). Lactase activity of microorganisms. *Folia Microbiologica, 23*(3), 210–215. https://doi.org/10.1007/BF02876581

Ramawat, K. G., & Mérillon, J.-M. (2015). *Polysaccharides: Bioactivity and biotechnology*. Springer. https://doi.org/10.1007/978-3-319-16298-0

Ray, A. (2012). Application of lipase in industry. *Asian Journal of Pharmacy and Technology, 2*(2), 33–37.

Saini, R., Singh Saini, H., Dahiya, A., & Harnek Singh Saini, C. (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry, 6*(4), 1865–1871.

Şanli, T., Sezgin, E., Deveci, O., Şenel, E., & Benli, M. (2011). Effect of using transglutaminase on physical, chemical and sensory properties of set-type yoghurt. *Food Hydrocolloids, 25*(6), 1477–1481. https://doi.org/10.1016/j.foodhyd.2010.09.028

Saqib, S., Akram, A., Halim, S. A., & Tassaduq, R. (2017). Sources of β-galactosidase and its applications in food industry. *3 Biotech, 7*(1), 1–7. https://doi.org/10.1007/s13205-017-0645-5

Sarmah, N., Revathi, D., Sheelu, G., Rani, K. Y., Sridhar, S., Mehtab, V., & Sumana, C. (2017). Recent advances on sources and industrial applications of lipases. *Biotechnology Progress, 34*, 5–28.

Schmid, R. D., & Verger, R. (1998). Lipases: Interfacial enzymes with attractive applications. *Angewandte Chemie International Edition, 37*(12), 1608–1633. https://doi.org/10.1002/(SICI)1521-3773(19980703)37:12<1608::AID-ANIE1608>3.0.CO;2-V

Schomburg, I., Chang, A., Placzek, S., Söhngen, C., Rother, M., Lang, M., Munaretto, C., Ulas, S., Stelzer, M., Grote, A., Scheer, M., & Schomburg, D. (2013). BRENDA in 2013: Integrated reactions, kinetic data, enzyme function data, improved disease classification: New options and contents in BRENDA. *Nucleic Acids Research, 41*(D1), 764–772. https://doi.org/10.1093/nar/gks1049

Seddigh, S., & Darabi, M. (2014). Comprehensive analysis of beta-galactosidase protein in plants based on Arabidopsis thaliana. *Turkish Journal of Biology, 38*(1), 140–150. https://doi.org/10.3906/biy-1307-14

Seitz, E. W. (1974). Industrial application of microbial lipases: A review. *Journal of the American Oil Chemists Society, 51*(2), 12–16. https://doi.org/10.1007/BF02545206

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances, 19*, 627–662.

Sitanggang, A. B., Drews, A., & Kraume, M. (2016). Recent advances on prebiotic lactulose production. *World Journal of Microbiology and Biotechnology, 32*(9). https://doi.org/10.1007/s11274-016-2103-7

Suchy, F. J., Brannon, P. M., Carpenter, T. O., Fernandez, J. R., & Gilsanz, V. (2010). National Institutes of Health Consensus development conference: Lactose intolerance and health. *Annals of Internal Medicine, 152*(12), 792–796.

Tafes, A. G. (2019). The application of novel inputs and advanced technology in dairy product processing a review. *Journal of Dairy and Veterniary Sciences, 14*(2). https://doi.org/10.19080/JDVS.2019.14.555882

Takata, H., Kuriki, T., Okada, S., Takesada, Y., Iizuka, M., Minamiura, N., & Imanaka, T. (1992). Action of neopullulanase. Neopullulanase catalyzes both hydrolysis and transglycosylation at α-(1→4)- and α-(1→6)-glucosidic linkages. *Journal of Biological Chemistry, 267*(26), 18447–18452.

Torres, J. K. F., Stephani, R., Tavares, G. M., de Carvalho, A. F., Costa, R. G. B., de Almeida, C. E. R., Almeida, M. R., de Oliveira, L. F. C., Schuck, P., & Perrone, Í. T. (2017). Technological aspects of lactose-hydrolyzed milk powder. *Food Research International, 101*(February), 45–53. https://doi.org/10.1016/j.foodres.2017.08.043

Trespalacios, P., & Pla, R. (2007). Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. *Food Chemistry, 100*(1), 264–272. https://doi.org/10.1016/j.foodchem.2005.09.058

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International, 89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Vaidya, S., Srivastava, P., Rathore, P., & Pandey, A. (2015). Amylases: A prospective enzyme in the field of biotechnology. *Journal of Applied Biosciences, 41*(1), 1–18.

van Scheppingen, W. B., van Hilten, P. H., Vijverberg, M. P., & Duchateau, A. L. L. (2017). Selective and sensitive determination of lactose in low-lactose dairy products with HPAEC-PAD. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 1060*, 395–399. https://doi.org/10.1016/j.jchromb.2017.06.024

Vandenplas, Y. (2015). Lactose intolerance. *Asia Pacific Journal of Clinical Nutrition, 24*-(December), S9–S13. https://doi.org/10.6133/apjcn.2015.24.s1.02

Verma, M. L., & Kanwar, S. S. (2008). Properties and application of poly(methacryclic acid-*co*-dodecyl methacrylate-*cl-N,N*-methylene bisacrylamide) hydrogel immobilized *Bacillus cereus* MTCC 8372 lipase for the synthesis of geranyl acetate. *Journal of Applied Polymer Science, 110*(2), 837–846. https://doi.org/10.1002/app.28539

Verma, N., Thakur, S., & Bhatt, A. K. (2012). Microbial lipases: Industrial applications and properties (a review). *International Research Journal of Biological Sciences, 1*(8), 88–92.

Whitcomb, D. C., & Lowe, M. E. (2007). Human pancreatic digestive enzymes. *Digestive Diseases and Sciences, 52*(1), 1–17. https://doi.org/10.1007/s10620-006-9589-z

Whitehurst, R. J., & Van Oort, M. (2009). *Enzymes in food technology* (2nd ed.). Wiley. https://doi.org/10.1002/9781444309935

Xavier, J. R., Ramana, K. V., & Sharma, R. K. (2018). β-galactosidase: Biotechnological applications in food processing. *Journal of Food Biochemistry, 42*(5). https://doi.org/10.1111/jfbc.12564

Yasueda, H., Kumazawa, Y., & Motoki, M. (1994). Purification and characterization of a tissue-type transglutaminase from Red Sea Bream (Pagrus major). *Bioscience, Biotechnology, and Biochemistry, 58*(11), 2041–2045. https://doi.org/10.1271/bbb.58.2041

Yu, Y. J., Wu, S. C., Chan, H. H., Chen, Y. C., Chen, Z. Y., & Yang, M. T. (2008). Overproduction of soluble recombinant transglutaminase from Streptomyces netropsis in Escherichia coli. *Applied Microbiology and Biotechnology, 81*(3), 523–532. https://doi.org/10.1007/s00253-008-1688-7

Zhou, Q. Z. K., & Chen, X. D. (2001). Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from Kluyveromyces lactis. *Biochemical Engineering Journal, 9*(1), 33–40. https://doi.org/10.1016/S1369-703X(01)00118-8

Enzymes in Brewing and Wine Industries

6

S. Pati and D. P. Samantaray

Abstract

Enzymes are the bio-catalysts used in breweries and wineries for the biochemical transformation of a substrate to produce finished products. Several enzymes such as amylase, glucanase, protease, glycosidase, pectinase, phytase and many more exogenous enzymes are used in different stages of brewing to improve the production level as well as the quality of beer. In contrast, lipase, lysozyme, pectinase, glucanase, glycosidase, urease, protease, phenoloxidase and ester hydrolase and synthetase are employed in oenology practices for the production of wine with enormous organoleptic properties. This chapter postulates a bird's eye view of different key enzymes used in breweries and wineries, their mechanism of action and their pros and cons.

Keywords

Bio-catalysts · Brewery · Winery · Exogenous · Oenology · Organoleptic

6.1 Background

Beer and wine have concealed a fascinating part of history and our social life. Although the discovery and development of brewing and wine processing signify a cornerstone achievement of humankind, its origin is still mysterious. In ancient civilization, people used to preserve fruits and grains in wooden containers for an extended period to produce beer and wine. The entire process of production is termed as fermentation, which came from theLatin word 'fervere', meaning 'to

S. Pati · D. P. Samantaray (✉)
Department of Microbiology, CBSH, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_6

boil'. The crushed fruits and grains in the wooden container produced bubbles due to microbial action or activities as if they were boiling. During that period, people didn't fully understand that a tiny, eukaryotic fungus was making the entire recipe work (Alba-Lois & Segal-Kischinevzky, 2010). Researchers took ten decades to find out how a diminutive microbe makes the entire fermentation successful and resulted in a potation that became a status symbol in the modern era. Nevertheless, the concept of brewing and wine processing was established prior to thousands of years, which was found in the graves and settlements of early civilizations. Ancient Egyptians made wine 8000 years ago from fruits and labelled it as such by pouring it in fruit-shaped flasks, while beer came to the limelight 7000 years ago from China. Initially, Germany, the United Kingdom, the United States, Belgium, Spain and Italy were the leading countries for beer and wine production. However, in the last five decades, beer and wine production has confronted significant changes as per the demand. Global beer production reached 1940 mhl in 2018, with China being the largest producer as well as a consumer (https://www.statista.com/statistics/270275/worldwide-beer-production). In contrast, as per the data of the International Organization of Vine and Wine 2018, the global wine production has reached 292.3 mhl, with 246 mhl consumption, where, Italy, France and Spain are the highest producers.

Enzymes are the bio-macromolecules that act as catalysts in specific reactions and play a vital role in food science and fermentation technology. Since 6000 BC, enzymes have been recognized as a biochemical substance to be utilized in the processing of various foods, such as cheese preparation, brewing, wine preparation and meat tenderizing to enhance the nutritional, sensorial and functional values of the finished products (Gomaa, 2018; Gurung et al., 2013). Amongst all, enzymes offer a heroic performance in brewing and winery by accelerating the release of digestive sugars and other nutrients used by the yeasts during fermentation (Spier et al., 2016). Brewing refers to the oldest fermentation process where complex starch is bio-transformed to ethanol by the action of yeasts. This traditional process involves a series of complex endogenous and exogenous enzymes that regulate the malting of grains, the mashing of grist and rate of fermentation to produce low-calorie beer with admirable flavour, aroma and texture (Oliver, 2011; Bamforth, 2009). While grapes bio-transformed to wine by yeasts under fermentation. This process is catalysed by a wide array of enzymes that not only convert complex sugar to ethanol but also release several volatile and non-volatile substances to enhance the quality and stability of the wine (Ottone et al., 2020). Traditionally, these enzymes of brewing and winery were produced naturally by yeast or present in the grains or grapes. However, recent trends in fermentation technology pave the way for commercial production of enzymes to enhance the quality and productivity of wine and beer (Claus & Mojsov, 2018). Hence, this chapter offers a comprehensive summary on the sequential processing of brewing and winery using different enzymes as well as their mechanism of action. Moreover, the effect of enzymes on the quality and quantity of the final product and pros and cons have been enlightened.

6.2 Enzymes Used in Breweries

The process of brewing evolved centuries ago as a result of the resourcefulness and artistry of the brewers. Traditionally, lager type is the most common method of brewing, comprising low-temperature fermentation of barley and pure water with an extended maturation period. However, variations have developed in the young beer style, where brewing is not possible without the application of enzymes that hydrolyse complex starch to simple fermentable sugar and then convert it to ethanol and CO_2 by yeast or bacteria. In brewing, the keystones, malted grains and barley are the sources of enzymes, including α- and β-amylase, exo-peptidase, carboxy-peptidase, proteases glucanases and cellulases (Sammartino, 2015). The detailed key role or mode of action of these enzymes is discussed below.

6.2.1 Enzymes Used in Different Stages of Brewing

The competitive brewing industry is repetitively sounding forward to improving the process in terms of quality and manufacturing costs. Brewing that refers to conversion of cereal to ethanol involves two steps: enzymatic hydrolysis of starch to fermentable sugars and conversion of fermentable sugar to ethanol and CO_2. Depending on the brewing process, raw materials and technical preferences, different steps such as malting, mashing, pitching and aging are directly affected by the enzymes, while others are indirectly affected. The detailed process of beer preparation is presented in Fig. 6.1. The extent and achievement of each step depends on the development and stability of native enzymes that ultimately affect the quality and quantity of the final product.

6.2.1.1 Malting

Malting is the initial and prime step of brewing, which stimulates complex carbohydrate and protein-reducing enzymes present in the barley grains. The most prominent endogenous enzymes of malt include α-glucosidase, α-amylase, β-amylase, carboxypeptidase, dextrinase, lipoxygenase, xylanase and glucanase. Besides these, peroxidase, acid or alkali phosphatases, catalase, polyphenol-oxidases and phytase enzymes are also responsible for malting reactions (Spier et al., 2016; Van Oort, 2010). The malting process comprises steeping, germination and drying. In steeping, barley grains are immersed in water for 48–60 h under oxic conditions that initiate germination and biosynthesis of amylase, glucanase, proteases and carboxypeptidase. Amylase acts on the modification of complex starch, whereas β-glucanase hydrolyses β-glucans for malting clarification and proteases act on the complex protein content of the grain (Van Oort, 2010). Then malt is subjected to drying with a temperature between 90 and 140 °C to enhance the flavour and colour of the finished product (Curtis, 2013). The challenge during this step is to maintain the quality of beer as the application of heat can adversely affect the functionality of enzymes.

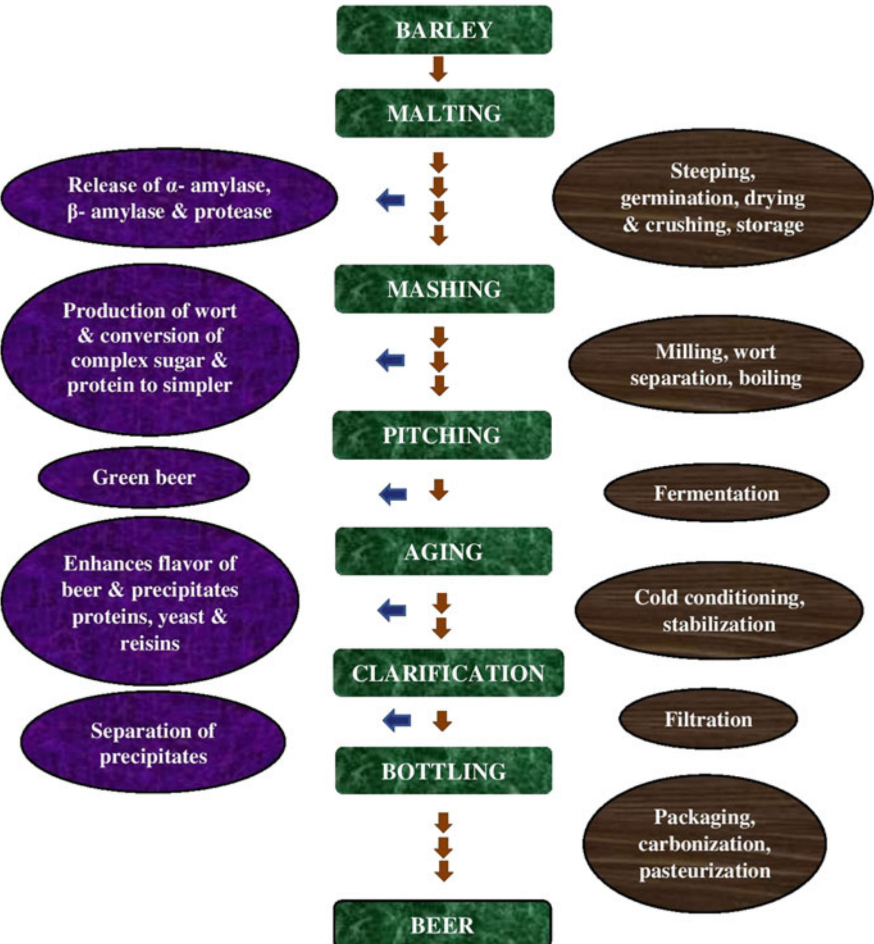


Fig. 6.1 Overview of brewing

6.2.1.2 Mashing

Mashing begins with boiling of malt with water, malt adjuncts and hops at different temperatures such as 50, 62, 72 and 78 °C for proteolysis, gelatinization, saccharification and inactivation of malting enzymes, respectively. Mashing embraces α- and β-amylases and protease, which degrade complex starch to maltose and dextrins, and the undesirable barley endosperm complex proteins to simple proteins, respectively (glutelin and hordein) (Dhillon et al., 2016; Spier et al., 2016). Additionally, exogenous enzymes, glucoamylases and pullulanases are added during mashing that break down the α-1,4 and α-1,6 linkages of starch to produce maltose, dextrin, maltotriose and glucose (Briess, 2013).

6.2.1.3 Pitching

Pitching refers to the addition of different strains of *Saccharomyces* such as *S. cerevisiae*, *S. uvarum* and *S. carlsbergensis* to the fermenter as inoculum. These strains own the MEL gene that synthesize α-galactosidases to cleave oligosaccharide melibiose to glucose and galactose. However, research is still on its way for genetically modified yeasts that can synthesize glucosidase, amylase and glucanase for metabolization of wort and soluble proteins (Stewart et al., 2013; Van Oort, 2010).

6.2.1.4 Aging

The beer obtained after completion of fermentation is called green beer due to its harsh taste and which can be removed by a process called aging. In aging, green beer is collected from a fermentation vat and stored in refrigerated conditions from a few weeks to several months. This process enhances the flavour of beer and precipitates yeasts, proteins and resins. During this period, diacetyl reductase is secreted by yeast, which reduces diacetyl to acetoin to develop an undesirable butterscotch flavour in beer. Thus, exogenous enzyme acetolactate decarboxylases is supplied that directly converts acetolactate to acetoin thereby reducing the undesirable flavour of beer during aging (Spier et al., 2016). In addition, several other enzymes actively involved in brewing are presented in Table 6.1.

6.2.2 Types of Enzymes in Brewing

The brewing process requires a prodigious knowledge of enzymology as it not only improves the quality of beer but also adds innovated attributes to the beer. Thus, enzymes with varied action and properties are used in the brewing industry. Enzymes involved in brewing can be either endogenous or exogenous. Endogenous enzymes are secreted naturally during the process of malting and mashing. Amongst all, the principal endogenous enzymes like amylase, protease, glucanase and peptidase are involved in brewing (Gomaa, 2018; Bamforth, 2009). On the other hand, at the pinnacle of enzyme technology, exogenous enzymes are applied to rectify the issues that evolved during brewing as well as to enhance the shelf life of beer by maintaining quality. Ficin, alpha-acetolactate decarboxylase (ALDC) and papain are commercially available enzymes typically used in brewing (Boulton, 2013). Details of all the enzymes are briefly discussed below.

6.2.2.1 Amylase

During brewing, both α- and β-amylases are involved in the biotransformation of complex starch to simple sugars such as dextrin, maltose, glucose and oligosaccharide (Bamforth, 2017). Endogenous α- and β-amylases are released from the barley, when the outer membrane of the granules is hydrolysed by xylanases and glucanases. During malting and mashing, amylase degrades complex starch, thereby increasing the availability of fermentable sugar. Amylase (α) strikes the internal α-(1–4) glycosidic linkage of α-glucose amylose and amylopectin to produce dextrin, whereas β-amylase cleaves the external α-(1–4) glycosidic bonds of amylose

Table 6.1 Sources and functions of enzymes involved in brewing

Enzymes	Process	Sources	Function
α-Amylase	Malting/ mashing	Endogenous/ exogenous (*Bacillus licheniformis*, *Bacillus subtilis*)	Improve starch hydrolysis and clarification
β-Amylase	Malting/ mashing	Endogenous/ exogenous (*Bacillus licheniformis*)	Improve starch hydrolysis, saccharification, malting and fermentation yield
Protease	Malting/ mashing/ storage	Endogenous/ exogenous (*Aperguillus* sp. and pineapple latex)	Boost fermentation and increase malting, clarification, chilling and storage quality
β-Glucanases	Malting/ mashing/ fermentation	Endogenous/ exogenous (*Trichoderma* sp. and *Orpinomyces* sp.)	Improve malting and clarification, lower viscosity and support clear wort production
Fungal α-amylase	Fermentation	Exogenous (*Aspergillus* sp.)	Increase fermentation yield
Amyloglucosidase	Mashing	Exogenous (*Aspergillus niger*)	Increase glucose content in wort
α-Acetolactate-decarboxylase (ALDC)	Fermentation	Exogenous (recombinant *Bacillus subtilis*)	Reduce fermentation time
Cysteine endopeptidases	Post-fermentation	Exogenous	Improve stability of beer and shelf-life of packaged beer
Glucoamylase	Mashing	Exogenous	Increase glucose content in wort
Phytase	Mashing	Endogenous/ exogenous (bacterial origin)	Reduce initial pH during mashing of lager beers
Pentosanases	Malting	Endogenous	Promotes conversion of pentosans into arabinose and xylose, also improves extraction and filterability of beer

and amylopectin to release maltose. Both the amylases function optimally at pH 5.2–5.5 and temperatures about 62–74 °C (Sammartino, 2015). Moreover, α-amylase is only used endogenously during brewing, while β-amylase is used endo as well as exogenously due to its vital role in saccharification.

6.2.2.2 Protease

Proteases have many reimbursements in brewing like digestion of peptide bonds of proteins to enhance clarification of malting, augmentation of protein solubility, lowering of beer viscosity and favour rapid yeast growth. Temperature labile protease has a key role in the malthouse, functions optimally at 52 °C and can be deactivated at 70–75 °C, however, it can function at a wide range of pH. Though

protease is endogenous, it is advisable to use exogenous protease to maintain the quality of beer. Brewers are acclaimed to use 0.3–1 kg of protease per ton of barley as an overdose of protease can cause foam instability leading to degradation of beer quality (Gomaa, 2018).

6.2.2.3 β-Glucanases

It is the key enzyme of malting and mashing involved in the digestion of the outer membrane of starch that allows other hydrolysing enzymes to act on starch granules. This enzyme not only lowers the viscosity of beer but also helps protease activity in the hydrolysis of a matrix of starch granules to make the kernel soften during germination. Though, β-glucanase are present naturally in barley, in the case of light beer it is supplied externally (0.3–1 kg per ton of wort) for improvement of light quality and texture of the beer (Bamforth, 2017; Lyven, 2016). β-Glucanase hydrolyses the cell wall at an optimum pH of 6 and temperature of 45–50 °C, however, this extremely heat-sensitive enzyme denatures at 60 °C (Bamforth, 2017). Thus, heat-stable microbial β-glucanases of *Bacillus subtilis*, *Aspergillus*, *Penicillium* and *Trichoderma* are used exogenously in the mashing stage of brewing.

6.2.2.4 Exogenous Enzymes

When the enzyme technology is at its zenith, other than endogenous many exogenous enzymes are also supplied to maximize the fine ability of beer during processing, storage and transportation. Some of the prime exogenous enzymes, bromelain, ficin and papain, belong to protease and alpha-acetolactate decarboxylase (ALDC) from lyases is involved in brewing (Boulton, 2013). Ficin, bromelain and papain are the chill-proof enzymes extracted from Pawpaw latex and fig plants and they are responsible for chilling haze by removing polyphenols and polypeptides leading to hydrolyzation of proteins as well as improvement of colloidal stability of beer. This chill-proof enzyme functions in a wide range of pH with temperatures ranging from 60 to 65 °C and supplies directly to the storing vessels at a concentration of 1–2 g/hl of beer (Gomaa, 2018; Boulton, 2013). Besides, ALDC is added to the beer during pilot-scale production for increasing the product yield at less fermentation time, without affecting the quality. ALDC attacks the C–C bond in the α-acetolactate for catalyzing the bioconversion of α-acetolactic acid to acetoin (Hans, 2008; Dulieu et al., 2000). It remains active at 25–40 °C temperature, 5–7 pH and is added directly during the initial period of fermentation at a concentration of 1–5 g/hl of wort and 0.4–1 g/hl of wort in post-fermentation period. Apart from these, many other exogenous enzymes such as pullulanases, phytase and diastase is also used efficiently during malting, mashing and post-fermentation period to enhance the quality of beer.

6.2.3 Effect of Enzymes on Quality of Beer

One of the most momentous advancements in brewing is to study the use and effect of different enzymes for beer production in order to fulfil the demand of consumers

for a beer with an appealing taste and flavour. Therefore, it is the need of the hour to know the effect of enzymes on the quality of beer. Malting is the sole source of endogenous enzymes like α- and β-amylases that are actively involved in the conversion of complex starch to simple fermentable sugar. These enzymes are also responsible for increasing the fermentability by boosting the saccharification, higher product yield and enhancing of thermal stability of mash during brewing, which ultimately removes the bitter taste from beer. The poor activity of β-glucanases can lead to deprived runoff, recovery, spent-grain drainage, filter performance and sedimentation in beer. However, surpassing β-glucanases activity makes the mash thermostable and boosts the fine ability and filterability of beer (Aehle, 2007). Malthouse enzyme protease provides foam stability in brewing. Moreover, the shelf life improvement, maturation and flavour of beer are also greatly affected by enzymatic activity. These enzymes ensure protein solubilization during mashing, together with sugar availability to yeast during fermentation. Enzymes like amylase also decrease the fermentation period during pilot-scale brewing. Furthermore, enzymes in combination with substrate and temperature affect the carbonation, aroma and flavour of the beer. Many commercial enzymes are also cast-off to boost the quality attributes (clarification, texture, colour and flavour) of beer. The enzymes utilized in brewing are assorted in their action and properties and are key factors for the improvement of each step of brewing. Hence, intensive research is highly essential regarding the mode of action and properties of these brewing enzymes.

6.3 Enzymes Used in Wineries

Biotechnological advances facilitate the combined use of fruit juice, yeasts and enzymes to make a surface for wine preparation. Wine is the partial or complete alcoholic fermentation of grape juice by yeasts where sugars are bio-transformed to ethanol and other metabolites. These metabolites, including volatile and non-volatile composites add significant flavour, colour, odour, taste and aroma to wine. Enzymes are either naturally present in the grapes or supplied exogenously to catalyse fermentation process as well as enhance the sensory properties of the wine. In this high-tech era of biotech, endogenous and exogenous enzymes are involved at different stages of wine preparation for smooth management of pre and post-fermentation conditions. Several enzymes such as pectinases, glucanases, glycosidases, xylanases, glucose oxidases, proteases, ureases, peroxidases and proteases are actively involved in the catalyzation of different reactions occurring during wine preparation. The role of these enzymes at various stages are momentarily discussed below.

6.3.1 Enzymes Used in Different Stages of Wine Preparation

Wine production begins with the collection of matured grapes followed by crushing for extraction of juice. Then, the juice is subjected to maceration for must formation, where enzymes start functioning as a part of pre-treatment. The must undergo fermentation by yeasts to obtain raw wine with a precise aroma due to the involvement of specific enzymes. In clarification, pectic enzymes are implied to reduce the viscosity and turbidity of the wine. Finally, suitable enzymes are used in the ageing process to get good quality wine, where pertinent physicochemical properties are incorporated into the wine. The implementation of various enzymes in different steps of wine preparation is presented in Fig. 6.2.

Fig. 6.2 Overview of wine preparation

6.3.1.1 Maceration

Desired enzymes of maceration not only expedite extraction of colour, aroma and antioxidant activity but also regulate ethanol concentration of the wine (Ottone et al., 2020). The endogenous cellulases and hemicellulases catalyse the lysis of grape cells, whereas pectinases lead to the degradation of polysaccharide to accelerate the extraction of juice. These enzymes cause activation, release and solubilization of derivatives of antioxidant activity and colour and fragrance precursors (Garg et al., 2016). Besides this, the addition of glucose oxidase to the must regulate the ethanol concentration of the wine, as it maintains the concentration of glucose in the must by using it as an electron acceptor. However, the by-product H_2O_2 released during maceration oxidized the phenolic components of the wine, resulting in reduced antioxidant activity. Thus, exogenous catalase enzyme is added to the must for conversion of toxic H_2O_2 to non-toxic H_2O.

6.3.1.2 Fermentation

The overall flavour and aroma of the wine are determined by the concentration of volatile components synthesized during bioprocess technology. Wine can be prepared with four types of aromas such as varietal, pre-fermentative, fermentative and post-fermentative. Production of varietal aroma is due to the partial metabolism of grapes depending on the variety, ripening percentage, soil and climate, while pre and post-fermentative aromas are developed during maceration and ageing (Samoticha et al., 2017). Moreover, the fermentative aroma is generated by acids, esters, alcohols and sulfur and carbonylated substrates produced by yeasts during fermentation. Oenology is incomplete without the involvement of yeasts like *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. These yeasts release certain enzymes that enhance the rate of fermentation and product yield and add flavour and aroma to the wine. Invertase is one of the most vital enzymes that causes hydrolysis of saccharose to fructose and glucose (Ribéreau-Gayon et al., 2006), whereas β-1,3-glucanases catalyses the synthesis of mannoprotein in fermentation medium and cell wall hydrolysis. Pectinases and β-glucosidase also found in yeasts catalyse the degradation of pectic components and improve the aroma of the wine respectively (Merín et al., 2014; Villena et al., 2007). Except for these endogenous enzymes, exogenous β-glucanases are also implemented during fermentation for better stability and structure of the wine (Spagna et al., 2002).

6.3.1.3 Clarification and Stabilization

In wine preparation, clarification steps make the way for bottling. The chemical reactions that occur during maceration and fermentation release many insoluble, floating materials that develop a cloudy appearance in the wine. Thus, in clarification and stabilization all the suspended materials are removed by processes like flotation, centrifugation, filtration, pasteurization, refrigeration, maturation and racking to improve the quality of the wine. Pectinase enzymes are added in the initial period of clarification to increase the juice yield, colour and flavour of the wine and to reduce the viscosity and turbidity of must (Martín & Morata de Ambrosini, 2014). It also significantly reduces the filtration time of wine preparation. Though pectinase is

highly indispensable for clarification, they are not synthesized naturally during wine preparation. Thus, commercially synthesized pectinase from microbes and plants is employed during bioprocess technology. On the other hand, protease is added to the medium to circumvent haze formation. Sometimes the reaction medium gets contaminated by *Lactobacillus* sp. and *Pediococcus* sp. causing acidity, mousy taint and buttery flavour in the wine (Bartowsky, 2009; Liburdi et al., 2014). Thus, lysozyme is supplemented, which not only inhibits the growth of these contaminants but also enhances malolactic fermentation (Ottone et al., 2020).

6.3.1.4 Ageing

Ageing potentially improves the quality of the wine, where it is aged for a significant time period till the availability of oxygen for the development of premium flavour, aroma and taste. However, throughout ageing the naturally occurring urea and ethanol react to form ethyl carbamate, which has carcinogenic activity. Therefore, acid urease is added in this period for removal of urea that effectively minimizes ethyl carbamate formation to reduce the toxicity in the wine.

6.3.2 Types of Enzymes in the Winery

Over the past decades enzymes have been established as a processing aid in oenology. The enzymes offer several advantageous bids during pre-fermentation, fermentation, clarification, stabilization and ageing steps that ultimately enhance the juice yield and add colour, odour and flavour to the wine, leading to the generation of extremely fine-quality wine. Pectinase, lipase, lysozyme, glucanases, glycosidases, ester hydrolases and synthetases, phenol oxidases and urease are the potential enzymes widely used in wine preparation (Table 6.2). The above-said enzymes are

Table 6.2 Enzymes of the winery and their functions

Enzymes	Functions
Lysozyme	Prevents the growth of undesirable lactic acid bacteria in the wine
Pectinase	Involved in degradation of pectin components, hydrolysis of cell wall, and enhances the quality, stability, colour and flavour of the wine
Glycosidase	Improves flavour and develops varietal aroma from odourless precursors in the wine
Glucanases	Liberate mannoproteins for the development of varietal flavour and cause lysis of the microbial cell wall to boost the clarification and filtration during wine preparation
Protease	Prevents haziness and reduces bentonite demand of the wine
Urease	Hydrolyses urea to prevent the synthesis of carcinogenic ethyl carbamate produced during the ageing stages of wine preparation
Lipase	Degrades lipids for better extraction of colour during wine preparation
Phenoloxidases	Enhance the stability and sensory attributes of the wine
Esterhydrolases and synthetases	Promote malolactic fermentation and develop fruity flavour in the wine

naturally synthesized during oenological practice; however, in some cases, they are also supplied externally.

6.3.2.1 Pectinase

The grape cell comprises cellulose, hemicellulose, mannan, pectin and xyloglucan linked with proteins. During wine processing, the highly viscous pectin impedes juice extraction, filtration, clarification and diffusion of aromatic components into the must (Claus & Mojsov, 2018). The enzyme pectinase hydrolyses pectin to enhance the juice yield and ease clarification and filtration. Pectinase treatment boosts the absorbance of phenols and anthocyanins by the must, as well as intensifies the colour and clarity of the wine (Mojsov et al., 2011). Polygalacturonase, pectin lyase, pectinesterase and acetylesterase are the pectinase enzymes actively involved in wine preparation. However, pectinase is neither present naturally in grapes nor secreted by the yeasts. Therefore, commercial pectinase solutions composed of 2–5% of active enzymes and additives (preservatives, sugar and salt) are used in wine preparation (Mojsov, 2013). The pectinase available in the market is generally of fungal origin or produced by *Rhodotorula mucilaginosa* and *Cystofilobasidium capitatum*. Nevertheless, high amounts of tannin, alcohol (above 17%) and SO_2 (above 500 mg/l) render the pectinase activity (Van Rensburg & Pretorius, 2000).

6.3.2.2 Lysozyme

Traditionally, SO_2 was added to the medium to prevent microbial contamination in the wine, but an allergic response caused by sulphites pushes the oenologist for an enzymatic solution (König & Fröhlich, 2017; Campos et al., 2016). Thus, lysozyme is used to prevent microbial contamination, which kills the bacteria by cell lysis. Lysozyme stabilizes the wine by preventing malolactic fermentation. As per the International Organization of Vine and Wine, hen's egg lysozyme at a concentration of 250–500 mg/l can be used in wine preparation. Moreover, after completion of bioprocess technology, this enzyme can be removed from the wine by the implementation of fining agents.

6.3.2.3 Glycosidase

Almost 90% of aroma precursors such as phenolic compounds, nerol, terpenes linalool and geraniol are present in grapes' skin in conjugated form as odourless compounds. Glycosidase hydrolyses these precursors to liberate volatile, aromatic terpenes that ultimately activate the organoleptic properties of the wine. These enzymes are naturally found in grapes and promote the liberation of aromatic compounds under optimized condition. However, these grape glycosidases are inactivated at high alcohol and glucose concentrations and at pH 5. Therefore, commercially available glycosidases extracted from the species of *Saccharomyces*, *Pichia*, *Candida* and *Rhodotorula* are also employed in wine preparation (Claus & Mojsov, 2018; Ugliano, 2009).

6.3.2.4 Glucanase

Lactic acid bacteria and some fungus associated with the skin of grapes release viscous polysaccharides, which hamper the wine filtration. Neither flocculants nor filtration can remove these polysaccharides; thus, glucanase is used for reduction of wine viscosity. Both endo andexo-glucanase release mannoproteins to enhance the varietal flavour in the wine. Endo-glucanase is naturally synthesized by *Saccharomyces* species during fermentation, while exo-glucanase is supplemented to the fermentation medium. Moreover, the commercially available exo-glucanase is extracted from fungus like *Taleromyces versatilis* and *Trichoderma* sp., different species of yeast like *Kloeckera*, *Zygosaccharomyces* and *Pichia* and lactic acid bacteria (Claus & Mojsov, 2018).

6.3.2.5 Protease

Proteins present in must or synthesized by starter culture cause allergic reactions in the consumer (Van Sluyter et al., 2015; Rizzi et al., 2016). Though proteins are precipitated after fermentation, the presence of acid, proteolytic and heat-resistant pathogenesis-related (PR) proteins causes undesirable turbidity in the wine. Generally, bentonite is used for the removal of PR proteins, but it can adversely affect the quality and quantity of the wine (Jaeckels et al., 2015). Thus, researchers are now focusing on protease as an attractive enzymatic solution for the removal of these undesirable proteins. Protease can not only enhance the quality and quantity of the wine but also reduce the haziness in the wine. *Saccharomyces* species doesn't show any protease activity; however, some fungus and non-*Saccharomyces* species depict the same. So, nowadays researchers are shifting their attention to selecting or developing protease positive starter culture for wine production.

6.3.2.6 Urease

In wine, fermentation yeast generates urea that is chemically converted to carcinogenic ethyl carbamate (Lonvaud-Funel, 2016). In 1997, urease was established as an enzymatic solution that cleaves urea into CO_2 and NH_3 and prevents ethyl carbamate synthesis. Generally, urease of *Lactobacillus* origin is used at a concentration of 25–50 mg/l in the fermentation medium (Pozo-Bayón et al., 2012).

6.3.2.7 Lipase

Lipids are released during wine fermentation as a result of the autolysis of yeast or grape cells, which cause significant changes in fermentation as well as in the finished product. Hence, lipase is employed in wine preparation to decompose the lipids present in the cell membrane for the improvement of the colour and texture of the wine. It is found in a few wild strains of *Lactobacillus* and yeasts.

6.3.2.8 Esterhydrolases and Synthetases

Esters are either present in grapes or synthesized by yeasts during fermentation, which contributes a significant fruity flavour to the wine. Esterhydrolases and synthetases regulate the concentration of the ester during malolactic fermentation by promoting its synthesis as well as hydrolysis (Ugliano, 2009).

6.3.2.9 Phenoloxidases

Phenoloxidases affect not only the sensory attribute of the wine but also the final phenol concentration of the wine. However, these oxygen-sensitive enzymes on exposure to O_2 cause enzymatic browning of wine. Phenoloxidases include tyrosinase, responsible for implementation of colour to the wine, and laccase, which causes phenol oxidation to enhance the organoleptic properties of the wine (Claus & Mojsov, 2018).

6.3.3 Effect of Enzymes on Quality of the Wine

Enzymes have a very controversial effect on wine preparation as they are highly selective. Several studies have been directed to know the effect of different enzymes on the organoleptic as well as quantitative properties of the wine. The colour extraction of the wine is greatly influenced by xylanase and glycosidase activities. During maceration, the enzymatic treatment enhances the extraction of phenolic compounds while depicting undesirable anthocyanin content and colour parameters. Similarly, glycosidase helps in aroma extraction in the wine; however, an abundance of glucose inhibits its action. Lysozyme causes total inhibition of lactic acid bacteria from wine, but the presence of the residual amount of lysozyme can cause an allergic reaction in the consumer (Liburdi et al., 2014). Furthermore, protease is essential to remove PR proteins from wine, but it is only functional under restricted alcohol concentration, pH and temperature (Espejo, 2020). It can't be ignored that enzymes are highly essential for wine preparation, but the sensitivity of these enzymes influences industry and academia to analyse the pros and cons before further application.

6.4 Pros and Cons of Enzymes Used in Brewing and Winery

Before taking any step forward towards the enzymology of brewing and wine preparation, it is highly recommended to study their pros and cons. The application of enzymes in brewing enhances the maturation of beer, catalyses low-calorie beer production, stabilizes the beer by improving mashing and clarification and reduces the viscosity, leading to fine-quality beer production (Gomaa, 2018). Nevertheless, the cost is the prime barrier in the enzymatic treatment of beer. Besides, the sensitivity of enzymes towards temperature, pH, alcohol and glucose concentration also significantly affects the use of enzymes in brewing. Like in brewing, enzymes also have a significant effect towards the enhancement of the flavour, aroma and taste of the wine. Some enzymes are used to prevent contamination of the wine by toxic microbes and compounds. Enzymes also ease the wine processing for higher yield and lower production cost, leading to a booming profit. On the other hand, it is always advisable to consider the drawbacks of these enzymes for a better future. Sometimes specific activity and side effects of a few enzymes have a detrimental effect on wine quality. Also, the use of commercial enzymes requires a lot of

pre-treatment, which is time-consuming as well as expensive. Intermittently, enzymes undergo unwanted chemical reactions with the components of the fermentation medium and cause an allergic response to the consumer. Hence, a detailed study of individual enzymes is highly essential before their use.

6.5 Conclusion

In the present scenario, breweries and wineries have established themselves as extremely lucrative businesses in the agriculture and alcohol industries. Earlier these two were produced by traditional methods, but in the modern era, the establishment of brewery and winery is not possible without the involvement of enzymes. On account of this, brewers and oenologists amalgamate traditional and advanced enzymological techniques for the development of modern methods for beer and wine production. Amylases, glucanases, proteases and cellulases are the foremost important enzymes being used in beer industries. Though the implementation of enzymes ensures faster and higher beer production, the brewer should screen the appropriate enzyme with eyes open because a slight modification in enzymatic treatment can cause devastating effects on the finished product. On the other hand, enzymes have a flourishing future in the wine industry and a wide array of enzymes are being used for the furtherance of different stages of wine preparation. Pectinases, protease, glucanases, lysozyme and lipase are the enzymes used during the different stages of wine processing for the production of superior-quality wine. These enzymes accomplish every goal to provide chemical, mechanical and thermal stability to wine. Moreover, recent advances in biotechnology pave the way for researchers to articulate a stringent plan of work or phenomenon to overcome various drawbacks of brewery and winery. This chapter intensely discussed several aspects of enzymes used in brewery and winery; however, further study is highly essential in this regard.

References

Aehle, W. (2007). Industrial enzymes. In *Enzymes in industry* (pp. 99–262). Wiley-VCH Verlag GmbH & Co. KGaA.

Alba-Lois, L., & Segal-Kischinevzky, C. (2010). Yeast fermentation and the making of beer and wine. *Nature Education, 3*(9), 17.

Bamforth, C. W. (2009). Current perspectives on the role of enzymes in brewing. *Journal of Cereal Science, 50*, 353–357.

Bamforth, C. W. (2017). Progress in brewing science and beer production. *Annual Review of Chemical and Biomolecular Engineering, 8*, 161–176.

Bartowsky, E. J. (2009). Bacterial spoilage of wine and approaches to minimize it. *Letters in Applied Microbiology, 48*(2), 149–156.

Boulton, C. (2013). *Encyclopedia of brewing* (Vol. 716). Wiley-Blackwell.

Briess. (2013). In E. Richard (Ed.), *Mashing for optimal yield*. Division Manager Brew East, Briess Malt and Ingredients Company.

Campos, F. M., Couto, J. A., & Hogg, T. (2016). Utilization of natural and by-products to improve wine safety. In M. V. Morena-Arribas & B. Bartolomé Sualdea (Eds.), *Wine safety, consumer preference and human health* (pp. 27–49). Springer International Publishing.

Claus, H., & Mojsov, K. (2018). Enzymes for wine fermentation: Current and perspective applications. *Fermentation, 4*(52), 1–19.

Curtis, T. (2013). *A London encyclopedia or universal dictionary of science, art, literature and practical mechanics* (Vol. 13, pp. 484–485). Forgotten Books.

Dhillon, G. S., Kaur, S., Oberoi, H. S., Spier, M. R., & Brar, S. K. (2016). Agricultural-based protein byproducts: Characterization and applications. In *Protein by-products: Transformation from environmental burden into value-added products*. Elsevier.

Dulieu, C., Moll, M., Boudrant, J., & Poncelet, D. (2000). Improved performances and control of beer fermentation using encapsulated alpha-acetolactate decarboxylase and modeling. *Biotechnology Progress, 16*, 958–965.

Espejo, F. (2020). Role of commercial enzymes in wine production: A critical review of recent research. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-020-04489-0

Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., & Mahajan, R. (2016). Microbial pectinases: An ecofriendly tool of nature for industries. *3 Biotech, 6*(1), 47.

Gomaa, A. M. (2018). Application of enzymes in brewing. *Journal of Nutrition and Food Science Forecast, 1*(1), 1–5.

Gurung, N., Ray, S., Bose, S., & Rai, V. (2013). A broader view: Microbial enzymes and their relevance in industries, medicine and beyond. *BioMed Research International, 2013*, 1–18.

Hans, S. O. (2008). *Enzymes in brewing*. Biokemisk Forening.

Jaeckels, N., Tenzer, S., Rosch, A., Scholten, G., Decker, H., & Fronk, P. (2015). β-Glucosidase removal due to bentonite fining during wine making. *European Food Research and Technology, 241*, 253–262.

König, H., & Fröhlich, J. (2017). Lactic acid bacteria. In *Biology of microorganisms on grapes, in must and in wine*. Springer International Publishing.

Liburdi, K., Benucci, I., & Esti, M. (2014). Lysozyme in wine: An overview of current and future applications. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 1062–1073.

Lonvaud-Funel, A. (2016). Undesirable compounds and spoilage microorganisms in wine. In M. V. Morena-Arribas & B. Bartolomé Sualdea (Eds.), *Wine safety, consumer preference, and human health* (pp. 3–26). Springer International Publishing.

Lyven. (2016). Brewing enzymes. Retrieved from lyven.com

Martín, M. C., & Morata de Ambrosini, V. I. (2014). Effect of a cold-active pectinolytic system on color development of Malbec red wines elaborated at low temperature. *International Journal of Food Science and Technology, 49*(8), 1893–1901.

Merín, M. G., Mendoza, L. M., & Ambrosini, V. I. M. (2014). Pectinolytic yeasts from viticultural and enological environments: Novel finding of *Filobasidium capsuligenum* producing pectinases. *International Journal of Basic Microbiology, 54*, 835–842.

Mojsov, K. (2013). Use of enzymes in wine making: A review. *International Journal of Technology Marketing, 3*, 112–127.

Mojsov, K., Ziberoski, J., & Bozinovic, Z. (2011). The effect of pectolytic enzyme treatments on red grapes mash of Vranec on grape juice yields. *Perspectives of Innovations, Economics and Business, 7*, 84–86.

Oliver, G. (2011). *The oxford companion to beer* (1st ed.). Oxford University Press.

Ottone, C., Romero, O., Aburto, C., Illanes, A., & Wilson, L. (2020). Biocatalysis in the winemaking industry: Challenges and opportunities for immobilized enzymes. *Comprehensive Reviews in Food Science and Food Safety, 19*, 595–621.

Pozo-Bayón, M. A., Monagas, M., Bartolomé, B., & Moreno-Arribas, M. V. (2012). Wine features related to safety and consumer health: An integrated perspective. *Critical Reviews in Food Science and Nutrition, 52*, 31–54.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2006). *Handbook of enology—The microbiology of wine and vinification* (Vol. 1, 2nd ed.). John Wiley & Sons, Ltd.

Rizzi, C., Mainente, F., Pasini, G., & Simonato, B. (2016). Hidden exogenous proteins in wine: Problems, methods of detection and related legislation—A review. *Czech Journal of Food Sciences, 34*, 93–104.

Sammartino, M. (2015). Enzymes in brewing. *Master Brewers Association of the Americas Technical Quarterly, 52*(3), 156–164.

Samoticha, J., Wojdyło, A., Chmielewska, J., Politowicz, J., & Szumny, A. (2017). The effects of enzymatic pre-treatment and type of yeast on chemical properties of white wine. *LWT—Food Science and Technology, 79*(Supplement C), 445–453.

Spagna, G., Barbagallo, R. N., Palmeri, R., Restuccia, C., & Giudici, P. (2002). Properties of endogenous β-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. *Enzyme and Microbial Technology, 31*, 1030–1035.

Spier, M. R., Nogueira, A., Alberti, A., Gomes, T. A., & Dhillon, G. S. (2016). Potential applications of enzymes in brewery and winery. In *Agro-industrial wastes as feedstock for enzyme production* (pp. 261–278). Elsevier.

Stewart, G. G., Hill, A. E., & Russell, I. (2013). 125th Anniversary review: Developments in brewing and distilling yeast strains. *Journal of the Institute of Brewing, 119*, 202–220.

Ugliano, M. (2009). Enzymes in winemaking. In M. V. Moreno-Arribas & M. C. Polo (Eds.), *Wine chemistry and biochemistry* (pp. 103–126). Springer Science Business Media LLC.

Van Oort, M. (2010). Enzymes in food technology—Introduction. In R. J. Whitehurst & M. Van Oort (Eds.), *Enzymes in food technology* (pp. 1–17). Wiley-Blackwell.

Van Rensburg, P., & Pretorius, I. S. (2000). Enzymes in winemaking: Harnessing natural catalysts for efficient bio-transformations: A review. *South African Journal for Enology and Viticulture, 21*, 52–73.

Van Sluyter, S. C., McRae, J. M., Falconer, R. J., Smith, P. A., Bacic, A., Waters, E. J., & Marangon, M. (2015). Wine protein haze: Mechanisms of formation and advances in prevention. *Journal of Agricultural and Food Chemistry, 63*, 4020–4030.

Villena, M. A., Iranzo, J. F. Ú., & Pérez, A. I. B. (2007). β-Glucosidase activity in wine yeasts: Application in enology. *Enzyme and Microbial Technology, 40*, 420–427.

Check for updates

7 Enzymes in Meat, Fish, and Poultry Products Processing and Preservation-I

Khadijeh Abhari and Hedayat Hosseini

Abstract

Enzymes are used in the food industry widespread to modify and enhance the nutritional value, and functional and sensorial characteristics such as color, smell, and flavor of different types of food products.

The technological applications of enzymes in the meat industry are a hot topic nowadays. The most common application of enzymes in red meat products is to increase the meat tenderness. The toughness of beef meat produced from dairy cattle is related to the volume of connective tissue, mainly collagen. Proteolytic enzymes have the potential to tenderize lower-grade meats and increase their market value. Consumers' demand for fresh meat without undesirable excessive fat and salt content leads the manufacturer to consider new possibilities of enzyme application to increase the carcass efficiency by using more effective slaughtering techniques.

The utilization of enzymes to maximize the by-products' efficiency is an undeniable possibility.

The common by-products of the poultry industry are feathers, which can be digested by enzymes and applied for animal feed and also nonfood industries such as films, coating, and packaging due to their high amount of keratins and hydrophobic amino acids. Fat, bone, and mechanical flesh are common by-products of meat processing, which can be digested by enzymatic reactions and used for their special meat properties. Enzymes also can be applied in seafood processing for deskinning and descaling, production of caviar, and recovery of

K. Abhari · H. Hosseini (✉)
Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
e-mail: hedayat@sbmu.ac.ir

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_7

by-products. In the current chapter, the potential application of enzymes in the meat, poultry, and seafood industry will be discussed.

Keywords

Meat · Poultry · Seafood · Enzymes · Preservation

7.1 Application of Enzymes in Red Meat Industry

7.1.1 Role of Enzymes in Meat Tenderization

Meat tenderness, which is determined by meat toughness, depends on many factors such as structure, nature, and the volume of connective tissues mainly collagen and elastin (Lepetit, 2008). Meat tenderness is recognized as one of the main criteria in scoring quality characteristics of meat. There are several methods and equipment to assess the tenderness of meat, such as determination of enzyme activity, scanning electron microscopic (Koohmaraie et al., 1988), analyzing the myofibrillar fragmentation index (Olson & Parrish, 1977), and measuring hydroxyproline (Ashie et al., 2002).

Tenderization is one of the substantial events in postmortem changes which can been affected by decreasing the pH value, temperature, time, and the condition of animal prior to slaughter. During the tenderization, endogenous proteolytic enzymes become active and attack all muscle proteins such as myofibrillar and connective tissues. As the result of proteolytic enzyme activity, the structural and biochemical changes occur, which lead to a significant increase in meat tenderness (Koohmaraie & Geesink, 2006; Herrera-Mendez et al., 2006; Hwang et al., 2003).

7.1.1.1 Endogenous Enzymes

There are several endogenous enzymes which play a role in postmortem tenderization, including calpains, lysosomal cathepsins, and proteasomes. Among these, the calpain system is the primary key of tenderization process. Cathepsins stands in the second level of protein degradation and proteasomes have no significant effect due to unsuitable role of myofibrils as its substrates (Koohmaraie & Geesink, 2006; Kemp et al., 2010).

Calpains

The calpain system, which is activated by calcium and thiol, contains three enzymes three protease enzymes: calpain I (μ), calpain II (m), calpain 3, and calpastatin, which is recognized as calpain inhibitor. Calpain I is linked to myofibrils of muscles, whereas calpain II and calpain 3 are situated in cytosol and sarcomere (near the Z and M line in myofibrillar structure), respectively.

It has been suggested that μ-calpain has an actual role on postmortem proteolysis. Many factors effect on calpain system activity, particularly the calcium ion

concentration; thus, any operation which increases the calcium ion can improve meat tenderness (Moudilou et al., 2010).

Studies revealed that injection of calcium or application of electrical stimulation causes an increase in meat tenderness due to increase in calcium level and decrease in calpastatin activity. The changes in Z line and degradation of myofibrillar proteins such as I troponins, T troponins, desmins, titins, and nebulins are the most common phenomenon during caplain system activity.

Cathepsins and Other Enzymes

Cathepsins contain three classes of enzymes according to the presence of different amino acids in their structure: cysteine, serine, and aspartic peptidase.

The role of cathepsins in tenderness of meat is still uncertain, due to lack of information regarding their release from lysosomes through postmortem storage of meat. It is suggested that small amount of myofibrillar proteins such as actin, myosin, and α-actinin can be affected by cathepsins.

Studies show that other enzymes such as endogenous collagenases, elastases, and proteases can cause the degradation of the structure of collagen and elastin.

7.1.1.2 Exogenous Enzymes

Furthermore, there are another group of enzymes which are derived from plants, animals, and microbial sources and have potential to effect on meat tenderization. The most common exogenous enzymes used for meat tenderization are proteases. The proteases are vital for cell growth as they are capable to catalyze and hydrolysis the peptide bond of proteins and provide amino acids for growth and differentiation.

Proteases have diverse variety due to their structure, protein characteristic, and mode of action. The exogenous proteases are commonly classified by their original source. The exogenous proteases that have been approved by Food and Drug Administration (FDA) and recognized as GRAS substances are as follows: plants (papain, ficin, bromelain), microbes (*Aspergillus niger*; *Bacillus subtilis*; *Rhizopus oryzae*), and animals (trypsin, pepsin).

Plant Enzymes

Papain

Papain is a protease enzyme which contains cysteine and extracted protease from the latex of the papaya plant (*Carica papaya*). This enzyme is a part of defense system of plant and protects the plant against insect. The optimum temperature and pH for enzyme activity is 65 °C and 5–8, respectively.

Commercial forms of papain contain various ratio of papain, papaya peptidase, and chymopapain and lead to different biochemical properties. Papain tenderizes the toughness of meat by acting on amino acids which have aromatic side chain like tyrosine and phenylalanine. Papain can effectively hydrolyze collagen and myofibrillar proteins. Investigations show that injection of papain approximately 30 min before slaughter can cause meat tenderness. As injection of papain to animal may

cause stress symptoms and severe shock, using inactive papain can be an alternative approach.

Ficin

Ficin is extracted from latex of fig fruits (*Ficus glabrata*, *Ficus anthelmintica*). Figs contain ten different proteases and ficin is most popular for its tenderizing effect. Studies revealed that application of ficin in temperatures of 60 and 70 °C has the most influence in tenderization process. It is suggested that ficin has the ability to effect on collagen, elastin, and myofibrillar proteins in addition to increase in water-holding capacity.

Cucumin

Cucumin is another proteolytic enzyme which is achieved from kachri (*Cucumis pubescens*). The researches show that cucumin can be effective on tenderizing buffalo meat, sheep meat, and chicken.

Bromelain

Bromelain is cysteine proteases which originated from pineapple. It has two sources: fruit bromelain and stem bromelain; it has been shown that fruit bromelain is more specific and more effective in comparison to stem bromelain. The optimum temperature for enzyme activity is 50–60 °C; however, the studies showed that the enzyme activity remains at 0 °C and it becomes inactive in temperatures more than 80 °C. The bromelain activity starts by degradation of collagen, and then attacks myosin.

Zingibain

Zingibain is a potent proteolytic enzyme which is obtained from ginger. The studies reported that Zingibain is more effective in meat tenderization when heated. Ginger is a great and economical source of proteases that begin degradation from the I band of each sarcomere and go through M line.

Actinidin

Actinidin is a natural enzyme extracted from kiwi fruit which has been recognized as an agent which can hydrolyze both connective tissue proteins specially collagen and myofibrillar proteins. Studies show that applying actinidin in brine solution can tenderize porcine muscles.

7.1.2 Role of Enzymes in Meat Flavor Development

Flavor has a complex definition which includes several phenomenons such as taste, odor, trigeminal senses, and texture. As well as the mentioned factors, tenderness significantly affects the flavor and the relationship of all these together creates consumer acceptability. Generally, the raw meat is tasteless due to absence of nonvolatile components; however, the flavor precursors are present in the meat

tissue and it is necessary to undergo enzymatic reaction to tastes appears in the meat (Fernandez et al., 2000; Toldra, 1998; Calkins & Hodgen, 2007).

As the results of several enzymes activity such as lipase, protease, glutaminase, and peptidase, biochemical reactions occur and lead to enhance the flavor. These enzymes may be endogenous natural lipases and protease, microbial originated enzymes or those which added to the product throughout the manufacturing procedure. Due to proteases activity mainly cathepsins, trypsin-like peptidases, and *Micrococcaceae* originated enzymes (Fernandez et al., 2000), nitrogenous complexes such as volatile compounds, small peptides, and free amino acids appear. As well as micrococci, other species of lactic acid bacteria, such as *Pediococcus* and *Lactobacillus*, are able to produce intracellular peptidases which contribute to increase the levels of free amino acids. These substances have principal effect on the taste of the product.

Another group of enzymes which takes part in flavor enhancement are lipases. Lipases produced by some microorganisms like lactobacilli or micrococci could effect on the lipids by their lipolytic activity and oxidation, leading to increase in the level of free fatty acids and short chain fatty acids. The substances produced by lipases are responsible to develop the flavor in final product. Although the optimum temperature for maximum activity of microbial lipases is 30–40 °C, some thermophilic microorganisms such as *Pseudomonas*, *Bacillus* sp., *Thermomyces lanuginosus,* and *Aspergillus niger* release lipases which can stand in the higher temperatures (50–65 °C).

Glutaminase also play a role in creating the flavor of sausages by deamidation of glutamine, leading to production of L-glutamic acid and ammonia through hydrolysis of the glutamine amide group. Glutamic acid is an amino acid which is responsible for the flavor enhancement and producing by starter cultures has an important role in flavor in sausage, seasonings such as soya sauce and miso, and pickles. Glutamic acid is the substance which increases "umami" taste in food products. The ammonia, produced by glutaminase, performs as an acid neutralizer. Glutaminase can be obtained from *Bacillus amyloliquefaciens*, *Aspergillus oryzae*, *Debaryomyces,* and *Rhizobium etli*.

7.1.3 Approaches for Utilization of Enzymes as in Meat Tenderizer

There are several traditional approaches to apply tenderizing substances into carcasses. These methods include injection; dipping and spraying the enzymes have both advantages and disadvantages. The main concern regarding utilization of traditional method is incomplete and insufficient distribution of the tenderizing agents into meat pieces. Neglected penetration of enzymes into the meat pieces causes surfaces to become over-tenderized while interior parts of meats remain unchanged (Rhodes & Dransfield, 1973; Lawrie, 1998).

7.2 Application of Enzymes in Seafood Industry

7.2.1 Deskinning

The most important application of enzymes in the seafood industry is to deskinning and descaling. In this procedure the skin is removed with the least damage to the flesh. If the deskinning process done suitable, the edible portion can increase. The main source of enzymes to use in the seafood industry is marine enzymes. Application of enzymes in combination with mechanical methods has been performed for processing of tuna, squid, herring, shrimp shells, Pollock, and skate (Simpson et al., 2012).

7.2.2 Production of Fish Protein Hydrolyzate

Fish protein hydrolyzates are recognized as one of the important by-products of the seafood industry which can be obtained by enzymatic or chemical reactions. The wasted of fish industry such as gills, head, skin, bones, viscera and liver are the main sources for producing fish hydrolyzates which are in the forms of peptides with 2–20 amino acids depends on the type of enzyme, kind of fish and the time of operation.

Fish hydrolyzates also can be obtained by alkali or acid reactions in high temperature and pressure in the traditional method. The major acids utilized in acid hydrolysis are sulfuric acid and hydrochloric acid. One of the disadvantages of acid and alkali hydrolysis is production of great amount of sodium chloride and sodium hydroxide, respectively, which decreases its functionality. Moreover, destruction of tryptophan, a critical amino acid, and production of several toxic compounds are other concerns regarding using acid hydrolysis approach.

On the other hand, enzymatic hydrolysis has several advantages, such as being economical, and required slight conditions of pressure, temperature, and pH. Fish hydrolyzates demonstrate functional properties in food formulation and can be applied as emulsifier or a substance which increase water-holding capacity, protein solubility, gelling activity, and oil-binding capability.

Fish hydrolyzates which are mostly produced by action of alcalase are more constructive compared to poultry by-products.

7.3 Application of Enzymes in Poultry Processing

Poultry industry with various products including chicken, egg, and turkey has a large portion in food sector and human diet. Due to side effects of red meat consumption, nutritional benefits of white meat and ease of preparation of such products for customers and of the poultry industry has been growing fast, as the USA, China, and Brazil have the maximum products, worldwide.

Consequence of such a large development in poultry industry is production of high amount of waste mainly feathers, deboning residue soft meat, blood and also dead birds on arrival. Due to several concern regarding converting the poultry waste to soil fertilizer such as accumulation of fat in soil, excessive levels of oxygen requirement and probable occurrence of pathogens in hatchery, dead and litter compost, utilization of enzymes to use wastes has been recommended. Nowadays, the waste materials of poultry industry are transformed to feather meal, meat and bone meal, blood meal, and fats/oils through rendering process. While the mentioned meals are rich source of protein, their application can be restricted due to their nutritional loss particularly essential amino acids, as well as high amount of lysine, calcium, and phosphorous in meat/bone meat and bone meal.

The significant portion of waste products is feathers which is 8% of the weight of an adult chicken.

Keratin is a protein which constituted approximately 90% of the feathers. Degradation of keratin is a complicated process due to disulfide bonds between polypeptide chains and also hydrophobic interactions and several hydrogen bonds.

Additional to feathers, other parts of the bird such as legs, heads, bones, skin, and viscera contain large amount of various proteins such as collagen, keratin, and elastin. The dead birds which on the arrival are also rich source of proteins. Utilization of poultry by-products helps to avoid environmental pollution; besides, the extracted and hydrolyzed protein can be used as a nutritious part of animal diet.

7.3.1 Utilization of Keratinase for Bioconversion of Feathers

Feathers are a great source of inexpensive protein; however, due to its low nutritional value and low digestibility, its application as a feedstuff has various concerns. Conventional hydrothermal method of degradation for feathers causes losing some essential amino acids, as well as production of non-nutritious amino acids such as lysinoalanine and lanthionine. To overcome the mentioned problems, biotechnological methods via microorganisms have been offered. The keratinase produced by microorganisms such as *Bacillus* spp. has the capacity to hydrolyze keratin and convert it to peptides which can be used as nitrogen fertilizers or animal feed. Controlled enzymatic hydrolysis is an appropriate method for production of bioactive peptides that have the potential to play role as ant antimicrobial, antioxidant and antihypertensive as Fontoura et al. (2014) reported DPP-IV and ACE inhibitor activities of hydrolyzed keratin obtained by feathers of raw chicken.

Another by-product of poultry industry is fat which is significantly located in the skin. Chicken fat contains both ω-3 and ω-6 polyunsaturated fatty acids which can be beneficial to reduce cholesterol. Lee and Foglia (2000) have indicated that *Candida rugosa* and *Geotrichum candidum* are able to produce lipases which act on triglyceride fractions that have been evoked from chicken fat by supercritical CO_2.

7.4 Application of Enzymes for Enhancement of Flavor

In Addition to marine sources for production of protein hydrolyzates, various meat by-products such as bones, bovine by-products, sheep visceral mass and chicken by-products can be treated with different enzymes for this purpose. These protein rich resources, depends on type of applied enzyme and condition have the capacity to enhance the meat flavor when not proper for food use. The main concern regarding using meat by-products as source of protein hydrolyzates is formation of bitter taste.

7.5 Future Prospective

Due to consumer demand for fresh tender meat with minimum fat and salt, and also the manufactures to produce the carcasses with sufficient profits, enzymatic techniques have been utilized to achieve these aims. One of these methods which recently become popular in meat industry is using TGase which is a cold binding is enzymatic technology.

In this method, the cut and timed pieces of meat from one or several animals can be attached together with TGase to form meat products with new shape and size, which can be stored in every temperature.

References

Ashie, I., Sorensen, T., & Nielsen, P. (2002). Effects of papain and a microbial enzyme on meat proteins and beef tenderness. *Journal of Food Science, 67*(6), 2138–2142.

Calkins, C. R., & Hodgen, J. M. (2007). A fresh look at meat flavor. *Meat Science, 77*(1), 6380.

Fernandez, M., et al. (2000). Accelerated ripening of dry fermented sausages. *Trends in Food Science & Technology, 11*(6), 201–209.

Fontoura, R., Daroit, D. J., Correa, A. P. F., Meira, S. M. M., Mosquera, M., & Brandelli, A. (2014). Production of feather hydrolysates with antioxidant, angiotensin-I converting enzyme- and dipeptidyl peptidase-IV-inhibitory activities. *New Biotechnology, 31*(5), 506–513.

Herrera-Mendez, C. H., et al. (2006). Meat ageing: Reconsideration of the current concept. *Trends in Food Science & Technology, 17*(8), 394–405.

Hwang, I., Devine, C., & Hopkins, D. (2003). The biochemical and physical effects of electrical stimulation on beef and sheep meat tenderness. *Meat Science, 65*(2), 677–691.

Kemp, C. M., et al. (2010). Tenderness—An enzymatic view. *Meat Science, 84*(2), 248–256.

Koohmaraie, M., & Geesink, G. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74*(1), 34–43.

Koohmaraie, M., et al. (1988). Factors associated with the tenderness of three bovine muscles. *Journal of Food Science, 53*(2), 407–410.

Lawrie, R. (1998). *Meat science* (6th ed.). Woodhead Publ. Ltd.

Lee, K., & Foglia, T. A. (2000). Synthesis, purification, and characterization of structured lipids produced from chicken fat. *Journal of the American Oil Chemists' Society, 77*, 1027–1034.

Lepetit, J. (2008). Collagen contribution to meat toughness: Theoretical aspects. *Meat Science, 80*(4), 960–967.

Moudilou, E., et al. (2010). Calpains expression during Xenopus laevis development. *Tissue and Cell, 42*(5), 275–281.

Olson, D., & Parrish, F., Jr. (1977). Relationship of myofibril fragmentation index to measures of beefsteak tenderness. *Journal of Food Science, 42*(2), 506–509.

Rhodes, D. N., & Dransfield, E. (1973). Effect of pre-slaughter injections of papain on toughness in lamb muscles induced by rapid chilling. *Journal of the Science of Food and Agriculture, 24*(12), 1583–1588.

Simpson, B. K., et al. (2012). *Food biochemistry and food processing*. John Wiley & Sons.

Toldra, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science, 49*, S101–S110.

8 Enzymes in Meat, Fish, and Poultry Product Processing and Preservation-II

Sandesh Suresh Karkal, Anushma Venmarath, Suresh Puthenveetil Velappan, and Tanaji G. Kudre

Abstract

The current chapter provides insights into the applications of enzymes in meat, fish, and poultry products' processing industries. The mechanical and chemical techniques mainly employed to process meat, fish and poultry products are not eco-friendly, consume more energy, and generate by-products. Hence, the substitution of these techniques with enzymes (biocatalysts) could be the best alternative. Besides, the application of enzymes in these industries is gaining momentum since they are eco-friendly, consume less energy, and also aid in obtaining good-quality products with better yield. The enzymes can catalyze the reaction process at mild temperature and pH and are highly specific toward substrate molecules. Enzymes such as proteases, lipases, transglutaminases from plant, animal, and microbial origin are suitable and have been extensively studied to process meat, fish, and poultry products. Currently, novel enzymes are also being developed as a result of technological advancements. The recombinant enzymes being developed through technical advancements in biotechnology for their application in meat, fish, and poultry products processing could be an excellent prospect. This chapter describes the potential enzymes used in the processing and preservation of meat, fish, and poultry products.

Keywords

Meat industry · Seafood industry · Poultry industry · Proteases · Lipases · Transglutaminases

S. S. Karkal · A. Venmarath · S. P. Velappan · T. G. Kudre (✉)
Meat and Marine Sciences Department, CSIR-Central Food Technological Research Institute, Mysuru, India
e-mail: tkudre@cftri.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_8

8.1 Introduction

Enzymes are termed as "biocatalysts" owing to their natural catalytic nature and are considered to be prime tools in biotechnology and related areas (Fernandes, 2016). Enzymes are mainly protein molecules known to speed up the biochemical reaction rate occurring in the cell (Singh, 2018). Enzymes have been utilized in the processing of food since ancient times, long before their catalytic function was discovered. Homer's epic poem, Iliad, written around the eighth century BC, describes the use of enzyme ficin for milk clotting (Copeland, 2000). Apart from this, the original inhabitants of the Pacific Islands exploited papaya juice for meat tenderization. The colonization of these islands by the British led to the growth of enzyme technology for food processing, especially meat tenderization and also wound healing in European nations (Gomes et al., 2018). At present, enzymes have found their potential application in food, leather, animal feed, biofuel, detergent, etc., industries. In these industries, enzymes have been employed as efficient catalysts (Singh, 2018). The increased demand for enzymes in industries, especially food industries, may be attributed to its high specificity towards the substrate molecule. Besides, enzymes can catalyze the reactions under mild pH and temperature with minimal by-product formation (Shahidi & Kamil, 2001).

Food is one of the necessities of life required for human survival. Despite being an important necessity, the global report on the food crisis (2019 Edition) by the European Commission illustrates that across 53 countries, a population of more than 113 million is experiencing a food shortage, nutritional deficiency, and also improper livelihood assistance (European Commission-GRFC, 2019). Due to the rapid population explosion, extensive utilization of natural resources, disputes across the globe, and variation in climate, there has been an increased demand for food worldwide (Singh, 2018). According to the United Nation Department of Economic and Social Affairs (UNDESA), New York, the population of the world is projected to be increased from 6.9 to 9.1 billion (i.e., 32% rise) by 2050 and this would trigger the hike in food demand up to 70% (UNDESA, 2017; Singh, 2018). Moreover, to mitigate food shortage, in the year 2015, the United Nations (UN) Member States adopted the 2030 Agenda, which mainly aims for the contribution of fisheries and aquaculture in the use of natural resources toward food security and nutrition, and thereby ensure sustainable development in economic, social, and environmental terms (FAO, 2016). A considerable increment in the production of meat and poultry products was also observed to meet the global food demands. According to the Food and Agriculture Organization of the United Nations (FAO), by 2050, the global production of meat is expected to increase to 455 million tons (Chandrasekaran et al., 2015).

At present, combating the current global scenario of the food crisis, elimination of malnutrition and hunger, and ensuring food security have been the most challenging research and development fields. Food processing could play a pivotal role in eradicating the persisting global food shortage (Singh, 2018). Food processing refers to the process where the agricultural raw material is processed into a consumer-ready product. The main objectives of modern food processing include (1) to prepare safe

food (microbiologically and chemically), (2) to deliver products enriched in organoleptic properties and nutrients, and (3) to make convenient food. Generally, chemical treatment methods are implemented to aid production while processing the food for commercial utilization. The substitution of chemicals with enzymes in food processing has resulted in quality products with better yields. Besides, it has also reduced energy consumption and doesn't emit greenhouse gases (Chandrasekaran et al., 2015). The application of enzymes in food industries such as meat, fish, and poultry product processing has been vital in obtaining better-quality products as well as their preservation. The demand for food enzymes in these industries has been growing alongside their increased production across the globe. Moreover, the recombinant enzymes, especially from microorganisms, are also being produced for their application in these industries. In meat and poultry products processing industries, enzymes have been predominantly used to improve the tenderness of beef, pork, sheep, and chicken meat, whereas enzymes in fish-processing industries find a wide array of applications, including the preparation of products, extraction of biomolecules, and preservation (Fernandes, 2016; Suresh et al., 2015). The current chapter primarily focuses on the application of enzymes in the processing of meat, fish, and poultry products, their significance, and also their future scope in the food industry.

8.2 Importance of Enzyme in Food Industry

Due to the rapid population explosion, extensive utilization of natural resources, disputes across the globe, and variation in climate, there has been an increased demand for food worldwide (Singh, 2018). In the food industry, the application of enzymes is promising for the processing of food and also in solving food-related problems (Venugopal et al., 2000; Singh, 2018). At present, enzymes have found their wide application in various food industries, including beverage, dairy, oils and fats, brewing, cereals, confectioneries, bakery, fruits and vegetables, juice, meat, seafood, and poultry (Chandrasekaran et al., 2015). The importance of enzymes in meat, fish, and poultry product processing is described below.

8.2.1 Current Scenario of Meat Production and Role of Enzymes in Meat Processing

Animals such as sheep, goat, pig, cattle, and other livestock are slaughtered, processed, and distributed in the meat industry (Chandrasekaran et al., 2015). According to FAO, in the year 2018, global meat production increased up to 1.2% compared to 2017, which could be easily interpreted in Fig. 8.1 (FAO, 2019). Figure 8.1 depicts the production of meat worldwide from the year 2016 to 2019. Increased production of pork and beef meat was observed in the year 2018, but there was a slight decrease in their production in 2019. However, sheep meat production increased from 2016 until 2019.

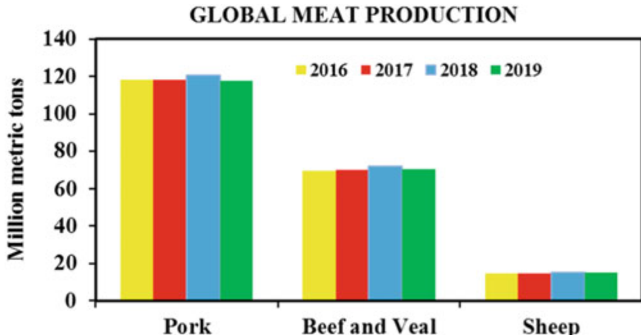


Fig. 8.1 Global meat production from the year 2016 to 2019. (Source: Statista, Production of meat worldwide, 2020)

In the past decades, global consumption of meat has increased due to population explosion and the economic boost in developing nations. Currently, the consumers consider the quality attributes of meat products rather than their price, which signifies that the greater the quality traits of meat products, the greater their importance to meet the increasing consumer demands (Gomes et al., 2018). The most appreciable quality trait of meat judged by consumers is "tenderness". The tenderness is a textural property of food associated with chewiness or cohesiveness. This quality trait of food during mastication manifests little resistance to fragmentation (Ashie et al., 2002; Gomes et al., 2018). The tenderness of the meat is primarily related to the structural integrity of connective tissue proteins and myofibrillar proteins. The key constituent of connective tissue is collagen, and the tenderness of the meat is determined by the amount, type, as well as the degree of intermolecular cross-linking in collagen. Studies have depicted an increased cross-linking in aged animals rather than young animals (Ashie et al., 2002). To improve the tenderness of the meat, the application of enzymes has been found to be desirable in terms of both producing quality products and the cost (Cazarin et al., 2015). There are two underlying mechanisms to obtain the desired tenderness in meat products using enzymes. The first mechanism involves the generation of amino acids or peptide fragments by breaking the covalent bonds of protein molecules. This would result in meat structure alteration. The second mechanism includes the formation of new covalent bonds, which would ultimately change the meat tenderness. The enzyme mainly involved in the new bond formation is transglutaminases (Cazarin et al., 2015). Both the endogenous enzymes (calpains, cathepsins, capsases, and proteosomes) and exogenous enzymes from the plant, animal, and microbial sources (ficin, bromelain, papain, actinidin, cucumin, zinzibain, collagenase, elastase, animal protease, and fungal protease) have been found to play a pivotal role in the tenderization of meat (Ashie et al., 2002; Cazarin et al., 2015; Gomes et al., 2018). Apart from the application of enzymes in meat tenderization, other techniques involve shock wave pressure, calcium chloride injection, mechanical tenderization, electrical stimulation, and elevated temperature storage (Ashie et al., 2002).

8.2.2 Current Scenario of Fish Production and Role of Enzymes in Fish Processing

Over the decades, a tremendous leap has been observed in fish production across the globe. The global fish production in the year 2018 was 178.8 million metric tons, and in the year 2019, it is projected to be 177.9 million metric tons (Statista, 2019; FAO-GLOBEFISH, 2020). Figure 8.2 displays the total global fish production from the year 2016 to 2019. The total global fish production includes aquaculture production, which includes the production of even crustaceans and mollusks (seafood). In the majority of the countries, fresh fish and seafood harvested are mainly sold for local consumption (Suresh et al., 2015). From the total fish and seafood processed, about 20–50% is available for edible purposes, and the rest of it is generated as non-edible by-products or discarded as waste. These by-products are rich in various biomolecules such as oil and lipids, proteins, bioactive peptides, pigments, polymers, vitamins, and minerals (Suresh et al., 2015).

The utilization of enzymes in the processing of fish and seafood has gained momentum due to the advancements in enzyme technology. Enzymes have been employed in fish-processing industries to obtain a diversified range and improved quality products. In fish-processing industries, enzymes have been used for descaling, deskinning, salted fish ripening, production of products such as surimi, fish sauce, and fish protein hydrolyzates, extraction of oil and biomolecules such as chitin, carotenoproteins, and flavor compounds, and removal of exoskeleton from shellfish (Suresh et al., 2015). The enzymes used for fish and seafood processing are mainly derived from microorganisms, plants, and animals (Fernandes, 2016; Suresh et al., 2015). Moreover, research has also been carried out on the enzymes isolated from fish discards and its application in the processing of fish and seafood (Suresh et al., 2015). This approach would help in waste management of fish and seafood by-products, the majority of which are mainly dumped into the environment. The enzymes—both natural and recombinants—derived from microorganisms have been used in fish and seafood processing. Further, the demand for microbial enzymes is more than that for enzymes isolated from plants and animals since the microbial enzymes exhibit better catalytic activity and high yield, and can be genetically

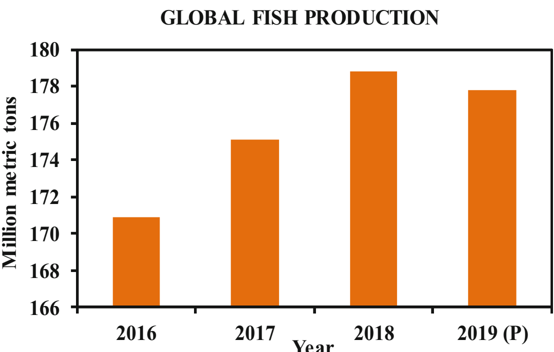


Fig. 8.2 Global fish production from 2016 to 2020. (Source: Statista, 2020; FAO-GLOBEFISH, 2020)

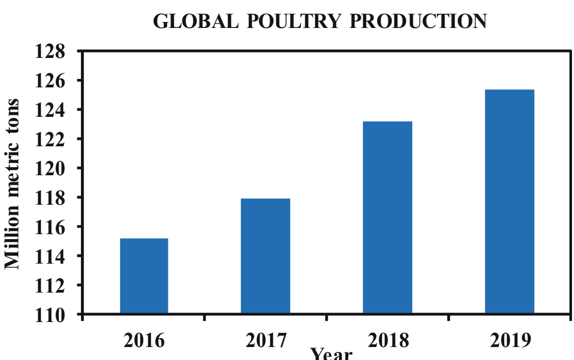


Fig. 8.3 Global poultry production from 2016 to 2019. (Source: Statista, Production of meat worldwide, 2020)

manipulated with ease, regular supply, and rapid microbial growth in low-priced culture media (Suresh et al., 2015). Besides fish and seafood processing, enzymes have also been used in the analysis of processed aquaculture and fish products (Fernandes, 2016).

8.2.3 Current Scenario of Poultry Production and Importance of Enzymes in Poultry Processing

Similar to meat and fish production worldwide, poultry production has also increased over the decades. Figure 8.3 displays the global poultry production from the year 2016 to 2019. From the figure, it is evident that the global poultry production is increasing every year and is higher than global sheep, pork and beef, and veal production. Global poultry production has been soaring to meet the global consumption demands due to overpopulation.

Besides the tenderization of pork, sheep, and beef meat, enzymes have also been used for the tenderization of poultry products. Studies have been carried out on improving the tenderness of poultry meat using exogenous enzymes such as papain, bromelain, proteases, etc., and also endogenous enzymes such as calpains (Bawa et al., 1981; Kang & Warner, 1974; Whipple et al. 1990).

8.3 Enzyme Sources and Types

As described in the previous sections, the enzymes used in meat, fish, and seafood as well as poultry products processing may be from plants, animals, or microorganisms. Apart from exogenous enzymes, the roles of endogenous enzymes are also crucial for the processing of food products. The enzymes from the microbial source are most widely preferred since they can be easily manipulated and microorganisms can be easily cultured. Besides, the trend towards the use of enzymes from marine microorganisms has been increasing from the last few years (Fernandes, 2016). Proteases such as ficin, bromelain, and papain, which are primarily plant-based,

have been widely used in meat tenderization. These proteases possess a sulfhydryl group in their active sites to carry out their catalytic activity. Apart from meat tenderization, these proteases have also been used in the preparation of the fish sauce. Moreover, papain has also been used in the preparation of fish protein hydrolyzates (Fernandes, 2016). Research has also been carried out on the extraction of enzymes from fish-processing discards and their application in the processing of fish products. Enzymes including pepsin, trypsin, gastricin, alkaline proteinase, acidic protease, calpain, lysosomal cathepsins type B, H, L, cathepsin and other lysosomal cathepsins, chymotrypsin, collagenases, chymosin, elastase, lipase, carbohydrases, and alkaline peptidase from fish-processing by-products have been tested for their application in the processing of fish and seafood products (Venugopal, 2016). Most of the enzymes used in meat, fish, and poultry product processing are proteases. Besides proteases, other enzymes (lipases and transglutaminases) play a major role in flavor modification, hydrolysis of fatty acids, and other processing methods. The different enzymes used in the processing of meat, fish, and poultry products, and also their sources are exhibited in Tables 8.1, 8.2 and 8.3, Fig. 8.4.

8.4 Enzymes Involved in Meat Processing and Preservation

8.4.1 Calpains

Calpains are calcium-dependent cysteine proteases mostly found in mammals. Calpain 1 (μ calpain), calpain 2 (m calpain), and calpain 3 are the major calpain proteases of skeletal muscle; this calpain system also consists of an inhibitor of calpain 1 and calpain 2, called calpastatin (Goll et al., 2003). They are activated by micromolar (1–100) and millimolar (0.1–1 mM) concentrations of calcium, respectively (Suzuki et al., 1992). The intracellular calcium concentration is 1 μm or less at most. Therefore, it always exists as a proenzyme until enough calcium is present for activation. Tenderness is an essential aspect of meat quality. The degradation of myofibrillar proteins (troponin T, troponin I, tropomyosin, connectin, C-protein, and desmin) results in the weakening of myofibers. This is a crucial event in the tenderization. μ-Calpain-induced degradation of key myofibrillar proteins is the cause of postmortem proteolysis, and this is the primary source of muscle tenderness (Koohmaraie & Geesink, 2006). This can be achieved by increasing the calcium concentration (Rees et al., 2002) or by lowering the calpastatin activity (Hope-Jones et al., 2010). Electrical stimulation (ES) can also lead to an increased flow of calcium ions into the cytoplasm and may lead to μ-calpain activation (Veeramuthu & Sams, 1999). Calpastatins inhibit calpains and thereby reduce tenderness, but after cooking calpastatin is inactivated, which leads to increased tenderness.

Table 8.1 Proteases used in the processing of meat, fish, and poultry products

Source	Enzyme	Source	Applications
Plants	Ficin	Fig (*Ficus glabrata*, *Ficus laurifolia*, *Ficus anthelmintica*)	Meat tenderization
	Papain	Papaya (*Carica papaya*)	Meat tenderization, preparation of fish sauce, and fish protein hydrolyzates
	Bromelain	Pineapple (*Ananas comosus*)	Meat tenderization and preparation of fish sauce
	Actinidin	Kiwi fruit (*Actinida deliciosa*)	Meat tenderization
	Cucumin	Kachri fruit (*Cucumis pubescens*, *Cucumis trigonas*)	Meat tenderization
	Zinzibain	Ginger (*Zingiber officinale*)	Meat tenderization
Animal	Porcine pancreatin	Pork	Meat tenderization
Microorganisms	Fungal protease	*Aspergillus oryzae*, *Penicilium chrysogenum* Pg222	Meat tenderization, preparation of fish protein hydrolyzates
	Alkaline protease	*Bacillus subtilis*	Preparation of fish protein hydrolyzates
	Thermophilic enzyme E	*Bacillus* strain E.A.1	Meat tenderization
	A1 protease	*Thermus* strain	Meat tenderization
	4-1 A protease	Rt4-1.A	Meat tenderization
	Caldolysin	*Thermus* strain T-35	Meat tenderization
	Elastase	*Bacillus* species EL31410	Meat tenderization
	Collagenase	*Vibrio* B-30	Meat tenderization

Source: Naveena et al. (2004), Venugopal et al. (2000), Cazarin et al. (2015)

8.4.2 Cathepsins

Cathepsins are lysosomal acid proteases found in almost all organisms. There are 13 types of lysosomal cathepsins, 7 of which exist in skeletal muscle (Goll et al., 1983). Cathepsins comprise both exoproteases and endoproteases, and are differentiated from the active site: aspartate (Cathepsin D and E), serine (Cathepsin G), and cysteine (Cathepsin B, H, L, and X) (Sentandreu et al., 2002). They exist as a proenzyme in living tissue. Due to pH fall after cell death, they are released into the cytoplasm or intercellular space due to lysosomal disruption (Duston, 1983). Cathepsins B and L are the major cathepsins involved in muscle proteolysis (Jamdar & Harikumar, 2002). Protease inhibitor cystatin regulates cathepsin B, H, and L in vivo (Turk & Bode, 1991).

Table 8.2 Lipases used in the processing of meat, fish, and poultry products

Source	Source	Applications
Animal	Porcine pancreas	Preparation of ω-3 PUFA concentrates
	Atlantic cod	Production of ω-3-enriched triglycerides, flavor improvement
	Sardine	Production of ω-3-enriched triglycerides, flavor improvement
	Indian mackerel	Production of ω-3-enriched triglycerides, flavor improvement
	Red sea bream	Production of ω-3-enriched triglycerides, flavor improvement
	Salmon	Production of ω-3-enriched triglycerides, flavor improvement
Microorganisms	*Pseudomonas* species	Enrichment of EPA and DHA in sardine oil
	Candida cylindracea	Increasing DHA content, hydrolysis of menhaden oil
	Lactobacillus plantarum	Synthesis of short-chain fatty acid esters

Source: Suresh et al. (2015), Venugopal et al. (2000), Cazarin et al. (2015), Uppada et al. (2017)

Table 8.3 Transglutaminase application in the processing of meat, fish, and poultry products

Source	Source	Applications
Microorganisms	*Streptoverticillum* sp., *Bacillus subtilis*, *Streptomyces* sp.	Fish meat sheet formation, fish meat film and mince formulations, surimi and restructured fishery products, texture modification of finfish, collagen and gelatin bond formation, improve the gel strength of chicken and beef sausages, improve the gel strength of chicken and beef meat

Source: Suresh et al. (2015), Cazarin et al. (2015)

Many research groups ignore cathepsins' contribution to meat tenderization due to various reasons like no large-scale degradation of the actin and myosin in postmortem conditioning (Koohmaraie et al., 1991). To access myofibril proteins and to increase the tenderness, cathepsins must be released from lysosomes (Hopkins & Taylor, 2002). Whipple et al. (1990) stated that there is only a minor association between meat tenderness and cathepsin activity. However, the activities of cathepsins B and L at 8 h postmortem have been found to positively correlate with beef tenderness (O'Halloran et al., 1997). In the myofibrils of rabbit, beef, and chicken, the main myofibrillar proteins (nebulin, tropomyosin, troponin T, I, C, titin) along with actin and myosin are hydrolyzed by Cathepsin L during the postmortem conditioning period (Mikami et al., 1987).

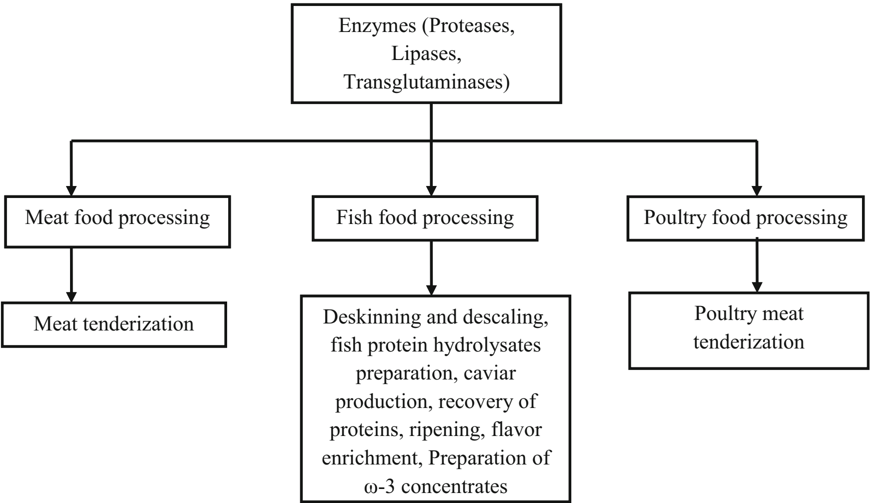


Fig. 8.4 Applications of enzymes in meat-, fish-, and poultry-processing industries

8.4.3 Papain

Papain is the most common enzyme used in meat tenderization. Papain, which belongs to the C1 family of cysteine protease, also called the papain family (Rawlings et al., 2010), is obtained from the latex of the papaya plant (*Carica papaya*), and it plays a major physiological role in protecting the plant from insects (Konno et al., 2004). Papain's commercial importance is primarily due to its good proteolytic activity against a broad variety of protein substrates, and because it is active in a wide range of operating conditions. Smith and Hong-Shum (2003) reported that it has a high optimal temperature (65 °C) and broad pH range (5–8) for its activity. Papain predominantly acts on the muscle structural component, which enhances the meat tenderization (Gracey & Thronton, 1985). It is a highly effective enzyme that is capable of inducing major degradation of collagen and myofibrillar proteins (Ashie et al., 2002), and the maximum tenderizing activity occurs during the cooking process (Tappel et al., 1956). Kang and Rice (1970) reported that papain showed strong activity for a myofibrillar fraction with greater solubilizing activity on the connective tissue. Use of papain leads to over tenderization of meat which resulted in mushy meat, hence the commercial use of papain is limited for meat tenderization (Han et al., 2009).

8.4.4 Ficin

Ficin, the proteolytic enzyme from fig trees (*Ficus* sp.), is a sulfhydryl protease. About ten proteases are present in crude ficin latex (Kramer & Whitaker, 1964). The mostly studied ficins are those from *Ficus glabrata* and *Ficus carica*. It is reported to

hydrolyze and increase the solubility of muscle proteins (El-Gharbawi & Whitaker, 1963). The solubility of meat protein might be increased by ficin by degrading the proteins into units of smaller molecular weight, which, when aggregated, form a three-dimensional network (Ramezani et al., 2006). El-Gharbawi and Whitaker (1963) reported that the optimum pH was around 7 for enzyme activity for collagen and myofibrillar proteins, and about 5.0–5.5 for elastin.

8.4.5 Bromelain

The proteolytic enzymes called bromelain are present in fruits, leaves, and stems of the Bromeliaceae family of which pineapple (*Ananas comosus*) is the most commonly used enzyme in the processing of meat (Doko et al., 1991). The enzyme extracted from the stem is called stem bromelain, and that extracted from the fruit is called fruit bromelain (Vanhoof & Cooreman, 1997). Fruit bromelain is said to have a higher proteolytic activity and specificity compared to stem bromelain (Barrett et al., 2004; Grzonka et al., 2007). This enzyme, like other proteases, results in over-tenderization by degrading myofibrillar proteins and collagen (Melendo et al., 1996). At first, it degrades about 40% of the collagen in the sarcolemma, and then it degrades myosin in the myofibrillar region (Kang & Rice, 1970; Wang et al., 1958). The application of this enzyme is easy and cheap and can be exploited on a household and industrial scale for tenderizing tough meat; it is also a better alternative to chemical tenderizers (Sunantha & Saroat, 2011). Ionescu et al. (2008) investigated the use of bromelain in adult beef with best results at 10 mg/100 g meat, with a tendering time of 24 h at 4 °C, followed by thermal treatment with an increase of 1 °C/ min to 70 °C (when enzyme inactivation occurs). These conditions improved the tenderness of the beef.

8.4.6 Cucumin

Cucumin obtained from kachri (*Cucumis pubescens*) is a protease. Initially, the dried coarsely ground fruits of *C. trigonus* Roxb (locally known as kachri) were traditionally used as a meat tenderizer in some parts of India (Naveena et al., 2004). Cucumin tenderizes meat to a significant degree, and may serve as a basis for commercial meat-tenderizing mixtures (Hujjatullah & Baloch, 1970).

8.4.7 Zingibain

Zingibain protease was isolated from the plant *Zingiber officinale roscoe* (ginger rhizome) by Thompson et al. (1973). This thiol protease shows optimum activity at 60 °C (Naveena et al., 2004). The ginger extracts contain two cysteine proteases with a molecular mass of 29 and 31 kDa, as reported by Su et al. (2009). This enzyme has a greater proteolytic activity when heated (Naveena & Mendiratta, 2001). Its

proteolytic activity produces more tender meat by degrading collagen and actinomycin (Naveena & Mendiratta, 2001; Thompson et al., 1973). Lee et al. (1986) reported that an extensive degradation of myofibrillar proteins is possible with a high concentration of ginger extract: the degradation appears to begin at the I band of each sarcomere and progresses to the M line.

8.4.8 Actinidin

Actinidin or actinidain is a cysteine protease obtained from the kiwi fruit (*Actinidai deliciosa*). Actinidin is stable at a pH range of 7–10 and has optimal activity at 58–62 °C (Yamaguchi et al., 1982). A pH range of 5–7 is also reported by Boyes et al. (1997), which reflects variations in the cultivar and assay used to estimate the proteolytic activity. Both myofibrillar proteins and connective tissue proteins are hydrolyzed by this enzyme (Christensen et al., 2009; Han et al., 2009). But a higher hydrolysis of collagen was reported by Wada et al. (2002).

8.4.9 Aminopeptidases

Aminopeptidases are the major enzymes involved in the characteristic flavor development in meat products. They hydrolyze amino acids from the N terminus of peptides and proteins during meat processing. This generates a large number of free amino acids that produce flavor. In porcine skeletal muscle, aminopeptidase B (BAP), leucyl (LAP), alanyl (AAP), and pyroglutamyl (PGAP) have been localized in the cytosol, and are named according to the preference of specific N terminal amino acid (Flores et al., 1993, 1996, 1997). Except for leucyl aminopeptidase, all are active at acidic pH. In the cytosol of skeletal muscle, around 86% of the total aminopeptidase consists of alanyl aminopeptidase (Lauffart & Mantle, 1998). As reported by Toldra (1992), aminopeptidases show good stability during the processing of dry-cured ham, with activity still recovered toward the end of the process.

8.4.10 Lipases

Lipolysis is the process of degradation of lipids in meat during processing (Toldra & Flores, 1998). Enzymes of fat cells and muscle fibers, as well as bacterial enzymes are involved in lipolysis. However, it is established that in dry fermented sausages, the contribution of bacterial lipase is weak because of many differences in the medium and optimum conditions (Molly et al., 1997). Intense lipolysis occurs in muscle and adipose tissues during the dry-curing of meat; most of the free fatty acid generation occurs during the initial 5 months (Motilva et al., 1993). Important lipolytic enzymes of adipose tissue are lipoprotein lipase, hormone-sensitive lipase, and monoacylglycerol lipase (Belfrage et al., 1984). Lipoprotein lipase, specific for

primary esters, is active at alkaline pH 8.5. Hormone-sensitive lipase hydrolyzes ester bond in triacylglycerols and diacylglycerols. Monoacylglycerol lipase hydrolyzes monoacylglycerols without any positional specificity and is active during salting and post-salting stages. Hormone-sensitive lipase is also called neutral lipase due to its high activity at neutral pH (Imanaka et al., 1984). The muscle lipase, lysosomal acid lipase, active at acidic pH hydrolyzes tri, di, and monoacylglycerol (Imanaka et al., 1984). Lipases can be used to remove fat in meat products (Uppada et al. 2017). Lipase produced from *Lactobacillus* sp. degrades meat in 72 h (Padmapriya et al., 2011).

8.4.11 Fungal Proteases

An exogenous serine protease (EPg222) obtained from *Penicillium chrysogenum* Pg222, an atoxigenic strain, was isolated from dry-cured ham (Núñez et al., 1996). This protease shows optimum activity at 45–55 °C, pH 6, and 0.25 M NaCl (Benito et al., 2002). Benito et al. (2003) reported that EPg222 hydrolyzed key myofibrillar proteins, supporting the tenderization of whole pieces of meat with 5% NaCl. The dry-cured meat product's texture could be improved by fungal proteases. The introduction of EPg222 in dry fermented sausages, ripened with starter cultures, is of great interest to boost sensory characteristics (Benito et al., 2004). An aspartic protease from *Aspergillus oryzae* shows optimal activity at a pH range of 2.5–6, and less than 20% of its activity remains after cooking at 75 °C (Ashie et al., 2002). It does not affect collagen. Its effect on myofibrillar proteins is limited compared to plant protease, with self-limiting hydrolyses at higher concentrations (Cronlund & Woychik, 1986; Ashie et al., 2002).

8.4.12 Elastase

Elastase is the enzyme that breaks down elastin in connective tissue. A new elastase, a protease with high proteolytic activity, was isolated from *Bacillus* sp. EL31410 by Chen and He (2002). It had a marked preference for elastin and collagen, which can contribute to meat hardness, over other myofibrillar proteins at the pH of meat, usually ranging from 5.5 to 6.0, and it has the same tenderization effect as papain on beef meat based on the results of texture, sensory, and structural analyses (Qihe et al., 2006).

8.4.13 Bacterial Protease

Many strains of bacteria play a major role in protein degradation in fermented meat and fish products (Bekhit, 2010). Because of relatively specific activity and low inactivation temperatures, proteases from bacterial sources make them useful for meat tenderization. Alkaline elastase from alkalophilic *Bacillus* sp. strain Ya-B

shows an optimum activity in the pH range of 5.5–6.0 and a temperature range of 10–50 °C (Takagi et al., 1992; Yeh et al., 2002). Compared to plant proteases, bacterial proteases have less hydrolytic activity towards myofibrillar proteins but their ability to degrade collagen was intermediate to that found in bromelain and papain (Yeh et al., 2002).

8.4.14 Animal Proteases

Animal proteases are less used for meat tenderization. Injection of pancreatin on beef semitendinosus tended to improve overall tenderness without affecting the taste (Pietrasik et al., 2010).

8.5 Enzymes in Fish Products Processing and Preservation

Various enzymes from different sources have been exploited for the processing of fish products and their preservation. The main enzymes, which play a key role in the processing of fish and seafood are described below.

8.5.1 Proteases

Proteases find their wide application in the seafood processing industry, especially in deskinning, descaling, salted fish ripening, fish sauce production, squid tenderization, etc. Besides, proteases have also been examined for the extraction of compounds (mainly pigments, oils and fats, and flavors) and the preparation of hydrolyzates from seafood processing wastes (Suresh et al., 2015). Proteases from plant, animal, microbial, and even aquatic sources have been used for the processing of fish.

1. Deskinning and descaling: Deskinning is the procedure involving the removal of fish skin without damaging the flesh. The deskinning process is carried out using rough mechanical procedures,which results in flesh damage and excess waste generation. The replacement of mechanical procedures with enzymes i.e., proteases could improve the edible yield. Commercial proteases such as Protease N and Proleather FG-F have been tested for the deskinning of catfish nuggets (Kim et al., 2014). Descaling of fish involves a harsh mechanical process, which might result in the tearing of fish skin and thereby lower the fillet yield. To avoid this loss, enzymatic descaling using the mixture of proteases is carried out (Fernandes, 2016).
2. Fish protein hydrolyzates: Over more than 50 years, the application of proteases in the fish protein hydrolyzates preparation has been widely studied. The fish protein hydrolyzates are generally prepared by treating fish meat or protein-rich fish processing by-products (fish head, skin, liver, trimmings, bones, and fish

viscera) with proteases at ideal pH and temperature for a few hours, followed by drying. The enzymes preferred for the production of fish protein hydrolyzates include pepsin, trypsin, chymotrypsin, alcalase, etc. The prepared fish protein hydrolyzates are amorphous powder and hygroscopic in nature. The fish protein hydrolyzates contain proteins (80–90%), fats (<5%), minerals (5–8%) and moisture (1–8%) (Venugopal et al., 2000; Venugopal, 2016). Traditionally, the preparation of fish protein hydrolyzates was carried out using either acids or bases. Hydrochloric acid or sulfuric acid (occasionally) was preferred for acid hydrolysis, whereas alkali hydrolysis was carried out using sodium hydroxide. Both acid and alkali hydrolysis are carried out at elevated temperature. Moreover, alkali hydrolysis produces toxic compounds during the reaction process, and acid hydrolysis destroys tryptophan. Besides, fish protein hydrolyzates preparation using low-cost proteolytic enzymes can be carried out at a mild temperature, pressure, and pH, and doesn't produce toxic compounds. Thus, the preparation of fish protein hydrolyzates using proteolytic enzymes is economical (Fernandes, 2016).

Gajanan et al. (2016) prepared protein hydrolyzates from threadfin breams (*Nemipterus japonicas*) a fish frame waste using two different plant proteases, papain and bromelain. The prepared protein hydrolyzates exhibited high Angiotensin-Converting Enzyme (ACE) inhibitory and anti-oxidative activity. The hydrolyzates also showed better functional properties. Similar results were also exhibited by protein hydrolyzates prepared from the muscle of small-spotted catshark (*Scyliorhinus anicula*) using esperase and alcalase (Vázquez et al., 2017).

3. Fish sauce: Fish sauce is a product produced by the fermentation process using endogenous or exogenous enzymes. The underlying process mainly involves the solubilization and digestion of fish proteins by enzymes. The pre-dominant enzymes involved in protein hydrolysis include chymotrypsin and trypsin, alongside cathepsins. When the pH of fish sauce drops from 7 to 5, the action of trypsin and chymotrypsin becomes complicated since they are active at near-neutral pH, whereas cathepsins are active at acidic pH. Fish sauce production using endogenous enzymes is a traditional process, and it's a very time-consuming process. Therefore, to hasten the process, exogenous enzymes such as papain, ficin, and bromelain, and commercial enzymes such as protamex (Protex 51FP) and neutrase have been used (Fernandes, 2016). About 80–90% of the Southeast Asian people consume fish sauce. It is being used as a prime condiment in Southeast Asian cuisines to improve the taste of food. Currently, Thailand is the leading producer of fish sauce in the world (Gowda et al., 2020).
4. Recovery of proteins from fish processing waste: Fish waste is a rich source of proteins such as collagen, myofibrillar proteins, and sarcoplasmic proteins. Proteases derived from the fish wastes (collagenases and pepsin) have been employed for the extraction of collagen from various fish offals, including skins, fins, bones, scales, swim bladders, and heads (Venugopal, 2016).
5. Ripening: Proteases have been known to be involved in the ripening of fermented fishery products as well as salted fish (Venugopal, 2016; Fernandes, 2016). The

exogenous proteases accelerate the development of soft texture and characteristic flavor in fermented fishery products. In Mediterranean countries, salted anchovy (*Engraulis encrasicholus*) is a heavily salted fish comprising around 14–15% of NaCl and has high nutritional value. In many European countries, salting of herring (*Clupea harengus*) is being carried out as a conventional preservation technique (Suresh et al., 2015). The ripening of salted fishes involves a complex biochemical process, wherein the muscle proteins undergo degradation by endogenous enzymes. The main endogenous enzymes involved in the ripening are trypsin, chymotrypsin, and cathepsins (Venugopal, 2016; Fernandes, 2016).

6. Caviar production: Caviar refers to the cured fish roes of salmon, trout, white sturgeon fish, etc. Traditionally caviar was used to refer to riddles and cured roe of sturgeon fish. The most difficult task in the preparation of caviar is the separation of roe from the roe sack. This process is generally termed riddling. Riddling process is generally carried out either manually or mechanically. Recently, the applications of enzymes in the preparation of caviar from various fish species have been examined (Suresh et al., 2015; Venugopal, 2016).
7. Other applications: The proteases have also been used in various other seafood processing operations. Some of these operations include (1) control of curd formation in canned fish, (2) preparation of bioactive peptides, (3) extraction of biopolymers, pigments, and compounds such as chondroitin sulfate and hyaluronic acid, (4) the production of aquafeed and seafood flavoring, (5) reduction of viscosity of stick water, and (6) control of enzymatic browning (Venugopal, 2016).

8.5.2 Lipases

Lipases (triacylglycerol acyl hydrolases EC 3.1.1.3) are the enzymes primarily involved in the hydrolysis of mono, di, and triglycerides and fatty acids. The hydrolysis reaction takes place in the presence of an excess of water, but when there is limited water, lipases promote the synthesis of esters. Lipases also exhibit other esterase type or phospholipase type activities. These activities of lipase have recognized industrial relevance. Lipases differ in their properties and also their origin (plant source, animal source, and microbial source). Lipases have been exploited for isolating oils and fats from seafood by-products. The lipases have also been utilized for ω-3 fatty acids' (eicosapentaenoic acid and docosahexaenoic acid) enrichment of fish oils. Lipases along with other muscle enzymes such as caplains, cathepsins, aminopeptidases, and peptidases have been known to influence the release of flavor compounds (Suresh et al., 2015; Fernandes, 2016; Venugopal, 2016).

8.5.3 Transglutaminase

Transglutaminase (TGAs, protein-glutamine γ-glutamyl transferase EC 2.3.2.13) enzymes primarily catalyze acyl transfer reactions. The reaction includes the

γ-carboxyamide group of peptide-bound glutamine acting as acyl donor and many primary amines acting as acyl acceptors. Simultaneously, many intermolecular and intramolecular covalent bond formation occurs, resulting in the cross-linking of proteins and peptides, and polymerization. In the absence of primary amines, water acts as an acyl acceptor, and the γ-carboxyamide group undergoes deamination to glutamic acid residues. TGase from plant and animal sources are Ca^{2+} dependent enzymes, whereas TGase from the microbial source is Ca^{2+} independent enzyme. Moreover, microbial TGase is active over a wide pH and temperature (0–70 °C). These features of microbial TGase and its cost-effective production make it the most widely preferred enzyme for processing of fish products. TGase has been produced by microorganisms such as *Streptomyces* sp., *Bacillus subtilis*, and *Streptoverticillium* sp. The application of TGases in the fish products processing industry includes preparation of surimi and restructured fishery products, fish meat film formulations, processing of shark fin, modification of texture of finfish, the formation of gelatin and collagen bonds, fish meat mince formulations, drip reduction in thawed fish, freeze-texturization of fishery products, and binding of ingredients with fish meat (Suresh et al., 2015; Venugopal, 2016).

8.5.4 Other Enzymes

Besides proteases, lipases, and transglutaminases, other enzymes have also been exploited for the fish products processing industries. Enzymes such as diamine oxidase, xanthine oxidase, nucleotide phosphorylase, putrescine oxidase, and glutamine dehydrogenase have been used for the evaluation of the quality of seafood and freshness. Restriction endonucleases and Taq polymerase have been used for the identification of fish species and adulteration detection. Furthermore, research works have also been carried out in increasing the shelf-life of seafood products using glucose oxidase enzymes.

8.6 Enzymes in Poultry Products Processing and Preservation

Various exogenous and endogenous enzymes have been exploited for the processing of poultry products. The enzymes used in the processing of pork, sheep, and beef meat are also being used in the processing of poultry meat. Mainly the enzymes are used to improve the tenderness of poultry meat. Papain, ficin, and bromelain enzymes derived from plants have been extensively utilized for the tenderization of poultry meat. The proteolytic enzymes derived from plants are considered to be superior to enzymes derived from bacteria (Ketnawa & Rawdkuen, 2011). The applications of commercial enzymes such as alcalase, neutrase, flavorzyme, protamex, collupulin, bromelain, and alphalase in softening the chicken meat (breast) texture have been studied. Of all the enzymes bromelain and collupulin have shown the marked softening of chicken breast. The exploitation of proteolytic enzymes from *Calotropis procera* latex has also shown to be effective in the

tenderization of poultry meat. Apart from the application of enzymes in poultry meat processing, the addition of enzymes in the improvement of barley and wheat-based feed has also been studied by Perić et al. (2011). They stated that incorporating the enzyme Allzyme SSF into poultry feed had a positive effect on the growth of chicken, and observed increased egg production in layers. Thus, enzymes find extensive application in the poultry industry to prepare poultry feed and poultry meat processing.

8.7 Future Prospects

Nature is a reservoir of several enzymes with promising potential for a wide application in human endeavors, but only a few of them have been exploited for over a century. This scenario's rationale includes that the enzyme might be from an undesirable or unreliable source (from unreachable environments such as deep-sea, or from microorganisms), challenges in production at a commercial scale and downstream processing, and the requirement of harsh conditions for activity. As a result, scientists and technologists continue their attempts to uncover novel enzymes from new origins and experiment with their potential applications, overcoming all obstacles through laboratory research efforts (Chandrasekaran, 2015).

Enzymes are known to play a crucial role in the food industry in producing both traditional and novel products. Enzymes have also found their application in meat, fish, and poultry processing to obtain products with better quality attributes, evaluate the products' quality and freshness, and extend the products' shelf-life. With the increase in the production of meat, fish and poultry products, the demand for enzymes has also increased. The enzymes from plants, animals, and microbial sources have been extensively studied for the processing of meat, fish or seafood, and poultry products. The complete substitution of the chemical processing methods with enzymatic methods would help overcome environmental problems arising due to chemical processing techniques. Further, the application of enzymes in the meat, fish, and poultry products processing industry has excellent prospects since the production of enzymes to be exploited in these industries is cheaper. Their utilization helps to obtain quality products with better yields.

Furthermore, advances in biotechnology, nanotechnology, molecular biology, instrumentation, and bioinformatics have opened up new horizons for designing hybrid catalysts and novel enzymes for diverse food industry applications, including meat, poultry, and fish processing industries. The novel enzymes or recombinant enzymes developed through the modern biotechnological approach would effectively carry out industrial processes at low temperatures, reduce the cost of production and energy consumption, and enhance the products' properties (Chandrasekaran, 2015). Besides, the enzymes derived from seafood processing waste have a promising future for their fish product processing application alongside the recombinant enzymes being developed for enzymatic processing and preservation of meat, fish, and poultry products.

8.8 Conclusion

Currently, the meat, fish, and poultry industries play a significant role in meeting the global food demand. The enzymatic processing of meat, fish, and poultry products processing is still in the early stage, and the introduction of new techniques is resulting in progress. The enzymatic processing technique has the potential to overthrow the chemical and mechanical techniques employed in the processing of meat, fish, and poultry products processing. This would help to overcome the environmental problems caused by chemical and mechanical processing techniques. The most suitable enzymes that could be employed in the processing of meat, fish, and poultry products are discussed in this chapter. The meat and poultry industries mainly utilize enzymes to improve the tenderness of the meat, whereas in fish products processing enzymes have a wide array of applications. Technological advancements have enabled the development of recombinant enzymes from microorganisms for their application in these industries. However, recombinant enzymes are yet to be used in the industries. Thus, the enzymes have a promising future for their application in the processing of meat, fish, and poultry products.

Acknowledgments The authors are grateful to the CSIR-Central Food Technological Research Institute (CSIR-CFTRI), Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, and the Department of Biotechnology (Govt. of India, New Delhi) for the support.

References

Ashie, I. N. A., Sorensen, T. L., & Nielsen, P. M. (2002). Effects of papain and a microbial enzyme on meat proteins and beef tenderness. *Journal of Food Science, 67*, 2138–2142.

Barrett, A. J., Rawlings, N. D., & Woessner, J. F. (2004). *Handbook of proteolytic enzymes*. Elsevier Academic Press.

Bawa, A. S., Orr, H. L., & Usborne, W. R. (1981). Enzymatic tenderization of spent white leghorn hens. *Poultry Science, 60*(4), 744–749.

Bekhit, A. E. D. (2010). Fermentation of fish roe. In D. R. Heldman, D. G. Hoover, & M. B. Wheeler (Eds.), *The encyclopedia of biotechnology in agriculture and food* (Vol. 1, pp. 251–256). Taylor & Francis Group.

Belfrage, P., Fredrikson, G., Stralfors, P., & Tornqvist, H. (1984). Adipose tissue lipases. In B. Borgström & H. L. Brockman (Eds.), *Lipases* (p. 365). Elsevier.

Benito, M. J., Rodríguez, M., Acosta, R., & Córdoba, J. J. (2003). Effect of the fungal extracellular protease EPg222 on texture of whole pieces of pork loin. *Meat Science, 65*(2), 877–884.

Benito, M. J., Rodríguez, M., Martín, A., Aranda, E., & Córdoba, J. J. (2004). Effect of the fungal protease EPg222 on the sensory characteristics of dry fermented sausage "salchichón" ripened with commercial starter cultures. *Meat Science, 67*(3), 497–505.

Benito, M. J., Rodríguez, M., Núñez, F., Asensio, M. A., Bermúdez, M. E., & Córdoba, J. J. (2002). Purification and characterization of an extracellular protease from *Penicillium chrysogenum* (Pg222) active against meat proteins. *Applied and Environmental Microbiology, 68*, 3532–3536.

Boyes, S., Strubi, P., & Marsh, H. (1997). Actinidin levels in fruit of Actinidia species and some Actinidia arguta rootstock scion combinations. *LWT—Food Science and Technology, 30*, 379–389.

Cazarin, C. B. B., Lima, G. C., da Silva, J. K., & Maroostica, M. R., Jr. (2015). Enzymes in meat processing. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 354–377). CRC Press.

Chandrasekaran, M. (2015). Emerging trends and future prospects. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 354–377). CRC Press.

Chandrasekaran, M., Basheer, S. M., Chellappan, S., Krishna, J. G., & Beena, P. S. (2015). Enzymes in seafood processing. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 354–377). CRC Press.

Chen, Q. H., & He, G. Q. (2002). Optimization of medium composition for the production of elastase by Bacillus sp. EL31410 with response surface methodology. *Enzyme and Microbial Technology, 5*, 667–672.

Christensen, M., Torngren, M. A., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A. H., & Ertbjerg, P. (2009). Injection of marinade with actinidin increases tenderness of porcine M. biceps femoris and affects myofibrils and connective tissue. *Journal of the Science of Food and Agriculture, 89*, 1607–1614.

Copeland, R. A. (Ed.). (2000). *Enzymes: A practical introduction to structure, mechanism, and data analysis* (pp. 1–10). Wiley-VCH.

Cronlund, A. L., & Woychik, J. H. (1986). Effect of microbial rennets on meat protein fractions. *Journal of Agricultural and Food Chemistry, 34*, 502–505.

Doko, M. B., Bassani, V., Casadebaig, J., Cavailles, L., & Jacob, M. (1991). *International Journal of Pharmaceutics, 76*, 199–206.

Duston, T. R. (1983). Relationship of pH and temperature to disruption of specific muscle proteins and activity of lysosomal proteases. *Journal of Food Biochemistry, 7*, 223–245.

El-Gharbawi, M., & Whitaker, J. R. (1963). Factors affecting enzymatic solubilization of beef proteins. *Journal of Food Science, 28*, 168–172.

European Commission-GRFC. (2019). Food shortage. Retrieved July 7, 2020 from https://ec.europa.eu/knowledge4policy/publication/global-report-food-crises-2019_en#:~:text=According%20to%20the%202019%20edition,3%20or%20above)%20in%202018

FAO. (2016). The state of world fisheries and aquaculture. Contributing to food security and nutrition to all, Fish production to combat food shortage, Rome, 200 pp. Retrieved July 7, 2020.

FAO. (2019). Food basic necessity of life. Retrieved July 7, 2020 from https://nutritionmeetsfoodscience.com/2019/01/03/food-more-than-an-essential-need/

FAO-GLOBEFISH. (2020). Information and analysis on world fish trade. FAO. Retrieved July 7, 2020 from http://www.fao.org/in-action/globefish/fishery-information/en/#:~:text=Global%20fish%20production%20growth%20is,have%20increased%203.9%20percent%20...

Fernandes, P. (2016). Enzymes in fish and seafood processing. *Frontiers in Bioengineering and Biotechnology, 4*, 59.

Flores, M., Aristoy, M. C., & Toldrá, F. (1993). HPLC purification and characterization of porcine muscle aminopeptidase B. *Biochimie, 75*, 861.

Flores, M., Aristoy, M. C., & Toldra, F. (1997). Curing agents affect aminopeptidase activity from porcine skeletal muscle. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A, 205*, 343–346.

Flores, M., Aristoy, M.-C., & Toldrá, F. (1996). HPLC purification and characterization of soluble alanyl aminopeptidase from porcine skeletal muscle. *Journal of Agricultural and Food Chemistry, 44*, 2578.

Gajanan, P. G., Elavarasan, K., & Shamasundar, B. A. (2016). Bioactive and functional properties of protein hydrolysates from fish frame processing waste using plant proteases. *Environmental Science and Pollution Research, 23*(24), 24901–24911.

Goll, D. E., Otusuak, Y., Nagainis, P. A., Shannon, J. D., Sathe, A. K., & Mururuma, M. (1983). Role of muscle proteinases in maintenance of muscle integrity and mass. *Journal of Food Biochemistry, 7*, 137–177.

Goll, D. E., Thompson, V. F., Li, H. Q., Wei, W., & Cong, J. Y. (2003). The calpain system. *Physiological Reviews, 83*, 7.

Gomes, H. A., Moreira, L. R., & Edivaldo Filho, X. F. (2018). Enzymes and food industry: A consolidated marriage. In A. M. Holban & A. M. Grumezescu (Eds.), *Advances in biotechnology for food industry* (pp. 55–89). Academic Press.

Gowda, S. G. S., Narayan, B., & Gopal, S. (2020). Antioxidant properties and dominant bacterial community of fermented Rohu (Labeo rohita) sauce produced by enzymatic and fermentation method. *Turkish Journal of Fisheries and Aquatic Science, 20*(8), 583–592.

Gracey, J. F., & Thronton, S. (1985). *Meat hygiene* (7th ed.). Bailliere Tindall, English Language Book Society.

Grzonka, Z., Kasprzykowski, F., & Wiczk, W. (2007). Cysteine proteases. In J. Polaina & A. P. MacCabe (Eds.), *Industrial enzymes* (pp. 181–195). Springer.

Han, J., Morton, J. D., Bekhit, A. E. D., & Sedcole, J. R. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science, 82*(3), 324–330.

Hope-Jones, M., Strydom, P. E., Frylinck, L., & Webb, E. C. (2010). The efficiency of electrical stimulation to counteract the negative effects of β-agonists on meat tenderness of feedlot cattle. *Meat Science, 86*, 699–705.

Hopkins, D. L., & Taylor, R. G. (2002). Post-mortem muscle proteolysis and meat tenderization. In M. te Pas, M. Everts, & H. Haagsman (Eds.), *Muscle development of livestock animals* (pp. 363–389). CAB International.

Hujjatullah, S., & Baloch, A. K. (1970). Proteolytic activity of *Cucumis trigonus* Roxb. Extraction, activity, characteristics. *Journal of Food Science, 35*(3), 276–278.

Imanaka, T., Amanuma-Muto, K., Ohkuma, S., & Takano, T. (1984). Characterization of lysosomal acid lipase purified from rabbit liver. *Journal of Biochemistry, 96*, 1089.

Ionescu, A., Aprodu, I., & Pascaru, G. (2008). Effect of papain and bromelin on muscle and collagen proteins in beef meat. *The Annals of the University Dunarea de Jos of Galati. Fascicle VI—Food Technology, New Series*, pp. 9–16.

Jamdar, S. N., & Harikumar, P. (2002). Sensitivity of catheptic enzymes in radurized chicken meat. *Journal of Food Science and Technology, 39*(1), 72–73.

Kang, C. K., & Rice, E. E. (1970). Degradation of various meat fractions by tenderizing enzymes. *Journal of Food Science, 35*, 563–565.

Kang, C. K., & Warner, W. D. (1974). Tenderization of meat with papaya latex proteases. *Journal of Food Science, 39*(4), 812–818.

Ketnawa, S., & Rawdkuen, S. (2011). Application of bromelain extract for muscle food tenderization. *Food and Nutrition Sciences, 2*, 393–401.

Kim, T., Silva, J. L., Parakulsuksatid, P., & Wang, D. S. (2014). Optimization of enzymatic treatments for deskinning of catfish nuggets. *Journal of Aquatic Food Product Technology, 23*(*4*), 385–393.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M., & Kohno, K. (2004). Papain protects papaya trees from herbivorous insects: Role of cysteine proteases in latex. *The Plant Journal, 37*, 370–378.

Koohmaraie, M., & Geesink, G. H. (2006). Contribution of post mortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74*, 34–43.

Koohmaraie, M., Whipple, G., Kretchmar, D. H., Crouse, J. D., & Mersmann, H. J. (1991). Postmortem proteolysis in longissimus muscle from beef, lamb and pork carcasses. *Journal of Animal Science, 69*, 617–624.

Kramer, D. E., & Whitaker, J. R. (1964). Ficus enzymes: II. Properties of the proteolytic enzymes from the latex of *Ficus caric*a variety Kadota. *Journal of Biological Chemistry, 239*, 2178–2183.

Lauffart, B., & Mantle, D. (1998). Rationalisation of aminopeptidase activities in human skeletal muscle soluble extract. *Biochimica et Biophysica Acta, 956*, 300.

Lee, Y. B., Kim, Y. S., & Ashmove, C. R. (1986). Antioxidant property of ginger rhizome and its application in meat products. *Journal of Food Science, 51*, 20–23.

Melendo, J. A., Beltrán, J. A., Jaime, I., Sancho, R., Roncalés, P., & P. (1996). Limited proteolysis of myofibrillar proteins by bromelain decreases toughness of coarse dry sausage. *Food Chemistry, 57*(3), 429–433.

Mikami, M., Whiting, A. H., Taylor, M. A. J., Maciewicz, R. A., & Etherington, D. J. (1987). Degradation of myofibrils from rabbit, chicken and beef by cathepsin l and lysosomal lysates. *Meat Science, 21*, 81–97.

Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinck, M., & Geenen, I. (1997). The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project. *Food Chemistry, 54*(4), 539–545.

Motilva, M.-J., Toldrá, F., Nieto, P., & Flores, J. (1993). Muscle lipolysis phenomena in the processing of dry-cured ham. *Food Chemistry, 48*, 121.

Naveena, B. M., & Mendiratta, S. K. (2001). Tenderization of spent hen meat using ginger extract. *British Poultry Science, 42*, 344–349.

Naveena, B. M., Mendiratta, S. K., & Anjaneyulu, A. S. R. (2004). Tenderization of buffalo meat using plant proteases from Cucumis trigonus Roxb (Kachri) and Zingiber officinale roscoe (Ginger rhizome). *Meat Science, 68*(3), 363–369.

Núñez, F., Rodríguez, M. M., Bermúdez, M. E., Córdoba, J. J., & Asensio, M. A. (1996). Composition and toxigenic potential of the mould population on dry-cured Iberian ham. *International Journal of Food Microbiology, 32*, 185–197.

O'Halloran, G. R., Troy, D. J., Buckley, D. J., & Reville, W. J. (1997). The role of endogenous proteases in the tenderisation of fast glycolysing muscle. *Meat Science, 47*, 187–210.

Padmapriya, B., Rajeswari, T., Noushida, E., Sethupalan, D. G., & Venil, C. K. (2011). *World Applied Science Journal, 12*, 1798–1802.

Perić, L., Sartowska, K., Milošević, N., Đukić-Stojčić, M., Bjedov, S., & Nikolova, N. (2011). The effect of enzymes on the economics of poultry meat and egg production. *Macedonian Journal of Animal Science, 1*, 113–117.

Pietrasik, Z., Aalhus, J. L., Gibson, L. L., & Shand, P. J. (2010). Influence of blade tenderization, moisture enhancement and pancreatin enzyme treatment on the processing characteristics and tenderness of beef semitendinosus muscle. *Meat Science, 84*(3), 512–517.

Qihe, C., Guoqing, H., Yingchun, J., & Hui, N. (2006). Effects of elastase from a Bacillus strain on the tenderization of beef meat. *Food Chemistry, 98*(4), 624–629.

Ramezani, R., Aminlari, M., & Fallahi, H. (2006). Effect of chemically modified soy proteins and ficin-tenderized meat on the quality attributes of sausage. *Journal of Food Science: Food Chemistry and Toxicology, 68*, 85–88.

Rawlings, N. D., Barrett, A. J., & Bateman, A. (2010). MEROPS: The peptidase database. *Nucleic Acids Research, 38*, D227–D233.

Rees, M. P., Trout, G. R., & Warner, R. D. (2002). Effect of calcium infusion on tenderness and ageing rate of pork m. longissimus thoracis et lumborum after accelerated boning. *Meat Science, 61*, 169–179.

Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science and Technology, 13*, 400–421.

Shahidi, F., & Kamil, Y. J. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science & Technology, 12*(12), 435–464.

Singh, S. B. (2018). Enzyme catalysis and its role in food processing industries. In M. Kuddus (Ed.), *Enzymes in food technology* (pp. 143–165). Springer.

Smith, J., & Hong-Shum, L. (2003). Enzymes. In *Food additives data book* (pp. 389–462). Blackwell Science.

Statista. (2019). Retrieved July 8, 2020 from https://www.statista.com/statistics/264577/total-world-fish-production-since-2002/#:~:text=The%20volume%20of%20global%20fish,million%20metric%20tons%20in%202010

Statista. (2020). Retrieved July 8, 2020 from https://www.statista.com/statistics/237632/production-of-meat-worldwide-since-1990/

Su, H. P., Huang, M. J., & Wang, H. T. (2009). Characterization of ginger proteases and their potential as a rennin replacement. *Journal of the Science of Food and Agriculture, 89*(*7*), 1178–1185.

Sunantha, K., & Saroat, R. (2011). Application of bromelain extract for muscle foods tenderization. *Food and Nutrition Sciences*. https://doi.org/10.4236/fns.2011.25055

Suresh, P. V., Nidheesh, T., & Pal, G. K. (2015). Enzymes in seafood processing. In M. Chandrasekaran (Ed.), *Enzymes in food and beverage processing* (pp. 354–377). CRC Press.

Suzuki, K., Saido, T. C., & Hirai, S. (1992). Modulation of the cellular signal by calpain. *Annals of the New York Academy of Sciences, 674*, 218–227.

Takagi, H., Kondou, M., Hisatsuka, T., Nakamori, S., Tsai, Y. C., & Yamasaki, M. (1992). Effects of an alkaline elastase from an alkalophilic Bacillus sytrain on the tenderization of beef meat. *Meat Science, 40*, 577–583.

Tappel, A. L., Miyada, D. S., Sterling, C., & Maier, V. P. (1956). Meat tenderization. II. Factors affecting the tenderization of beef by papain. *Food Research, 21*, 375–383.

Thompson, E. H., Wolf, I. D., & Allen, C. E. (1973). Ginger rhizome: A new source of proteolytic enzyme. *Journal of Food Science, 38*, 652–655.

Toldra, F. (1992). The enzymology of dry curing of meat products. In F. J. M. Smulders, F. Toldra, J. Flores, & M. Prieto (Eds.), *New technologies for meat and meat products* (p. 209).

Toldra, F., & Flores, M. (1998). The role of muscle proteases and lipases in flavor development during the processing of dry-cure Ham. *Critical Reviews in Food Science and Nutrition, 38*(*4*), 331–352.

Turk, V., & Bode, W. (1991). The cystatins: Protein inhibitors of cysteine proteinases. *FEBS Letters, 285*(2), 213–219.

United Nations-Department of Economic and Social Affairs (UN DESA). (2017). World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100. Retrieved July 7, 2020, from https://www.un.org/development/desa/en/news/population/worldpopulation-prospects-2017.html

Uppada, S. R., Akula, M., Bhattacharya, A., & Dutta, J. R. (2017). Immobilized lipase from *Lactobacillus plantarum* in meat degradation and synthesis of flavor esters. *Journal of Genetic Engineering and Biotechnology, 15*(*2*), 331–334.

Vanhoof, G., & Cooreman, W. (1997). Bromelain. In A. Lauwers & S. Scharpe (Eds.), *Pharmaceutical enzymes* (pp. 131–154). Marcel Dekker.

Vázquez, J. A., Blanco, M., Massa, A. E., Amado, I. R., & Pérez-Martín, R. I. (2017). Production of fish protein hydrolysates from *Scyliorhinus canicula* discards with antihypertensive and antioxidant activities by enzymatic hydrolysis and mathematical optimization using response surface methodology. *Marine Drugs, 15*(10), 306.

Veeramuthu, G. I., & Sams, A. R. (1999). Post mortem pH, myofibrillar fragmentation, and calpain activity in Pectoralis from electrically stimulated and muscle tensioned broiler carcasses. *Poultry Science, 78*, 272–276.

Venugopal, V. (2016). Enzymes from seafood processing waste and their applications in seafood processing. In S. Kim & F. Toldra (Eds.), *Advances in food and nutrition research* (Vol. 78, pp. 47–94). Academic Press.

Venugopal, V., Lakshmanan, R., Doke, S. N., & Bongirwar, D. R. (2000). Enzymes in fish processing, biosensors and quality control: A review. *Food Biotechnology, 14*(1–2), 21–77.

Wada, M., Suzuki, T., Yaguti, Y., & Hasegawa, T. (2002). The effects of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *Food Chemistry, 78*, 167–171.

Wang, H., Weir, C. E., Birkner, M. L., & Ginger, B. (1958). Studies on enzymatic tenderization of meat. III. Histological and panel analyses of enzyme preparations from three distinct sources. *Food Research, 23*, 423–438.

Whipple, G., Koohmaraie, M., Dikeman, M. E., Crouse, J. D., Hunt, M. C., & Klemm, R. D. (1990). Effect of fasting on energy metabolism and tenderizing enzymes in chicken breast muscle early postmortem. Evaluation of attributes that affect longissimus muscle tenderness in *Bos taurus* and *Bos indicus* cattle. *Journal of Animal Science, 68*(9), 2716–2728.

Yamaguchi, Y., Yamashita, Y., Takeda, I., & Kiso, H. (1982). Proteolytic enzymes in green asparagus, kiwifruit and mint: Occurrence and partial characterisation. *Agricultural and Biological Chemistry, 46*, 1983–1986.

Yeh, C. M., Yang, M.-C., & Tsai, Y.-C. (2002). Application potency of engineered G159 mutants on P1 substrate pocket of subtilisin YaB as improved meat tenderizers. *Journal of Agricultural and Food Chemistry, 50*, 6199–6204.

Check for updates

Enzymes in Functional Food Development

9

Iran Alemzadeh, Asma Sadat Vaziri, Kianoush Khosravi-Darani, and Pierre Monsan

Abstract

Enzymes are ubiquitous biocatalysts, represent a consolidated strategy in various food industries for the transformation of one biological compound to another. In contrast to conventional synthesis, enzymatic synthesis is an important technological advancement as a consequence of sustainability, substrate selectivity, nontoxicity, and low energy input. Therefore, there has been a growing trend in the use of free and immobilized enzymes in various food industrial applications, including the manufacturing of several functional foods and ingredients. Functional foods with health benefits beyond nutrition have gained much attention due to some factors such as interest in health-consciousness and the growing market. A variety of functional foods can be considered to be those fortified, wholes, enriched, or enhanced foods that represent physiological health benefits beyond the essential nutrients provision (e.g., vitamins and minerals) when consumed in adequate amounts on a regular basis. The initial components in functional food supplements naturally consist of fruit, food, beverage, grains, and supplement sectors at a low level. Nutraceuticals or functional food could be produced from the enzymatic reaction utilizable in one of the several areas of food science and technology, that is experiencing fast growth in recent years. Functional food

I. Alemzadeh (✉) · A. S. Vaziri
Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, Iran
e-mail: alemzadeh@sharif.edu

K. Khosravi-Darani
National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

P. Monsan
Département de Génie Biochimique et Alimentaire, INSA, Toulouse Cedex 4, France

White Biotechnology Center, Parc Technologique du Canal, Ramonville Saint Agnes, France

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_9

could be enzymatically derived from various reactions: L-asparaginase, an intercellular enzyme, is used in the baking industry which suppresses acrylamide production. Addition of asparaginase is a recommended strategy to decrease the formation of acrylamide, as it catalysis the hydrolysis of asparagine to aspartic acid an amino acid with high nutritive value. Carbohydrate active enzymes such as β-glucanase which is able to proceed on glucose polysaccharides and to further develop the production of β-glucans, the saccharide made up of multiple sugar molecules. β-Glucan may offer a number of health benefits, including lowering cholesterol, improving blood sugar management, and boosting the immune system which is produced by the enzymatic process of beta D-glucose polysaccharide naturally occurring in the cell wall of cereals like oat. Chlorophyllase (Chlase) is widely distributed in the chloroplast, thylakoid membrane, and etioplast of higher plants, such as ferns, mosses, brown and red algae, and diatoms. Chlase catalysis is the degradation of chlorophyll to chlorophyllide (also called Chlide), which is introduced as a food colorant. L-Glutaminase is a catalyst for the production of L-theanine, a free amino acid with many health benefits. L-Theanine synthesis occurs from L-glutamine and ethylamine substrates through the transpeptidation reaction of L-glutaminase, and a demand for this amino acid is expected to grow rapidly in the industry. Lipids such as CLA (conjugated linoleic acid) are in the group of functional food, a result of CLA production from natural oil in the presence of a lipase. CLA is a natural derivative of essential fatty acid, linoleic acid which shows many beneficial effects on the immune system, role in obesity control and anticarcinogenic behavior phytases may find application in food processing to produce functional foods and phytic acid degradation. Phytase is able to decrease the antinutritional effect of phytate (inorganic phosphate complex) adverse impact of inorganic P excretion to the environment while increasing the digestibility of phosphorous (P), calcium, amino acids, and energy. γ-Amino butyric acid (GABA) is an active protein, in rice germ using protease or additives: produced by the decarboxylation of glutamic acid, GABA is catalyzed by glutamate decarboxylase, GABA functions as the main inhibitory neurotransmitter in the central nervous system. Tannase is able to catalyze the production of tea beverages while maintaining the tea's original deep flavor. Engineering of enzymes is a potential strategy in the design of food development according to the specificity and enzyme structural changes. The next future step toward the commercialization of the enzymatic approach in food processing is to enhance their efficiency and mitigate adverse effects on the sensory properties of food products. In this chapter, we mainly highlighted the importance of selected enzymes on food development.

Keywords

Enzymes · Functional food · L-Asparaginase · β-Glucanase · Chlorophyllase · Lipase · L-Glutaminase · Phytase · Protease · Tannase

9.1 Introduction

Enzymes are ubiquitous biocatalysts, represents a consolidated technique in green chemistry for the transformation of one biological compound to another for biotechnological purposes. In contrast to conventional synthesis, enzymatic synthesis is an important technological advancement as a consequence of sustainability, substrate selectivity, nontoxicity, and low energy input. Therefore, there has been a growing trend in the use of free and immobilized enzymes in many industrial applications, including the manufacturing of several functional foods and ingredients. Functional foods with health benefits beyond nutrition have increased rapidly due to factors such as interest in health-consciousness and the growing market. A variety of functional foods can be considered to be those fortified, wholes, enriched, or enhanced foods that represent physiological health benefits beyond the essential nutrients provision (e.g., vitamins and minerals) when consumed in adequate amounts on a regular basis (Alemzadeh et al., 2020). The initial components in functional food supplements naturally consist of fruit, food, beverage, grains, and supplement sectors at low levels (Granato et al., 2020). Nutraceuticals or functional foods involve extraction, purification, concentration, and activation processes in order to be utilizable in one of the several areas of food science and technology, which could be processed by an enzymatic reaction. The discovery, characterization, and stabilization of various enzymes employed in the production of functional foods have been attended increasingly in many researches.

Food and stress are two major factors that have a direct impact on the gut microbiome and are responsible for inflammatory reactions in the human gastrointestinal. Any dysbiosis in the gut microbiota results in several gastrointestinal-associated diseases like diarrhea, irritable bowel syndrome (IBS), and colorectal cancer. The use of functional foods has been increasingly applied as a complementary therapy to rejuvenate and enrich the beneficial gut microorganisms, thereby, promoting the health, alleviate, and relief the symptoms associated with gut-related diseases and side effects of conventional diseases, reduce the risk of cardiovascular diseases, and improve overall well-being. Microbial enzyme engineers or enhance the production of certain naturally occurring dietary substances through fermentation technology (Melini et al., 2019). Functional foods, enriched with prebiotics, synbiotics, and probiotics, as well as other plants- and animal-derived food compounds, are also considered beneficial additives to improve the consumers health and well-being (de Paulo Farias et al., 2019; Vaziri et al., 2019).

Functional food could be enzymatically derived from various reactions; the L-asparaginase enzyme produced by the enzymatic reaction is used in the baking industry, which suppresses acrylamide production. The addition of asparaginase is a recommended approach to decrease the formation of acrylamide since it results in the hydrolysis of asparagine to aspartic acid, an amino acid with high nutritive value (Momeni et al., 2015; Torang & Alemzadeh, 2016).

Lactic acid bacteria strains have been shown to produce glucose, galactose, and oligosaccharides (prebiotics), as a result of lactose hydrolysis and transgalactosidal activities of beta-galactosidase. Another illustration is the production of

prebiotic-based compounds from cereals, a kind of non-starch polysaccharides (NSP), such as β-glucan and arabinoxylan as dietary fiber constituents (You et al., 2016). β-Glucan consists of multiple sugar molecules, which has a significant role in lowering cholesterol level, boosting blood sugar management, and improving the immune system. This essential fiber is synthesized by an enzymatic process through the action of carbohydrase on beta D-glucose polysaccharide, which basically occurs in the cell wall of cereals like barely and oat (Kumar et al., 2020).

Chlorophyllase enzyme (Chlase, chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14) is a hydrophobic enzyme in the chlorophyll hydrolysis to become degraded into chlorophyllide (Childe) and phytol. Chlorophyllide can be further considered as natural water-soluble food colorant alternative to synthetic colorants. Moreover, they possess biological and therapeutic activities, such as anti-inflammatory, antioxidant, antibacterial, wound healing, anticarcinogenic, and deodorizing properties (Hosikian et al., 2010).

Incorporation of lipase enzyme in the esterification of glycerol with CLA and production of enriched fatty acid derivatives with more health benefits and reduction in free fatty acids accumulation is a solution for functional food development. Lipids such as CLA (conjugated linoleic acid) are in the group of functional food, resulting from CLA production from natural oil in the presence of a lipase. CLA is a natural derivative of essential fatty acid, linoleic acid, which shows many beneficial effects on the immune system, obesity control, and anticarcinogenic behavior. Catalytic action of lipase enzyme in the esterification of glycerol with CLA causes the production of enriched fatty acid derivatives with more health benefits and reduction in free fatty acids accumulation. Incorporation of lipase enzyme in the esterification of glycerol with CLA and production of enriched fatty acid derivatives with more health benefits and reduction in free fatty acids accumulation is a solution for functional food development (Kouchak Yazdi & Alemzadeh, 2016a, b; Kouchak Yazdi et al., 2017).

Synthesis of theanine was examined in the presence of the L-glutaminase enzyme produced by *Trichoderma koningii* fungus. Production of L-glutaminase enzyme was first concerned by *T. koningii* using sesamum oil cake under solid-state fermentation (SSF). Then L-theanine production by L-glutamine and ethylamine as substrates and extracted enzyme solution were evaluated (Sakhaei & Alemzadeh, 2017).

Enzyme-catalyzed degradation to enhance the bioavailability of metal ions like iron and zinc in cereal-based foods is an example of enzyme-hydrolysis by phytase. The effects of phytase functionality on amino acid digestibility and energy utilization, and the in vivo functional food specificity have been monitored (Dersjant-Li et al., 2015). Phytases application in functional foods development is recommended for the increment of phytic acid dephosphorylation and degradation. Phytase can increase mineral bioavailability and decrease the anti-nutritional effect of phytate (inorganic phosphate complex) by improving phosphorous (P) digestibility, as well as reducing the negative impact of inorganic P excretion on the environment (Song et al., 2019; Handa et al., 2020).

Proteolytic enzymes are ubiquitous in innumerable biological systems and led to improve functional and biological characteristics of protein byproducts, which can

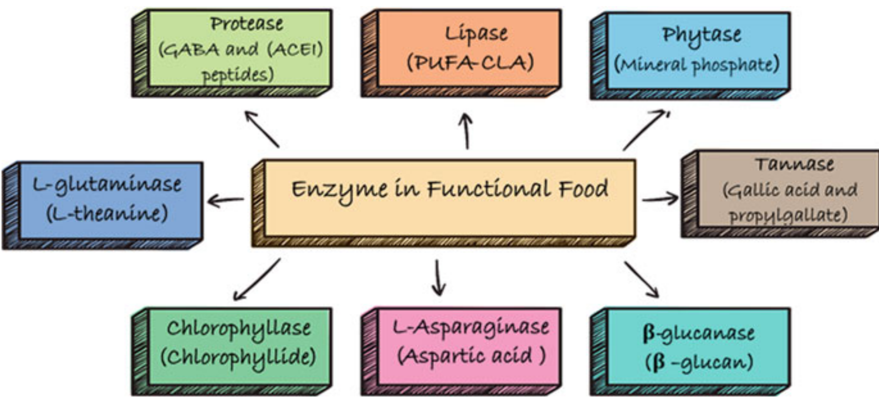


Fig. 9.1 Enzymes accessible in functional food development and the selected bioactive components

be further utilized as protein substrates for food applications (Pokora et al., 2013). Bioactive peptides (BP) are one of the most important bioactive compounds generated from protease hydrolysis. The protein fragments of BPs act as peptide inhibitors of the angiotensin-converting enzyme (ACE), and antimicrobial, anti-aggregating, antithrombotic, anticancer and antihypertensive, and immune-modulatory peptides. The proteolysis of BPs is required for their release and activation since they are hidden and encrypted within the parent proteins, such as casein, lactalbumin, beta-lactoglobulin, and lactoferrin (De Leo et al., 2009). Gamma-aminobutyric acid (GABA) is another potent bioactive protein catalyzed by glutamate decarboxylase. GABA functions as a major inhibitory neurotransmitter in the mammalian brain and central nervous system that strongly effects the behavior and stress management. It is widely distributed in nature and demand for microbial GABA supply has raised to satisfy the needs of GABA-enriched foods (Cui et al., 2020).

Tannase is another group of enzymes that are suitable candidates as biocatalysts in the treatment of phenolic-rich foods. It catalysis the biotransformation of phenolic compounds in beverages to generate a product with enhanced clarity and color appearance, as well as promoting its functional activity. As an example, the process of browning in tea beverages occurs due to the presence of caffeine and catechins in the tea, which results in the formation of insoluble compounds in cold water. Biotransformation by tannase reduces the gallic acid-binding and inhibits the browning effect (Srivastava & Kar, 2009; Cao et al., 2019; Gligor et al., 2019). The enzyme accessible in functional food development and bioactive components is categorized in Fig. 9.1. In this chapter, we describe the functional food components, functional food in health, the role of selected enzymes in functional foods manufacturing, or food improving quality circumstances. Enzymes, such as asparaginase, carbohydrase, chlorophyllase, lipase, L-glutaminase, phytase, protease, and tannase are introduced, and the beneficial health effects are discussed.

9.2 Functional Food Components

Functional foods are enriched or dietary materials that may offer several health benefits beyond basic nutrition. Functional foods for health are an essential factor of an overall healthful lifestyle that consists of a balanced diet from various sources. People should strive to consume a wide variety of foods and food components including the examples listed in Table 9.1. These food components could naturally exist in natural food, such as whole grains, fruits, vegetables, beverages, enriched compounds, and dietary supplements, extracted by chemical approaches or produced by enzymatic processes.

9.2.1 Functional Food in Health

Obesity and malnutrition are major drawbacks associated with diet habits, which are strongly influenced by urbanization, and lifestyle alterations (Tanna & Mishra, 2018). Functional food could inherently include bioactive ingredients or nutraceuticals and could be added from various sources to produce fortified food. The bioactive ingredients are in the group of carbohydrates, enzymes, protein, phytochemicals, vitamins, and lipids (Suleria et al., 2015; Maqsood et al., 2020). These bioactive compounds could be derived from different sources like cereals, seeds, marine-based, microorganisms, plants, animals, and fishery. Since bioactive compounds in natural food products are in low amounts, the bioactive ingredients could be produced by a microbial or enzymatic process in high quantity. The bioactive ingredients have antibiotic, antiparasitic, antiviral, anti-inflammatory, anti-fibrotic, and anticancer activities. Health benefit of the bioactive compounds affects metabolic syndrome (Mets), such as CVD, elevated triglycerides, cholesterol, and blood pressure, besides insulin resistance (Sirtori et al., 2017). Nutraceutical ingredients have specificity such as antimicrobial, antiviral, antioxidant, anti-obesity, anticancer, antidiabetic, anti-inflammatory, nephrotoxic, and arthritis.

9.3 Enzyme Application in Functional Food

Enzymes are catalytic biomolecules responsible for the catalyzing diverse biochemical reactions in several food industries. Enzymatic processes can be a promising alternative to conventional organic synthesis, as they provide eco-friendly, more viable and sustainable conditions, safer products, and use of renewable substrates. Enzymes are therefore possessing a significant role in many biotechnological applications including food quality improvement, as well as food manufacturing operations. Selected enzymes representing these concerns are defined and evaluated respectively.

Table 9.1 Examples of functional food sources selected nutraceuticals, and their biological activity

Functional food sources	Selected nutraceutical	Biological activity	Reference
Coriander fruits (seed and pericarp)	Fatty oil	Antimicrobial, antioxidant, antidiabetic	Sahib et al. (2013)
Non-starch polysaccharides (NPS)	Dietary fiber	Cholesterol reduction	Sirtori et al. (2017)
Pumpkin seeds	Traditional medicine	Antioxidative, hypoglycemic, anticancer	Patel and Rauf (2017)
Yeasts	Various bioactive components	preventing oxidative stress	Rai et al. (2019)
Probiotics	Traditional medicine	Intervention of IBD	Al Mijan and Lim (2018), Grom et al. (2020)
Seaweed	Metabolite	Chronic diseases such as cancer, arthritis, diabetes	Tanna and Mishra (2018)
Mediterranean diet	Various bioactive components	Antidiabetes	Alkhatib et al. (2017)
Plant sterols and stanols	Phytosterols	Plasma cholesterol control	Poli et al. (2018)
Phytochemicals	Various bioactive components	Cancer, CVD, anti-obesity	Gul et al. (2016)
Higher polysaccharide	Dietary fibers	Anti-cardiovascular and cerebrovascular disease	Venkatakrishnan et al. (2020)
Food bioactive compounds, plant foods	Polyphenol, flavonoids	Anticancer	Adefegha (2018)
CLA	PUFAs	Anti-obesity, antidiabetic	Kouchak Yazdi et al. (2017), Yazdi and Alemzadeh (2017)
Cereals	Polyphenols	Anti-chronic, diabetes, anticancer, anti-obesity	Arab et al. (2011)
Marin microorganisms	Bioactive polysaccharides	Anticoagulant, anti-inflammatory, antiviral	Dewapriya and Kim (2014), Suleria et al. (2015)
Date fruit and seed	Phytochemical	Antioxidant, antifungal and antiviral, anticancer	Maqsood et al. (2020)

9.3.1 L-Asparaginase

L-Asparaginase, also known as asparagine amino hydrolase (EC 3.5.1.1), is an intercellular enzyme broadly used in various pharmaceutical and food applications for catalyzing the degradation of L-asparagine into ammonia and L-aspartate. In the medicinal aspect, the asparaginase role is to inhibit cell starvation by reducing asparagines availability (Asselin & Rizzari, 2015). The L-asparaginase has been regarded to be a safe and favorable additive for the mitigation of acrylamide levels during food processing (Baskar et al., 2019).

Acrylamide is a main component in many carbohydrate/starchy-based foods, such as cereals, peanuts, potatoes, vegetables, crips, chips, coffee, biscuits, and lentil, particularly when exposed to high temperatures up to 120 °C. Several food processes including frying, baking and roasting, and process conditions, such as temperature, type of product, humidity, time, and content of reducing sugars and free amino acids result in the formation of acrylamide, and the processing variables influence directly the level of acrylamide content (Zuo et al., 2015). Acrylamide is generated during Millard reactions in the presence of reduced sugars and asparagine. The main consequences of acrylamide formation in food products are the strong color and flavors.

The potential toxicity and adverse effects of this compound on human health were declared (WHO/FAO). L-Asparaginase has remarkable attention in research from past decades, because of its vital role in the metabolism of amino acids, and thus, can be considered for the treatment of different types of cancers and diseases including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), non-Hodgkin's lymphoma, and food applications (Alam et al., 2019). According to the Global Asparaginase Sales Market Report, the asparaginase market has growing demand. The reported market value for asparaginase was 380 million USD in 2017 and is estimated to reach 420 million USD by 2025 (Global Medical Asparaginase Sales Market Report, 2018). L-Asparaginase is isolated from various plants and animal sources, such as animal cells, algae, chicken liver, serum of rodents, and microorganisms including fungal, yeast, and bacteria. *Bacillus* sp. and *Pseudomonas* sp. are the two main microbial sources of asparaginase. Bacterial sources have gained more attention for industrial application due to their versatile characteristics, easy maintenance, and high efficiency (Shi et al., 2017). There are two types of L-asparaginase; type I and type II. For food and medical applications, type II asparaginase is more favorable. Type II asparaginase shows a high affinity toward L-asparagine and hence it has been implicated for the removal of asparagine before processing the raw ingredients to reduce acrylamide formation (Hendriksen et al., 2016). Although several types of research emphasized the allergic reactions due to the high immunogenicity of the asparaginase and its clinical toxicity, such as headache, dyslipidemia, diarrhea, nausea, mucositis, pancreatitis, liver dysfunction, central nervous system dysfunction, and abdominal pain, type II asparaginase proved to be less immunogenic, and therefore, is more suitable for medical applications (Mitchell et al., 1994; Rizzari et al., 2000; Zuo et al., 2015). Other microorganisms with fewer adverse effects such as eukaryotic microorganisms have

been explored (Patro et al., 2014). Fungal sources are also considered safe asparaginase producers (generally recognized as safe, GRAS). It has been reported that fungic L-asparaginase utilization during food manufacturing leads to acrylamide reduction without any negative impact on the final appearance and nutritional value of the product. This approach mainly occurs from two aspects, interference with Millard reactions and removal of L-asparagine as the main precursor of acrylamide formation (Batool et al., 2016). However, the efficacy of asparaginase formation is not sufficient for the industrial demands (FAO/WHO, 2002; Jha et al., 2012). Two commercial samples of fungal asparaginase in the food industry, PreventAse (DSM) and Acrylaway (Novozymes) are obtained from *Aspergillus niger* and *Aspergillus oryzae*, respectively (Pedreschi et al., 2011; Xu et al., 2016; DSM, 2018). PreventAse presents an optimum pH in the range of 4–5 and temperature at 50 °C, while these values for Acrylaway are 6–7 and 60 °C. The activity of L-asparaginase during food preparation and hydrolysis should be high, thus producing a stable enzyme is of great importance.

The cultivation conditions including media composition, pH, temperature, oxygen levels, and the fermentation technique, namely solid-state fermentation or submerged fermentation, have considerable influence on the production of an ideal enzyme (Brumano et al., 2019). Carbon and nitrogen are the most important substrates in the culture medium used for L-asparaginase production (Cachumba et al., 2016). Glucose is considered the most appropriate carbon source to achieve L-asparaginase (Doriya & Kumar, 2016). L-Asparagine, L-proline, urea, yeast extract, and peptone are some of the important sources of nitrogen for high-yield asparaginase (de Moura Sarquis et al., 2004).

Determination of the optimum pH and temperature of the fermentation medium is also an important step for producing high-yield enzymes. The emergence of engineered enzymes according to the structural and mechanistic methods and direct evolution is needed to enhance the efficiency of enzymes for the synthesis of various products (Bornscheuer et al., 2012). Bakery products, French fries, and roasted coffee are the most widely consumed products that have high acrylamide content.

9.3.2 Carbohydrate Active Enzymes

Glucans are non-starch glucose polymers and one of the most abundant polysaccharides found in the cell wall of fungi, plants, and bran cereal grains (Wu et al., 2018). The glucose moieties link through either or both alpha (α) or beta (β) linkages, so their structures can be either linear or branched, and microfibrillar or amorphous. α-1,3-Glucans sensu strictu (pseudonigerans) is known as the most abundant α-glucans in the cell wall of fungi. Their structure is in the form of microfibrils which make resistance to the cell wall. On the other hand, the structure of β-glucans is a complex of linear or branched. In most cases, the structure is in the form of microfibrils with mostly β-1,3-linear, and β-1,6-branched linkages. One such example is a water-soluble low molecular weight polymers (LMW), which contains β-1,2 besides β-1,6 joined branches and has been isolated from black yeast

Table 9.2 The classification of β-glucans and β-glucanases enzymes from various sources

β-Glucans type	Structure	Description	EC number	References
Bacterial (*Euglena gracilis*)	Linear	β-1,3-glucan	Exo: EC 3.2.1.58 Endo: EC 3.2.1.39	Wu et al. (2018)
Fungal (*Schizophyllum commune*)	Branched	β-1,6-branched, and β-1,3-glucan	Endo: EC 3.2.1.6	Sutivisedsak et al. (2013)
Yeast	Long branched	β-1,6-branched and β-1,3-glucan endo-β-1,3-glucanases	EC 3.2.1.39 EC 3.2.1.6	Sato et al. (2012)
Cereal (barley)	Linear	β-1,3/1,4-glucan	Endo 3.2.1.6	Rodríguez-Mendoza et al. (2019)
Lichen (lichenase, licheniase)	Linear	1,3–1,4-β-glucan	Endo 3.2.1.73	You et al. (2016)

(Aerobasidiumpillulans). β-1,3–1,6-D-glucan represents antitumor, anti-metastatic, and anti-stress effects (Sato et al., 2012). β-Glucans together with chitin are known to be the most important structural constituents of fungal cell walls (Ruiz-Herrera & Ortiz-Castellanos, 2019).

As an example of a β-glucans-producer fungal, *Schizophyllum*, produces schizophyllan, which is a homoglycan natural polysaccharide with a β-1,3 linked backbone and single-1,6-linked glucose side chains at each residue. Its role as a biological response modifier and a stimulator of the immune system provides a variety of applications in the therapeutic and cosmetics industry. This biodegradable polymer has been also used for food preservation by forming oxygen impermeable films (Sutivisedsak et al., 2013).

β-Glucanase is an enzyme classified in the glycosyl hydrolase family that catalyzes the release of glucose from the reducing and nonreducing ends of β-glucan. The glucose released from the catabolic activity of β-glucanase applied in bioethanol and biofuels manufacturing (Lopez-Casado et al., 2008). Selected β-glucanases have an essential role in the pharmaceutical and medical industry by biologically controlling fungal pathogens, synthesizing drug carriers, and producing in vivo immunomodulatory anticancer drugs (You et al., 2016). β-1,3-Glucanases have been used in the food industry, mainly for alcoholic beverages processing. The classification of β-glucans and β-glucanases enzymes is represented in Table 9.2.

The modification of polysaccharides through β-D-glucans hydrolysis results in gluco-oligosaccharides production which provides several biological activities such as immunomodulation (Giese et al., 2012).

Another advantage of exogenous β-glucanase is to incorporate it into wheat and barley-based diets to inhibit the anti-nutritive effect of water-soluble NSP. Benefits of using exogenous enzymes for nutrient digestibility have been reported previously; however, the mechanism of action of these enzymes is not fully recognized. One other application of these exogenous enzymes is to enhance the microbial activity of the poultry intestine (Giese et al., 2012).

β-1,3-Glucanases have been recommended to apply in the food industry to improve the organoleptic properties of the final products. Besides, they have a positive role in the production of feed and animal nutrition (Rodríguez-Mendoza et al., 2019). The sources of glycoside hydrolase β-1,3-glucanase are extended among fungi, bacteria, and plants. The hydrolysis position of structural β-1,3-glucans is on 1,3-β-glucosidic bonds to transfer or hydrolyze glycosides. β-1,3-Glucanases are normally classified into endo-enzymes (EC 3.2.1.39) and exo-enzymes (EC 3.2.1.58). β-1,3-Glucanases are able to act as a protective agent against fungal pathogens and provide immune-modulating and antitumor properties in plants. They are also capable to suppress fungal growth in fermentation media by the convention of β-1,3-glucans into (1→3)-linked β-D-glucan oligosaccharides (Dewi et al., 2016; Wu et al., 2018).

Endoglucanases are generally classified into three categories based on their modes of action: Endo-1,3–1,4-β-glucanase (EC 3.2.1.73), endo-1,3-β-glucanase (laminarinase; EC 3.2.1.39), and endo-1,3(4)-β-glucanase (EC 3.2.1.6). They specifically cleave the blended (1→3) or (1→4) linkages of β-D-glucan. β-Glucanases are important commercial biocatalysts in various industries, from exogenous β-glucanase, which are applicable specifically in reducing the unwanted effects of barley β-glucan in the mashing operation as a step in the brewing industry, and to improve the β-glucan digestibility in poultry feedstuffs, to endoglucanase to convert biomass into bioethanol in and xylanase in bioenergy production (You et al., 2016).

9.3.3 Chlorophyllase

Chlorophyllase enzyme (Chlase, chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) is a hydrophobic enzyme leading to chlorophyll degradation into chlorophyllide (Chlide) and phytol. Chlorophyllase activity is the most important step in the chlorophyll (Chl) breakdown and synthesis (Hörtensteiner & Kräutler, 2011). Another Chl hydrolysis pathway is through pheophytinase, which leads to pheophorbide (Schelbert et al., 2009). The chlorophyll breakdown pathway determines the Chl accumulation and various intermediate catabolites in chloroplasts. Chlorophyll hydrolysis is most evident in fruit ripening, and seasonal changes as leaf senescence and the color of plants convert from green to yellow. Chlorophylls are one of the most important plant pigments that determine the absorption of solar radiation in leaves (Filella et al., 1995). They are a brilliant source of natural green color and can be an alternative to synthetic colorants. Moreover, they possess biological and therapeutic activities, such as anti-

Fig. 9.2 The initial steps of chlorophyll catabolism (CLH and MCS are refer to chlorophyllase and magnesium-dechelating substance, respectively) (Hu et al., 2015)

inflammatory, antioxidant, antibacterial, wound healing, anticarcinogenic, and deodorizing properties (Hosikian et al., 2010).

The activity of chlorophyllase in fruits and vegetables prolongs during storage and processing conditions. Chlorophyllide is obtained through enzymatic conversion of chlorophyll by the cleavage of the phytol group and is used for the preservation of green color in various canned vegetable products. By the aid of magnesium-chelating substrates, the induction of chlorophyll breakdown leads to pheophytins hydrolysis and more pheophorbide formation (Fig. 9.2). A well-known application of chlorophyllide formation is the formation of pheophorbide during olive fermentation. For example, the activity of chlorophyllase in ripe olives of arbequina and picual varieties was studied and demonstrated that the process of oil extraction and propitiating the pheophytization of chlorophylls in the fresh fruit cause a significant reduction in the chlorophyll's pigments (Gandul-Rojas & Mínguez-Mosquera, 1996).

As shown in Fig. 9.2, the catabolism of chlorophyll to synthesize chlorophyllide triggers chlorophyllase. In both decoloration processes and the homeostasis of chlorophylls, chlorophyllase takes part as an effective catalyst in the early steps of chlorophyll breakdown. The role of chlorophyllase in plant development and

survival is vital because it leads to faster degradation of chlorophyll and its intermediates, and further detoxification process (Carle & Schweiggert, 2016).

Experimental evidence in epidemiology has shown the association of green fruits and vegetables with chronic disease prevention, thus, phytochemical foods have established as bioactive dietary compounds. Chlorophyll and its lipophilic derivatives are composed of chlorophyll a and b (fresh fruits and vegetables), metal-free pheophytins and pyropheophytins (thermally processed fruits and vegetables), and Zn-pheophytins and Zn-pyropheophytins (thermally processed green vegetables) are believed to be responsible for such associations, as they are phytochemical compounds. Chlorophyllides, pheophorbides, and sodium copper chlorophyllin (SCC), a commercial-grade derivative, are hydrophilic compounds with the predominant contribution to the dietary chlorophyll role. Antioxidant and antimutagenic activity, mutagen trapping, modulation of xenobiotic metabolism, and induction of apoptosis are attributed to useful biological activities of chlorophyll derivatives which assimilate cancer prevention.

Although the medical evidence of chlorophyll derivatives are mainly focused on commercial-grade SCC, there is significant attention to the cancer-preventive potential of chlorophyll. Further research is required to define the extent to which chlorophyll derivatives are responsible for modulating cancer-risk biomarkers (Ferruzzi & Blakeslee, 2007).

Chlorophyllase catalyzes the hydrolysis of chlorophylls to chlorophyllide and phytol. This catalytic activity of chlorophyllase has potential industrial and agricultural applications. In this context, the application of chlorophyllase could be useful in the removal of green pigments (chlorophyll) from edible oils to improve their oxidative stability. In addition, the products of chlorophyllase, chlorophyllides, and their derivatives (such as pheophytin) have been demonstrated to have antiviral, antioxidant, antimutagenic, and anticarcinogenic activities in vitro. Accordingly, in recent years, trends for purification of natural chlorophyllases or recombinant production of the enzyme has been increased (Sharafi et al., 2017).

9.3.4 L-Glutaminase

L-Glutamine catalysis is the enzymatic reaction of thiamine. Thiamine (γ-glutamyl ethyl amide) is a nonprotein amino acid and is known to be responsible for the sense of relaxation in green tea. L-Thiamine is generally recognized as safe (GRAS), and apart from its umami taste and unique flavor, has several pharmaceutical and physiological effects, such as anticancer, anti-obesity, anti-hypertensive and neuroprotective effects. It may further improve learning ability and mind performance. Thus, thiamine is an essential compound for the human body that should be administrated orally, as it is not synthesized in the body (Haskell et al., 2008; Panesar et al., 2016).

The natural sources of thiamine include plants, animals, and microorganisms. Microbial-derived is preferable sources compared to plant and animal-derived thiamine, mainly due to the low cost of operation, as well as other technical advantages

Fig. 9.3 Theanine synthesis reaction catalyzed by L-glutaminase in the presence of ethanolamine ($EtNH_2$) (Sakhaei & Alemzadeh, 2017)

such as higher yields within a limited period of fermentation time. The enzymatic biosynthesis of L-thiamine takes place in the presence of ethylamine and L-glutamine as substrate and catalyst (glutaminase). L-Thiamine production was carried out under solid-state fermentation by the fungal strain *Trichoderma koningii*. The enzymatic synthetic reaction for theanine production is presented in Fig. 9.3.

9.3.5 Lipase

Lipases, also known as triacylglycerol acyl ester hydrolases (EC 3.1.1.3), are capable to catalyze cleavage of carboxyl ester bonds in tri-, di-, and monoacylglycerols. Most lipases are generally recognized as safe (GRAS) and can be employed in the food industry, mainly for diary applications with the aim of ripening acceleration in Italian-type cheeses, and hydrolysis of milk fat to further be used as a flavor enhancer in cheese products (Afarin et al., 2018).

Further applications of lipases in pharmaceutical and fine chemical preparation processes include (but are not limited to) lipase-mediated synthesis of optically pure and optically active polymers, lipase-catalyzed resolution of racemic aqueous mixtures, monoglycerides production (as an emulsifier in the food and cosmetic products), fatty alcohols (as lubricants in hydrophobic waste treatment), and sugar esters as biosurfactants (Tarahomjoo & Alemzadeh, 2003).

Another advantage of lipase incorporation in dairy products is fat and oil-tailoring for enhancing functional and nutritional properties. One such example is employing lipase in conjugated linoleic acid (CLA) large-scale production for the modification of triglycerides and upgrading of low-value oils and fats like human milk fat replacers, cocoa butter substitutes, and enrichment of nutraceutical fats with poly-unsaturated fatty acids (Paiva et al., 2000; Yazdi & Alemzadeh, 2017). CLA is one of the most physiological active fatty acids a natural derivative of linoleic acid, an essential fatty acid, that possesses a conjugated double-bond system for describing octadecadienoic acids mixtures (18:2) (Jiang et al., 1998; Lin, 2006; Gofferjé et al., 2014).

Due to the global rising trend concerning diseases, such as obesity, hyperlipid-emia, atherosclerosis, diabetes, cancer, and high blood pressure and the established association of lifestyle on health management, the quality of dietary fat is an important factor that should be considered for prevention and treatment of early stage diseases. In this regard, conjugated fatty acids have gained much attention for their potential health effects on lowering the risk of these diseases, such as

anticarcinogenic activity, immune system enhancement, immune stimulation, lowering atherosclerosis symptoms, and cholesterol reduction (Niezgoda & Gliszczyńska, 2019; Kouchak Yazdi & Alemzadeh, 2016a, b; Afarin et al., 2018).

Natural sources of CLA include dairy products (0.40–0.55% of total dairy lipids) and beef (0.43% of total beef lipids). *Lactobacillus plantarum* is a microbial-derived source of nutritious and valuable components, such as CLA. CLA contains a variety of positional and geometrical conjugated double bonds of linoleic acid (C18:2) isomers, including all possible geometric configurations of trans–cis, cis–trans, cis–cis, and trans–trans isomers (Paiva et al., 2000; Philippaerts et al., 2013).

The 9c, 11t-18:2 and 10t, 12c18:2 isomers of CLA are believed to be responsible for the beneficial physiological effects of CLA. The 9c, 11t-18:2 isomer (rumenic acid), is the main isomer, consists of 90% of the total CLA found in dairy and beef lipids; so, the focus of most research on CLA employment is on cis-9, trans-11 CLA. Moreover, the multitude health-promoting effects of CLA, such as anti-atherogenic activity, are associated with c9, t11 CLA (ANDO, 2004). Other isomers, namely, trans-10, cis-12 (t10, c12CLA), are found in much smaller quantities. c9, t11, and t10, c12 isomers are determined to represent the highest bioactive property (Ogawa et al., 2005). Further investigations on optimizing the experimental design for a safe and selective biological process for CLA preparation to achieve enriched food with high CLA concentration are required (Afarin et al., 2018).

Although the exact mechanism of anticarcinogenic and atherosclerosis risk-reduction benefits is still needed to be studied, clinical evidence demonstrates that the management and prevention of these diseases are achievable through CLA-functional food on regular basis. It has been recommended that the consumption of 3 g/day of CLA is essential to provide essential health factors. It is noteworthy that the attainable CLA from natural resources is approximately 0.3 g/day; therefore, CLA-based dietary supplements and CLA-rich vegetable oils and dairy products should be considered as part of a healthy lifestyle. Main commercial approaches for CLA production consisting of chemical, microbial, and enzymatic methods are represented in Table 9.3. Each technique implements a different protocol and results in a different mixture of CLA isomers. Among them, the alkali isomerization of linoleic acid has been reported to be the most potential and cost-effective method (Yang et al., 2002; Afarin et al., 2018).

According to the potential health benefits demonstrated for CLA, it is a preferable additive for the preparation of functional foods. The synthesis of CLA can be performed either by isomerization of linoleate-rich oils with the aid of an alkaline catalyst or by dehydration of ricinoleic acid-rich oils or their esters using an acid catalyst.

CLA isomers, either in their natural or synthetic forms, have been shown considerable physiological effects on human health. CLA free fatty acid may cause undesirable reactions in the human gut. Thus, it has been recommended to be consumed in the form of triglycerides. One way is to apply sunflower oil for transesterification of lipid, mainly due to its beneficial health effect, via lipase-catalysis. Enzymatic process is an alternative method to catalyze the transesterification reaction biologically to produce triacylglycerols with enriched

Table 9.3 CLA production by various techniques

Method	Catalyst	Substrate	References
Chemical	Alkaline	Linoleic acid	Afarin et al. (2018)
Chemical	Alkaline	Ricinoleic acid	Yang et al. (2002)
Chemical	Metal catalyst ($SnCl_2$)	Methyl linoleate	Philippaerts et al. (2013)
Microbial	*Lactobacillus plantarum* PTCC 1058	Castor oil	Kouchak Yazdi and Alemzadeh (2016a, b)
Microbial	Dairy starter cultures	Linoleic acid	Jiang et al. (1998)
Microbial	*Lactobacillus acidophilus*	Linoleic acid	Ogawa et al. (2005)
Microbial	*Lactobacillus plantarum* A6-1F	Linoleic acid	Zhao et al. (2011)
Microbial	*Lactobacillus reuteri*	Linoleic acid	Lee et al. (2003)
Microbial	*Bifidobacteria* and *Propionibacteria*	Linoleic acid	Hennessy et al. (2012)
Microbial	Lactic acid bacteria	Linoleic acid, castor oil	Ogawa et al. (2005)
Microbial	*Lactobacillus plantarum* JCM 1551	Castor oil	ANDO (2004)
Enzymatic	Crude enzyme from *Lactobacillus delbrueckii* ssp. *bulgaricus* (CCRC14009)	Linoleic acid, oleic acid, linolenic acid	Lin (2006)

residues. Generally, the alteration of fatty acids composition results in promoted nutritional properties of triacylglycerol molecules. Identifying an environmentally friendly and less energy-intensive strategy for oil esterification by using an enzyme as a catalyst should be investigated (Ogawa et al., 2005; Hennessy et al., 2012).

9.3.6 Phytase

Phytic acid, designated as phytate when in salt form, is regarded as a primary reservoir of organic phosphorus in pollen and plant seeds like nuts, cereals, oil seeds, and legumes. It has a high negative charge and is, therefore, able to form ionic complexes with essential cationic minerals (Ca^{2+}, K^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+}), proteins, and trace elements (Greiner & Konietzny, 2006). A main anti-nutritional consequence of this interaction is the considerable reduction in dietary minerals bioavailability. Phytase, also known as myo-inositol (1,2,3,4,5,6)-hexakisphosphate phosphohydrolase, act as a dephosphorylating enzyme to transfer phytic acid to more soluble and lower chelating products to inhibit anti-nutritional factors of phytic acid in food products and increase mineral absorption (Kuddus, 2018). Depending on their catalytic mechanism, phytases can be divided into histidine acid phytases, cysteine phytases, β-propeller phytases, or purple acid phytases (Chu et al., 2004) and based on their optimum pH, into acid and alkaline phytases. Dephosphorylation is initiated at the carbon in the myo-inositol ring of phytic acid. There are three different types based on the carbon; 3-phytases (EC 3.1.3.8), 5-phytases

(EC 3.1.3.72), and 6-phytases (EC 3.1.3.26) (Greiner & Konietzny, 2006). Enzymatic dephosphorylation of phytates was originally a strategy to improve the nutritional value of feed by liberating phosphate, especially for mono-stomached animals like fish, broilers, shrimp, pigs, and poultry diets (Konietzny & Greiner, 2004).

Recently, there have been numerous studies showing the potential use of these enzymes for functional food development. One of the major concerns in phytate hydrolysis content during food manufacturing is the selection of an ideal phytase. The activity of added phytase has to be high enough during food preparation. Therefore, a high capability of phytate degradation over a wide range of temperatures and pH is essential (Greiner & Konietzny, 2006). According to previous studies the optimum pH range for acid phytase is 3.5–6.0, and for alkaline phytase is 7.0–8.0. The optimum temperature for high phytate-degrading activity is in the range of 35–80 °C. The optimum temperature and pH of the added enzyme are strongly affected by the source of phytase.

Phytase has been found in plants, microorganisms, and animal tissues. There are already available commercial phytases from different microbial sources, such as *Escherichia coli*, *Peniophora lycii*, *Aspergillus niger*, *Schizosaccharomyces pombe*. Commercial production of phytase from *Aspergillus*, a soil fungus, has several prominent drawbacks, such as fungal enzymes are catalytic efficiency, substrate specificity, and resistance to proteolysis. Bacterial phytases have been distributed in various aerobic and anaerobic species, including *Bacillus* strains, *Pseudomonas* strains, *Raoultella* strains, *Escherichia coli*, *Citrobacter braakii*, *Prevotella* strains, *Selenomonas ruminantium*, *Mitsuokella jalaludinii*, *Megasphaera elsdenii*, *Lactobacillus sanfranciscensis*. The expression of phytase from these bacteria sources is different and it can be regulated by the nature of the culture composition used for growth and the nutrient or energy limitations. The level of inorganic phosphate on the phytase synthesis induction was reported to be effective for all microbial sources, except *Raoultella terrigena* and the rumen bacteria. In some phytase producers, the nature of the carbon source is a significant inducible factor by involving in the catabolism pathway (Konietzny & Greiner, 2004).

Generally, microbial-derived phytases are more favorable economically compared to plant-derived ones, normally due to their thermostability and pH tolerance as well as a broad specific activity. The specific activity of characterized phytasea, is approximately in the range of 10–1000 U/mg at 37 °C and various pH optimums (Greiner & Konietzny, 2006). The highest specific activities are reported for microbial-derived enzymes. In food applications, it is important that the enzymatic properties of phytase to hydrolyze phytate in the gastric conditions be stable under acidic pH and pepsin enzyme. Likewise, for food supplements, an enzyme with acidic pH optimum and highly resistant to digestive enzymes is indisputably attractive.

However, when exposed to pH values above pH = 7.5 or below pH = 4.0, most of the plant enzymes dramatically lose their stability (Greiner & Konietzny, 2006). Besides, the activation of these enzymes is irreversibly affected when exposed to temperatures above 70 °C within a few minutes. Despite the fact that most of the

plant enzymes are inactivated at pH values lower or higher than the pH optima and high temperatures, they are more acceptable among consumers as they are rarely reported as an allergen.

The use of agro-industrial byproducts in the culture media has been suggested as low-cost substrates for the production of phytase (Pires et al., 2019). Corn meal and rice bran were used for the production of phytase from the fungus *A. zeae* B, and the yeast *K. marxianus*. The activity of phytases from agro-industrial products strongly depends on the species of the microorganism. For example, the determination of total phytase activity in the mentioned study revealed tenfold higher values in the yeast extraction compared to those of the fungus extraction (Pires et al., 2019).

Engineered phytases via genetic engineering are a promising type of enzyme that show optimized catalytic features, thermal stability, and broad specific activity for food application. Enhancement of thermal stability by a shift from 80 to 90 and optimum temperature from 55 to 65 for *Escherichia coli* was achieved by using mutagenesis technology. The basis of the phytase synthesis is to mutation of gene site saturation by comparing the amino acid sequences derived from both the available standard program and the homologous proteins (Rodriguez et al., 2000; Garrett et al., 2004).

The incorporation of phytase in the food industry is a growing trend, mainly for two purposes; increasing the mineral bioavailability of a given food by reducing its phytate content and exerting nutritional health benefits in a supplemental product (Konietzny & Greiner, 2004).

Natural phytate-degrading activity, observed in most plants, results in phytate dephosphorylation during food processing such as soaking, germination, and fermentation. This capability of intrinsic phytate-degrading activity in plant seeds exhibits over a wide range of pH values from 3 to 10, and the maximum activity peak occurs at pH = 5–5.5 (Greiner & Konietzny, 2006). Therefore, phytate hydrolysis by a certain phytase at optimum pH and temperature might differ greatly among various plants and microorganisms. Some examples of optimum conditions for maximum phytase activity are represented in Table 9.4.

9.3.7 Protease

Enzymatic hydrolysis for modification of food proteins in functional food applications has been explored for several decades. Proteolytic action of an enzyme results in alteration of molecular conformation of the native protein, and thus, leads to the production of functional and bioactive components as texture enhancers in beverages, infant formula, and pharmaceutical ingredients (Adebiyi et al., 2009). The resultant properties are highly affected by the degree of hydrolysis (DH). Variations in functionality factors such as solubility, stability, gelling behavior, water absorption, emulsion, and foaming capacity are best observed following a low degree of protein hydrolysis (<10%). Low-DH protein hydrolysates are appropriate for enhancing food texture, while high-DH protein hydrolysates are suitable

Table 9.4 The effect of phytase sources on the specific activity and pH and temperature optimum for various applications

Phytase source	Specific activity	pH optimum	Temperature optimum (°C)	Application	References
Yersinia intermedia	3960 U/mg	4.5	55	Feed industry	Huang et al. (2006)
Streptomyces sp. (NCIM 5533)	–	2.5	70	Plant growth	Puppala et al. (2019)
Sporotrichum thermophile	1190 U/mg	5.0	60	Dietary supplement	Maurya et al. (2017)
Aspergillus aculeatus APF1	13.82 U/mg	3.0	50	Biofortified cereals	Saxena et al. (2020)
Enterobacter sp. ACSS	805.98 U/mg	2.5	60	Dephytinizing animal feeds, baking industry	Chanderman et al. (2016)
Pichia anomala	–	4.0	60	Fractionation of allergenic soya proteins	Joshi and Satyanarayana (2017)
Rhizopus oligosporus MTCC 556	31.3 U/gds	5.5	50	Food and feed industry	Suresh and Radha (2016)
Aspergillus niger NCIM 563	160 IU/mL	2.5	50	Organophosphorus pesticides degradation	Shah et al. (2017)
Penicillium oxalicum EUFR-3	12.8 U/g	7.0	40	Food industry	Kaur (2017)
Aspergillus ficuum	5.17 U/gds	6.1	37	Food industry	Tian and Yuan (2016)

for utilization in medical diets, such as hypoallergenic foods or protein supplements (Pokora et al., 2013).

The scientific community has declared the important role of dietary compounds as health-promoting ingredients for food and pharmaceutical applications. Dietary components are mainly considered bioactive substances if their consumption in adequate amounts shows beneficial biological effects on human health. Therefore, the desirable consequences of bioactive compounds incorporation in food production are represented provided that their utilization is in the real sense and without the exertion of side effects (De Leo et al., 2009). Short peptides including 3–20 amino acids are examples of bioactive peptides (BP) that usually encrypted within the primary structure of a larger protein. BPs are encrypted within the parent proteins; so in order to exert their physiologically positive effects on human health, their proteolysis is required.

Biologically active peptides in the protein sequence are defined as "fragments that remain inactive in precursor protein sequences, but when released by the action of proteolytic enzymes, they may interact with selected receptors and regulate the body's physiological functions" (Mohanty et al., 2016). Bovine milk contains approximately 3–3.5% protein of which 80% and 20% are casein and whey proteins, respectively. It exerts a wide range of nutritional, functional and biological activities (Fox, 1989). Many milk proteins possess specific biological properties that make them potential ingredients for health-promoting foods. Many researches have been carried out on bioactive peptides from casein hydrolysates. The list health benefits of biologically activity of milk bioactive peptides are radical scavenging, lipid oxidation inhibition, antiproliferation, stimulate proliferation, antimutagenicity, immunostimulant, immunosuppressant, and antimicrobial (Phelan et al., 2009). These peptides can be liberated by:

1. Gastrointestinal digestion of milk
2. Fermentation of milk with proteolytic starter cultures or
3. Hydrolysis by proteolytic enzymes like pepsin, trypsin, chymotrypsin, etc.

A great variety of peptides are formed during cheese ripening which has been shown to exert biological activities. Furthermore, secondary proteolysis during cheese ripening may lead to the formation of bioactive peptides (Korhonen & Pihlanto, 2006). The occurrence of the bioactive peptides which are naturally formed in cheese depends on the equilibrium between their formation and the degradation of the peptide during cheese ripening. Ripening is a time-consuming process during cheese making. To overcome these problems, some economic and technological methods for acceleration of cheese ripening have been reported (Fox, 1989). Acceleration of ripening may help to increase bioactive peptides. Encapsulation of enzymes in liposome can accelerate cheese ripening (Jahadi et al., 2016; Mohammadi et al., 2015; Zoghi et al., 2018). V_{max} and K_m and stability of the enzyme were increased by encapsulation of the Flavourzyme in liposome (Vafabakhsh et al., 2013; Jahadi et al., 2012, 2015, 2020; Jahadi & Khosravi-Darani, 2017).

Gamma-amino butyric acid (GABA) is another amino acid that consists of four carbons widely found in microorganisms, animals, and plants. GABA has been used in pharmaceuticals and functional foods due to its blood pressure-lowering activity and its role as a neurotransmitter signal in the central nervous system including the mammalian brain (Takahashi et al., 2012). It can also be used for the synthesis of nylon 4 as a monomer (Kim et al., 2007; Park et al., 2013). Its production has been reported to occur by decarboxylation of glutamate, a reaction catalyzed by glutamate decarboxylase (Pham et al., 2016).

The ever-increasing demand for GABA has generated the need for efficient microbial production. Lactic acid bacteria (LAB) are one of the main microbial sources for GABA production, mainly due to their food-grade property and high ability in the direct production of GABA-rich components. The progress toward culture conditions optimization and genetic engineering to achieve physiology-oriented engineering approaches and co-culture methods for the GABA-producing LAB species, the biosynthesis pathway of GABA by LAB, and the glutamate decarboxylase (GAD), as the key enzyme in GABA biosynthesis, is growing rapidly (Cui et al., 2020). The biosynthesis of GABA is known to exhibit superior performance due to high product efficiency, mild reaction conditions, simple process, and low environmental burden (Gavrilescu & Chisti, 2005; Alcalde et al., 2006; Carvalho, 2017). Irreversible α-decarboxylation of L-glutamate is an important pathway in GABA biosynthesis, which is catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent glutamate decarboxylase (GAD) (Xu et al., 2017).

It has been indicated that the enzymatic hydrolysis of collagen provides structural cleavage and better functionality. Enzymatic hydrolysis of bovine collagen and beef bone extract, which were undertaken by Flavourzyme® and Protamex®, respectively, have shown better functional (antioxidant and antimicrobial activity) and physicochemical properties of the final hydrolysates (Vidal et al., 2018).

Peptides have been proved to offer health-promoting benefits for hypertension, a primary risk factor in cardiovascular disease. Short-chain peptides are frequently favored compared to other protein resources like free amino acids combinations, due to their enhanced absorption kinetics (Jahan-Mihan et al., 2011). Thus, the proteolysis process to achieve short-chain peptides is of paramount importance. Angiotensin-converting enzyme inhibitory (ACEI) peptides are among the most recognized peptides. The proteolysis of ACEI peptides is necessary for their bioactivity and bioaccessibility, as they are encrypted within the parent proteins (De Leo et al., 2009).

Protein hydrolysis in peptides occurs both in vitro and in vivo. In vitro degradation takes place during food preparation processes by the addition of isolated or microbial enzymes. The in vivo process occurs within the gut, in the presence of digestive and microbial enzymes of the gut flora. Employing a blend of diverse proteases and a step-by-step procedure should be carried out for consecutive proteolysis (Boutrou et al., 2013).

Recently, dietary supplements based on the protein hydrolysates have already gained much attention in case of being unable to assimilate native proteins due to specific illnesses, such as Crohn’s disease or pancreatitis (Posovszky, 2016). The

incorporation of protein hydrolysates in sports nutrition has heightened its significance (Holm et al., 2017). Another consequence of protein hydrolysates is muscle anabolic due to their insulin atrophic effect (Yuan et al., 2017; Rittig et al., 2017). The combination of several effects would couple their nutritional effects with other "bioactivities," an area of study which has received increasing attention nowadays (Tavano et al., 2018).

The enzymatic hydrolysis of soybean protein products has resulted in reducing allergenicity and improving functional properties. The allergenicity of hydrolyzed soybean protein isolates (SPI) by means of alcalase, trypsin, chymotrypsin, bromelain, or papain was investigated. Assessment of chymotrypsin hydrolyzed samples indicated a higher allergenicity potential of digested SPI due to the β-conglycinin protein fragments (Panda et al., 2015).

Allergenicity of food products is regarded as adverse health effects arising from a specific immune response when exposed to a given food reproducibly (Sicherer & Sampson, 2014). Both IgE-mediated and non-IgE-mediated allergenic reactions are included in adverse immune responses (Berin, 2015). In this regard, the selection of a fit protease with desirable specificity and proper hydrolysis procedure can significantly reduce protein allergenicity (Burton et al., 2002). It has been reported that the allergenicity of milk proteins and gluten decreased significantly by proteolysis utilization in food process engineering (Rizzello et al., 2007). Another study confirmed the amplification of food allergenicity by IgE immunoblots measurement when chymotrypsin or bromelain was used for the hydrolysis of soybean protein isolate (SPI) (Panda et al., 2015). A 65% reduction in IgE reactivity was shown for roasted peanut protein hydrolysis using Flavourzyme for 300 min (Berin, 2015). SPI hydrolysis was carried out using pepsin, alcalase, and papain. The results showed the suitability of these enzymes in the destruction of the main soybean allergens. However, the application of papain and Flavourzyme were more beneficial in view of less marked bitter taste production (Meinlschmidt et al., 2016). The effect of enzymatic hydrolysis of SPI by Corollas PP on antihypertensive and antioxidant activities was evaluated (Guan et al., 2018).

It has been proved that the gluten uptake, from various sources like *Triticum* species, rye and barley proteins, and their crossbred varieties, can notably distress genetically susceptible individuals to inflammation of the small intestine, namely celiac and nonceliac diseases (Rizzello et al., 2007).

The effect of proteolytic enzyme modification of egg-yolk protein and white protein during the preparation processes was evaluated and resulted in the production of the bioactive peptide with enhanced properties like antioxidant activity, ACE-inhibitory activity, ferric reducing power, scavenging capacity, water-holding capacity, and chelating capacity (Pokora et al., 2013).

9.3.8 Tannase

Polyphenolic compounds are naturally present in a variety of plant sources, namely tea, nuts, and fruits. These compounds are considered to be health-promoting and

disease-preventing bioactive molecules. They have an important role in decreasing degenerative diseases, such as diabetes, cardiovascular, and cancers by mutagenesis and carcinogenesis-preventive activities (Kuddus, 2018). Therefore, it seems these bioactive compounds are a two-edged sword. However, the bioavailability and bioefficacy of polyphenolic compounds are affected by their capacity to form complexes with macromolecules like proteins, cellulose, pectin, starch, and minerals, generally found in polymeric, glycosidic, and esteric compounds (Arshad et al., 2019). As a result of the formation of complexes with macromolecules, the absorption rate in the small intestine will reduce dramatically (Kuddus, 2018). The higher the molar mass of polyphenolics, the lower the bioavailability activities and fewer nutritional effects (Aharwar & Parihar, 2019).

Hydrolyzable tannins are commonly known as polyphenolic secondary metabolites, in the form of galloyl esters or their derivatives. The attachment of galloyl moieties or their metadepsidic derivatives of tannin to a variety of polyol, catechin, and triterpenoid units is called gallotannins. Through hydrolysis, glucose and gallic acid are produced, which are more beneficial for human health (Chung et al., 1998; Khanbabaee & van Ree, 2001).

Tannin acyl hydrolase, also called tannase (EC 3.1.1.20), is an inducible extracellular enzyme belonging to the esterase superfamily. The molecular weight of tannase is in the range of 31–310 kDa, depending on their source. Generally, fungal tannases have a higher molecular weight range (45–310 kDa) compared to bacterial tannases (31–90 kDa) (Aharwar & Parihar, 2018). The ranges of pH and temperature optima of tannases are 4–8 and 30–70 °C, whereas the stability ones are 3–8 and 20–90 °C, respectively (Selvaraj et al., 2019). Tannase catalysis is the hydrolysis of depside and ester bonds, including gallyol ester of gallic acid and alcohol components of hydrolyzable tannins, like chlorogenic acid, epigallocatechin gallate, epicatechin gallate, and tannic acid. Through hydrolysis, other oligomeric tannins and other useful and absorbable compounds were yielded. A famous example is a gallic acid which is an essential intermediary constituted in the synthesis of trimethoprim antibacterial drug and is applied in the pharmaceutical industry. Propyl gallate is another substrate for the synthesis of antioxidants in the food industry, or even other components applied for clarifying, as well as treating tannery effluents in the juice industry (Aharwar & Parihar, 2018, 2019).

Generally, tannase has extensive applications in the chemical, brewing, cosmetic, beverage, pharmaceutical, and food industries. Tannase is able to degrade plant cell walls, cleaving cell-wall cross-links, and facilitate the absorption of polyphenolic compounds in the small intestine by enzymatically hydrolyzing them into simpler and smaller phenolic compounds (García-Conesa et al., 2001; Shoji et al., 2006; Georgetti et al., 2009). Increasing the antioxidant, anti-inflammatory, immunomodulatory, antiproliferative, and anticarcinogenic activities are the consequences of these modifications (Kuddus, 2018). It is also a well-known commercial enzyme in the elaboration of instantaneous, gallic acid production, and acorn liquor (Aharwar & Parihar, 2018).

Tannase production has been studied extensively. Several microorganisms (fungi, yeast, and bacteria) are used for microbial tannase production; among them, several

fungal genera, including *Aspergilli*, *Penicillium*, *Trichoderma*, and *Rhizopusare* are more typical. Among different microbial sources, fungi, such as *Aspergillus ficuum*, *Aspergillus oryzae*, *Arxula adeninivorans*, and *Aspergillus fumigates*, are the most common sources of the tannase-producer genus in the absence of tannic acid (Cavalcanti et al., 2020). Based on the strain type and culture conditions, submerged fermentation (SmF), or solid-state fermentation (SSF), the enzyme can be inducible or constitutive. Tannase synthesis is induced in the presence of several phenolic compounds such as gallic acid, pyrogallol, tannic acid, and methyl gallate (Aharwar & Parihar, 2018). Agricultural residues have also been used as alternative substrates for economic tannase production (Selvaraj et al., 2019).

However, the synthesis mechanism involved is a controversial issue that should be addressed. SmF has several advantages in sterilization, incubation time, process control, and ease of extracellular recovery (Kumar et al., 2016). In contrast, the advantages of SSF are related to its cost-effective downstream and upstream processes, water-dependent conditions, ease of agro-waste substrates usage, the minimum amount of effluents, and high efficiency, so in most cases, SSF is preferable (Selvaraj et al., 2019). The potential of *Lasiodiplodia plurivora* CAN-10 for tannase production was compared under these two fermentation methods by utilizing *Terminalia catappa* (almond leaves) and *Magnifera indica* (mango leaves) as agro-waste substrates to provide carbon and nitrogen sources (Ire & Nwanguma, 2020). The results of this study revealed that SSF yielded higher tannase production compared to SmF. SmF was used for the production of tannase in the presence of tannic acid (2% w/v) from *Aspergillus* sp. and culture conditions like temperature, inoculums size, moisture content, aeration rate, and substrate consecutively affect the process and the fermentation products (Pandey et al., 1999). More examples of tannase producing sources and the corresponding mechanism that results in the functional enhancements are shown in Table 9.5. More studies are required for the identification of high-yield strains as well as low-cost and efficient processing development (Aharwar & Parihar, 2018). Recombinant DNA technology and genetic engineering for tannase production have been investigated in several new researches. Tannase-encoding genes of *Aspergillus oryzae* were used for producing a wide variety of hydrolytic enzymes, namely proteolytic and amylolytic enzymes. In a study, AotanB (AO09002300047), one of the encoding genes of *A. oryzae*, was overexpressed using a glucoamylase gene promoter, P*glaA142*, and a recombinant enzyme was characterized. Based on the results, the recombinant enzyme produced by *A. oryzae* showed better thermal stability and enzyme activity as compared to *Pichia pastoris*-tannase producer strain (Koseki et al., 2018). In another study, transferring tannase gene into *Aspergillus niger* resulted in better yield of enzyme production and improved characteristics (Liu et al., 2018).

Enzyme stability maintenance during shelf life, handling, transport, and storage at room temperature is an essential property for enzyme commercialization in the food industry. According to the current literature, several techniques such as hydration by spray drying and freeze-drying, immobilization through nanoparticles, with the aid of various additives (adjuvants/carriers), such as carbohydrates, proteins, lipids, gums, and surfactants, as protectors against oxidative denaturation, have been

Table 9.5 Some examples of tannase production from various microorganisms and their related properties (tannase activity, pH, and temperature)

Source	Activity	pH optimal stability	Substrate	A half-life ($t_{1/2}$) temperature	Application	Reference
Aspergillus niger rAntan1	390.4 U/mL	6.0 3.0–8.0	Catechingallate	5.4 h at 60 °C 0.5 h at 70 °C	Green tea extraction	Shao et al. (2020)
Bacillus subtilis PAB2	2868.75 U/mg	5.0 3.0–8.0	Tamarind seed	4.5 h at 60 °C	Bacterial tannase production	Jana et al. (2013)
Penicillium atramentosum	34.7 U/mL 32.8 U/mL	7.5 5.5	Keekar leaves (3% w/v) Amla leaves (2% w/v)		–	Selwal and Selwal (2012)
Pestalotiopsis guepinii	98.6 U/mL	6.9 –	Pomegranate seeds		–	Reges de Sena et al. (2014)
Aspergillus niger Bde14	111.5 U/mL	7.0 –	Propyl gallate	–	–	Liu et al. (2018)
Aspergillus fumigates CAS21	28.5 U/mL (B-cyclodextrin)	5–6 –	2% tannic acid	40–60 °C (in the presence of B-cyclodextrin as an adjuvant)	Sorghum and leather effluent treatment Propyl gallate production	Cavalcanti et al. (2020)
Talaromyces verruculosus	32.18 U/gds	8.0 4–8	Acacia nilotica bark	60 °C	Fruit juice detannification	Aharwar and Parihar (2019)
Bacillus gottheilii M2S2	0.265 U/gds	– –	Triphala (11.532 g)	–	–	Selvaraj et al. (2019)
Aspergillus niger *Aspergillus fumigatus*	21.94 31.89	4.0 4.0	1% tannic acid	30 °C 30 °C	Industrial applications	Cavalcanti et al. (2017)

(continued)

Table 9.5 (continued)

Source	Activity	pH optimal stability	Substrate	A half-life ($t_{1/2}$) temperature	Application	Reference
Klebsiella pneumoniae	39.72	5.52 –	Indian gooseberry leaves	39.72 °C	–	Kumar et al. (2016)
Serratia marcescens	42 U/mL	– –	2% tannic acid	–	Antibacterial activity	Nsayef Muslim et al. (2017)
Bacillus gottheilii M2S2	49.32 U/L	4.74 –	4% tannic acid	–	Clarification of acidic beverages	
Aspergillus niger PN1	148.7 U/g 116.52 88.64	– –	Rice bran and spent coffee ground Coconut residue	–	–	Mansor et al. (2019)
Enterobacter cloacae	4 U/mL	6.0 –	Glucose	50 °C	–	Govindarajan et al. (2019)
Aspergillus UCP1284	12.26 U/g	5.5 –	Cashew bagasse	30 °C	Tannase production	Tatiana et al. (2016)
Aspergillus oryzae	139.22 IU/mL	4.89 –	Pomegranate rind, *Cassia auriculata* flower, black gram husk, and tea dust	34.91 °C	–	Varadharajan et al. (2017)

employed to enhance tannase activity and stability. The properties of the additive compounds have a great influence on the properties of the final enzyme powder, like glass transition temperature, density, particle size, and solubility. Therefore, the adjuvants should be chosen considerably (Cavalcanti et al., 2020). The immobilization method in multi-walled carbon nanotubes for tannase has increased tannase stability (Ong & Annuar, 2018). Apart from the processing method, statistical tools, mainly response surface methodology, have been considered for the optimization of tannase production to reduce the cost and the time (Bhoite & Murthy, 2015).

Five main tannase applications are tea processing, fruit juices clarification and de-bittering, animal feed treatment for nutritional improvement, gallic acid, and propyl gallate production used in food, leather, dye, and chemical industries, and wastewater treatment in agro-industries for decolorization and polyphenolic compound reduction (Aharwar & Parihar, 2018; Cao et al., 2019).

9.4 Conclusion

In this chapter, we have reviewed some of the main enzymes incorporated in food development, influencing the bioavailability and bioactivity of nutraceuticals. Worldwide companies have been established to do high-tech research and development of functional foods that have potentially positive effects on human health beyond basic nutrition. However, scientists should bear in mind that in vitro and in vivo experiments and clinical trials are essential steps that should be considered to guarantee any health claims based on the major factors limiting their bioaccessibility. According to traditional knowledge as well as scientific evidence, enzymes have a dominant role in food biotechnology for the development and commercialization of functional foods. Enzymes utilization in the production of bioactive metabolites and development of bioprocesses has shown an increasing trend. Several bioprocesses which involve enzymes have been established for the development of commercially important nutraceuticals. Yet, many traditional fermented foods have remained unexploited from technological aspects of functional foods and nutraceuticals development. Enzymes, such as L-asparaginase, chlorophyllase, beta-glucanase, L-glutaminae, lipase, and proteases are key enzymes in nutraceutical and functional food production.

Exploring for high-efficient and cost-effective technologies should be concerned in parallel with optimizing the experimental conditions to enhance the yield and physiological stability of these bioactive compounds. Recently, food and agricultural wastes have intensely attended as potential substrates to produce high-value products, namely free and immobilized enzymes. They represent ideal sources of proteins, lipids, and carbohydrates, and therefore, offer remarkable advantages from economic and eco-sustainability aspects. Nevertheless, the large-scale applicability, potential repeatability, and operation costs in real-industrial implementation are some of the challenges that need to be considered more systematically. Another emerging aspect of food enzyme application is the alteration of DNA encoding native enzymes for the development of genetically modified enzymes. Although the

safety of genetic engineering approaches for food processing is a severe concern, there have been promising results for the beneficial employment of genetically modified enzymes in the modern food industry.

More investigations should be done on the development of efficient and sustainable protocols, as well as enhanced properties such as pH and temperature stability, multifunctionality, surface property, catalytic efficiency, eco-friendly and economically efficient to achieve precise and sophisticated food matrixes. In addition, their impact on the resultant food characteristics such as sensory, rheological, physicochemical, and functional properties. All in all, there is a far distance to thoroughly comprehend the relationships between functional chemical compounds, and their dosage, stability, safety, cost, and delivery vehicles. In fact, further considerations should be taken into account for investigating the marketing standpoints of the functional food market based on the current scientific advancements in food science.

References

Adebiyi, A. P., et al. (2009). Purification and characterization of antioxidative peptides derived from rice bran protein hydrolysates. *European Food Research and Technology, 228*(4), 553–563.

Adefegha, S. A. (2018). Functional foods and nutraceuticals as dietary intervention in chronic diseases; Novel perspectives for health promotion and disease prevention. *Journal of Dietary Supplements, 15*(6), 977–1009.

Afarin, M., Alemzadeh, I., & Yazdi, Z. K. (2018). Conjugated linoleic acid production and optimization via catalytic reaction method using safflower oil. *International Journal of Engineering, Transactions B: Applications, 31*(8), 1166–1171.

Aharwar, A., & Parihar, D. K. (2018). Tannases: Production, properties, applications. *Biocatalysis and Agricultural Biotechnology, 15*, 322–334.

Aharwar, A., & Parihar, D. K. (2019). Talaromyces verruculosus tannase production, characterization and application in fruit juices detannification. *Biocatalysis and Agricultural Biotechnology, 18*, 101014.

Al Mijan, M., & Lim, B. O. (2018). Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends. *World Journal of Gastroenterology, 24*(25), 2673–2685.

Alam, S., et al. (2019) Recent development in the uses of asparaginase as food enzyme (pp. 55–81).

Alcalde, M., et al. (2006). Environmental biocatalysis: From remediation with enzymes to novel green processes. *Trends in Biotechnology, 24*(6), 281–287.

Alemzadeh, I., et al. (2020). Encapsulation of food components and bioactive ingredients and targeted release. *International Journal of Engineering, Transactions A: Basics, 33*(1), 1–11.

Alkhatib, A., et al. (2017). Functional foods and lifestyle approaches for diabetes prevention and management. *Nutrients, 9*(12), 1310.

Ando, A. (2004). Conjugated linoleic acid production from castor oil by Lactobacillus plantarum JCM 1551. *Enzyme and Microbial Technology, 35*, 40–45.

Arab, F., Alemzadeh, I., & Maghsoudi, V. (2011). Determination of antioxidant component and activity of rice bran extract. *Scientia Iranica, 18*(6), 1402–1406.

Arshad, R., et al. (2019). Optimized production of tannase and gallic acid from fruit seeds by solid state fermentation. *Tropical Journal of Pharmaceutical Research, 18*(5), 911–918.

Asselin, B., & Rizzari, C. (2015). Asparaginase pharmacokinetics and implications of therapeutic drug monitoring. *Leukemia and Lymphoma, 56*(8), 2273–2280.

Baskar, G., Aiswarya, R., & Renganathan, S. (2019). Applications of asparaginase in food processing. In *Green bio-processes* (pp. 83–98). Springer.

Batool, T., et al. (2016). A comprehensive review on L-asparaginase and its applications. *Applied Biochemistry and Biotechnology, 178*(5), 900–923.

Berin, M. C. (2015). Immunopathophysiology of food protein-induced enterocolitis syndrome. *Journal of Allergy and Clinical Immunology, 135*(5), 1108–1113.

Bhoite, R. N., & Murthy, P. S. (2015). Biodegradation of coffee pulp tannin by Penicillium verrucosum for production of tannase, statistical optimization and its application. *Food and Bioproducts Processing, 94*, 727–735.

Bornscheuer, U. T., et al. (2012). Engineering the third wave of biocatalysis. *Nature, 485*(7397), 185–194.

Boutrou, R., et al. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition, 97*(6), 1314–1323.

Brumano, L. P., et al. (2019). Development of L-asparaginase biobetters: Current research status and review of the desirable quality profiles. *Frontiers in Bioengineering and Biotechnology, 6*(January), 212.

Burton, S. G., Cowan, D. A., & Woodley, J. M. (2002). The search for the ideal biocatalyst. *Nature Biotechnology, 20*(1), 37–45. https://doi.org/10.1038/nbt0102-37

Cachumba, J. J. M., et al. (2016). Current applications and different approaches for microbial L-asparaginase production. *Brazilian Journal of Microbiology, 47*, 77–85.

Cao, Q. Q., et al. (2019). Improving the taste of autumn green tea with tannase. *Food Chemistry, 277*, 432–437.

Carle, R., & Schweiggert, R. (Eds.). (2016). *Handbook on natural pigments in food and beverages: Industrial applications for improving food color*. Woodhead Publishing.

Carvalho, C. C. C. R. (2017). Whole cell biocatalysts: Essential workers from nature to the industry. *Microbial Biotechnology, 10*(2), 250–263.

Cavalcanti, R. M. F., et al. (2017). Screening, selection and optimization of the culture conditions for tannase production by endophytic fungi isolated from Caatinga. *Journal of Applied Biology & Biotechnology, 5*(1), 1–9.

Cavalcanti, R. M. F., et al. (2020). Stabilization and application of spray-dried tannase from Aspergillus fumigatus CAS21 in the presence of different carriers. *3 Biotech, 10*(4), 1–14.

Chanderman, A., et al. (2016). Production, characteristics and applications of phytase from a rhizosphere isolated Enterobacter sp. ACSS. *Bioprocess and Biosystems Engineering, 39*(10), 1577–1587.

Chu, H. M., et al. (2004). Structures of Selenomonas ruminantium phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure, 12*(11), 2015–2024.

Chung, K. T., Wei, C. I., & Johnson, M. G. (1998). Are tannins a double-edged sword in biology and health? *Trends in Food Science and Technology, 9*(4), 168–175.

Cui, Y., et al. (2020). Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *International Journal of Molecular Sciences, 21*(3), 995.

De Leo, F., et al. (2009). Angiotensin converting enzyme (ACE) inhibitory peptides: Production and implementation of functional food. *Current Pharmaceutical Design, 15*(31), 3622–3643.

de Moura Sarquis, M. I., et al. (2004). Production of L-asparaginase by filamentous fungi. *Memórias do Instituto Oswaldo Cruz, 99*(5), 489–492.

de Paulo Farias, D., et al. (2019). Prebiotics: Trends in food, health and technological applications. *Trends in Food Science and Technology, 93*, 23–35.

Dersjant-Li, Y., et al. (2015). Phytase in non-ruminant animal nutrition: A critical review on phytase activities in the gastrointestinal tract and influencing factors. *Journal of the Science of Food and Agriculture, 95*(5), 878–896.

Dewapriya, P., & Kim, S. K. (2014). Marine microorganisms: An emerging avenue in modern nutraceuticals and functional foods. *Food Research International, 56*, 115–125.

Dewi, R. T. K., Mubarik, N. R., & Suhartono, M. T. (2016). Medium optimization of β-glucanase production by Bacillus subtilis SAHA 32.6 used as biological control of oil palm pathogen. *Emirates Journal of Food and Agriculture, 28*(2), 116–125.

Doriya, K., & Kumar, D. S. (2016). Isolation and screening of l-asparaginase free of glutaminase and urease from fungal sp. *3 Biotech, 6*(2), 239.

DSM. (2018). DSM introduces PreventASe® XR to extend use of enzymatic acrylamide-reduction solution to more snacks and baked goods. Retrieved from https://www.dsm.com/corporate/media/informationcenter-news/2018/07/2018-07-10-dsm-introduces-preventase-xr-to-extend-use-of-enzymatic-acrylamide-reduction-solution-to-more-snacks-and-baked-goods1.html

Ferruzzi, M. G., & Blakeslee, J. (2007). Digestion absorption and cancer preventative activity of dietary chlorophyll derivatives. *Nutrition Research, 27*(1), 1–12. https://doi.org/10.1016/j.nutres.2006.12.003

Filella, I., Serrano, L., Serra, J., & Peñuelas, J. (1995). Evaluating wheat nitrogen status with canopy reflectance indices and discriminant analysis. *Crop Science, 35*(5), 1400–1405. https://doi.org/10.2135/cropsci1995.0011183X003500050023x

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science, 72*(6), 1379–1400.

Gandul-Rojas, B., & Mínguez-Mosquera, M. I. (1996). Chlorophyllase activity in olive fruits and its relationship with the loss of chlorophyll pigments in the fruits and oils. *Journal of the Science of Food and Agriculture, 72*(3), 291–294.

García-Conesa, M. T., et al. (2001). Hydrolysis of diethyl diferulates by a tannase from Aspergillus oryzae. *Carbohydrate Polymers, 44*(4), 319–324.

Garrett, J. B., et al. (2004). Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. *Applied and Environmental Microbiology, 70*(5), 3041–3046.

Gavrilescu, M., & Chisti, Y. (2005). Biotechnology—A sustainable alternative for chemical industry. *Biotechnology Advances, 23*(7–8), 471–499.

Georgetti, S. R., et al. (2009). Enhanced in vitro and in vivo antioxidant activity and mobilization of free phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. *Journal of Applied Microbiology, 106*(2), 459–466.

Giese, E. C., Dekker, R. F. H., Barbosa, A., & da Silva, M. (2012). Production of β-(1,3)-glucanases by Trichoderma harzianum Rifai: Optimization and application to produce gluco-oligosaccharides from paramylon and pustulan. *Fermentation Technology, 1*(1), 1–5.

Gligor, O., et al. (2019). Enzyme-assisted extractions of polyphenols—A comprehensive review. *Trends in Food Science and Technology, 88*, 302–315.

Global Medical Asparaginase Sales Market Report 2018. (2018). Retrieved from https://www.themarketreports.com/report/global-medical-asparaginase-sales-market-report-2018

Gofferjé, G., et al. (2014). Kinetics of enzymatic esterification of glycerol and free fatty acids in crude Jatropha oil by immobilized lipase from Rhizomucor miehei. *Journal of Molecular Catalysis B: Enzymatic, 107*, 1–7.

Govindarajan, R. K., et al. (2019). Purification, structural characterization and biotechnological potential of tannase enzyme produced by Enterobacter cloacae strain 41. *Process Biochemistry, 77*, 37–47.

Granato, D., et al. (2020). Functional foods: Product development, technological trends, efficacy testing, and safety. *Annual Review of Food Science and Technology, 11*(1), 93–118.

Greiner, R., & Konietzny, U. (2006). Phytase for food application. *Food Technology and Biotechnology, 44*(2), 125–140.

Grom, L. C., et al. (2020). Probiotic dairy foods and postprandial glycemia: A mini-review. *Trends in Food Science and Technology, 101*, 165–171.

Guan, H., et al. (2018). The enzymatic hydrolysis of soy protein isolate by Corolase PP under high hydrostatic pressure and its effect on bioactivity and characteristics of hydrolysates. *Food Chemistry, 245*, 89–96.

Gul, K., Singh, A. K., & Jabeen, R. (2016). Nutraceuticals and functional foods: The foods for the future world. *Critical Reviews in Food Science and Nutrition, 56*(16), 2617–2627.

Handa, V., et al. (2020). Biotechnological applications of microbial phytase and phytic acid in food and feed industries. *Biocatalysis and Agricultural Biotechnology, 25*, 101600.

Haskell, C. F., Kennedy, D. O., Milne, A. L., Wesnes, K. A., & Scholey, A. B. (2008). The effects of L-theanine caffeine and their combination on cognition and mood. *Biological Psychology, 77*(2), 113–122. https://doi.org/10.1016/j.biopsycho.2007.09.008

Hendriksen, H. V., Puder, K., & Goldbech Olsen, A. (2016). *Method for reducing the level of asparagine in a food material*. U.S. Patent Application 14/781,760.

Hennessy, A. A., et al. (2012). The production of conjugated α-linolenic, γ-linolenic and stearidonic acids by strains of bifidobacteria and propionibacteria. *Lipids, 47*(3), 313–327.

Holm, L., et al. (2017). Contraction mode and whey protein intake affect the synthesis rate of intramuscular connective tissue. *Muscle and Nerve, 55*(1), 128–130.

Hosikian, A., Lim, S., Halim, R., & Danquah, M. K. (2010). Chlorophyll extraction from microalgae: A review on the process engineering aspects. *International Journal of Chemical Engineering*, 1–11. https://doi.org/10.1155/2010/391632

Hörtensteiner, S., & Kräutler, B. (2011). Chlorophyll breakdown in higher plants. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1807*(8), 977–988. https://doi.org/10.1016/j.bbabio.2010.12.007

Hu, X., et al. (2015). Reexamination of chlorophyllase function implies its involvement in defense against chewing herbivores. *Plant Physiology, 167*(3), 660–670.

Huang, H., et al. (2006). A novel phytase with preferable characteristics from Yersinia intermedia. *Biochemical and Biophysical Research Communications, 350*(4), 884–889.

Ire, F. S., & Nwanguma, A. C. (2020). Comparative evaluation on tannase production by Lasiodiplodia plurivora ACN-10 under submerged fermentation (SmF) and solid state fermentation (SSF). *Asian Journal of Biotechnology and Bioresource Technology*, 39–49. https://doi.org/10.9734/ajb2t/2020/v6i130074

Jahadi, M., & Khosravi-Darani, K. (2017). Liposomal encapsulation enzymes: From medical applications to kinetic characteristics. *Mini-Reviews in Medicinal Chemistry, 17*, 366–370.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., Mozafar, M. R., Saboury, A. A., & Pourhosseini, P. S. (2015). The encapsulation of flavourzyme in nanoliposome by heating method. *Journal of Food Science and Technology, 52*(4), 2063–2072.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., Mozafari, M. R., Saboury, A. A., Zoghi, A., & Mohammadi, M. (2016). Modeling of proteolysis in Iranian brined cheese using proteinase-loaded nanoliposome. *International Journal of Dairy Technology, 69*(1), 57–62.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., et al. (2012). Evaluating the effects of process variables on protease-loaded nano-liposome production by Plackett-Burman design for utilizing in cheese ripening acceleration. *Asian Journal of Chemistry, 24*(9), 3891–3894.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., et al. (2020). Accelerating ripening of Iranian white brined cheesesusing liposome-encapsulated and free proteinases. *Biointerface Research in Applied Chemistry, 10*(1), 4966–4971.

Jahan-Mihan, A., et al. (2011). Dietary proteins as determinants of metabolic and physiologic functions of the gastrointestinal tract. *Nutrients, 3*(5), 574–603.

Jana, A., et al. (2013). Structural characterization of thermostable, solvent tolerant, cytosafe tannase from *Bacillus subtilis* PAB2. *Biochemical Engineering Journal, 77*, 161–170.

JECFA. (2002). Evaluation of certain food additives. Fifty-ninth report of the joint FAO/WHO expert committee on food additives. World Health Organization technical report series, 913.

Jha, S. K., et al. (2012). Microbial L-asparaginase: A review on current scenario and future prospects. *International Journal of Pharmaceutical Sciences and Research, 3*(9), 3076–3090.

Jiang, J., Björck, L., & Fondén, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *Journal of Applied Microbiology, 85*(1), 95–102.

Joshi, S., & Satyanarayana, T. (2017). Characteristics and multifarious potential applications of HAP phytase of the unconventional yeast Pichia anomala. In *Developments in fungal biology and applied mycology* (pp. 265–278). Springer.

Kaur, R. (2017). Production and characterization of a neutral phytase of *Penicillium oxalicum* EUFR-3 isolated from Himalayan region. *Nusantara Bioscience, 9*(1), 68–76.

Khanbabaee, K., & van Ree, T. (2001). Tannins: Classification and definition. *Natural Product Reports, 18*(6), 641–649.

Kim, S. H., et al. (2007). Cloning and expression of a full-length glutamate decarboxylase gene from *Lactobacillus brevis* BH2. *Biotechnology and Bioprocess Engineering, 12*(6), 707–712.

Konietzny, U., & Greiner, R. (2004). Bacterial phytase: Potential application, in vivo function and regulation of its synthesis. *Brazilian Journal of Microbiology, 35*(1–2), 11–18.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945–960.

Koseki, T., et al. (2018). Characterization of a novel *Aspergillus oryzae* tannase expressed in Pichia pastoris. *Journal of Bioscience and Bioengineering, 126*(5), 553–558.

Kouchak Yazdi, Z., & Alemzadeh, I. (2016a). Lipase catalyzed incorporation of conjugated linoleic acid by transesterification into sunflower oil applying immobilized lipase (lipozyme thermomyces lanuginosus and rhizomucor mehei). *International Journal of Engineering, Transactions A: Basics, 29*(4), 436–443.

Kouchak Yazdi, Z., & Alemzadeh, I. (2016b). Lipase catalyzed incorporation of conjugated linoleic acid by transesterification into sunflower oil applying immobilized lipase (Lipozyme TL im and im RM). *International Journal of Engineering, Transactions A: Basics, 29*(4), 549–556.

Kouchak Yazdi, Z., Alemzadeh, I., & Vossoughi, M. (2017). Comparison and optimization of conjugated linoleic acid production by *Lactobacillus plantarum* and *Lactobacillus plantarum* subsp. plantarum. *Scientia Iranica, 24*(3), 1272–1280.

Kuddus, M. (2018). *Enzymes in Food Biotechnology: Production, Applications, and Future Prospects*. Academic Press.

Kumar, D., et al. (2020). Barley grain beta glucan enrichment: Status and opportunities. In *Wheat and barley grain biofortification* (pp. 295–308). Woodhead Publishing.

Kumar, M., et al. (2016). Improved production of tannase by *Klebsiella pneumoniae* using Indian gooseberry leaves under submerged fermentation using Taguchi approach. *AMB Express, 6*(1), 46.

Lee, S. O., et al. (2003). Bioconversion of linoleic acid into conjugated linoleic acid during fermentation and by washed cells of *Lactobacillus reuteri*. *Biotechnology Letters, 25*(12), 935–938.

Lin, T. Y. (2006). Conjugated linoleic acid production by cells and enzyme extract of *Lactobacillus delbrueckii* ssp. *bulgaricus* with additions of different fatty acids. *Food Chemistry, 94*(3), 437–441.

Liu, F., et al. (2018). High level expression and characterization of tannase tan7 using *Aspergillus niger* SH-2 with low-background endogenous secretory proteins as the host. *Protein Expression and Purification, 144*, 71–75.

Lopez-Casado, G., et al. (2008). Plant glycosyl hydrolases and biofuels: A natural marriage. *Current Opinion in Plant Biology, 11*(3), 329–337.

Mansor, A., et al. (2019). Evaluation of selected agri-industrial residues as potential substrates for enhanced tannase production via solid-state fermentation. *Biocatalysis and Agricultural Biotechnology, 20*, 101216.

Maqsood, S., et al. (2020). Bioactive compounds from date fruit and seed as potential nutraceutical and functional food ingredients. *Food Chemistry, 308*, 125522.

Maurya, A. K., Parashar, D., & Satyanarayana, T. (2017). Bioprocess for the production of recombinant HAP phytase of the thermophilic mold Sporotrichum thermophile and its structural and biochemical characteristics. *International Journal of Biological Macromolecules, 94*, 36–44.

Meinlschmidt, P., Ueberham, E., Lehmann, J., Reineke, K., Schlüter, O., Schweiggert-Weisz, U., & Eisner, P. (2016). The effects of pulsed ultraviolet light cold atmospheric pressure plasma and gamma-irradiation on the immunoreactivity of soy protein isolate. *Innovative Food Science & Emerging Technologies, 38*, 374–383. https://doi.org/10.1016/j.ifset.2016.06.007

Melini, F., et al. (2019). Health-promoting components in fermented foods: An up-to-date systematic review. *Nutrients, 11*(5), 1189.

Mitchell, L., et al. (1994). Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: Risk of thrombotic complications in L-sparaginase-induced antithrombin III deficiency. *Blood, 83*(2), 386–391.

Mohammadi, R., Mahmoudzade, M., Atefi, M., Khosravi-Darani, K., & Mozafari, M. R. (2015). Applications of nanoliposomes in cheese technology. *International Journal of Dairy Technology, 68*(1), 11–23.

Mohanty, D. P., Mohapatra, S., Misra, S., & Sahu, P. S. (2016). Milk derived bioactive peptides and their impact on human health—A review. *Saudi Journal of Biological Sciences, 23*, 577–583.

Momeni, V., Alemzadeh, I., & Vosoughi, M. (2015). Enhancement of L-asparaginase production by candida utilis in a 13L fermenter and its purification. *International Journal of Engineering, Transactions B: Applications, 28*(8), 1133–1141.

Niezgoda, N., & Gliszczyńska, A. (2019). Lipase catalyzed acidolysis for efficient synthesis of phospholipids enriched with isomerically pure cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid. *Catalysts, 9*(12), 1012.

Nsayef Muslim, D. S., Abbas Dham, Z., & Mohammed, D. N. J. (2017). Synthesis and characterization of nanoparticles conjugated tannase and using it for enhancement of antibacterial activity of tannase produced by *Serratia marcescens*. *Microbial Pathogenesis, 110*, 484–493.

Ogawa, J., et al. (2005). Production of conjugated fatty acids by lactic acid bacteria. *Journal of Bioscience and Bioengineering, 100*(4), 355–364.

Ong, C. B., & Annuar, M. S. M. (2018). Immobilization of cross-linked tannase enzyme on multiwalled carbon nanotubes and its catalytic behavior. *Preparative Biochemistry and Biotechnology, 48*(2), 181–187.

Paiva, A. L., Balcão, V. M., & Malcata, F. X. (2000). Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme and Microbial Technology, 27*(3–5), 187–204.

Panda, R., et al. (2015). Enzymatic hydrolysis does not reduce the biological reactivity of soybean proteins for all allergic subjects. *Journal of Agricultural and Food Chemistry, 63*(43), 9629–9639.

Pandey, A., et al. (1999). Fermentation for the production of industrial enzymes. *Fermentation Science and Technology, 77*(1), 1–14.

Panesar, P. S., Kaur, R., Singla, G., & Sangwan, R. S. (2016). Bioprocessing of agro-industrial wastes for production of foodgrade enzymes: Progress and prospects. *Applied Food Biotechnology, 3*(4), 208–227.

Park, S. J., et al. (2013). Synthesis of nylon 4 from gamma-aminobutyrate (GABA) produced by recombinant *Escherichia coli*. *Bioprocess and Biosystems Engineering, 36*(7), 885–892.

Patel, S., & Rauf, A. (2017). Edible seeds from Cucurbitaceae family as potential functional foods: Immense promises, few concerns. *Biomedicine and Pharmacotherapy, 91*, 330–337.

Patro, K. R., et al. (2014). Development of new medium composition for enhanced production of L-asparaginase by *Aspergillus flavus*. *Journal of Environmental Biology, 35*(1), 295–300.

Pedreschi, F., et al. (2011). Acrylamide reduction in potato chips by using commercial asparaginase in combination with conventional blanching. *LWT—Food Science and Technology, 44*(6), 1473–1476.

Pham, V. D., et al. (2016). Engineering the intracellular metabolism of *Escherichia coli* to produce gamma-aminobutyric acid by co-localization of GABA shunt enzymes. *Biotechnology Letters, 38*(2), 321–327.

Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. (2009). Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal, 19*, 643–654.

Philippaerts, A., Van Aelst, J., & Sels, B. (2013). Conjugated linoleic acids and conjugated vegetable oils: From nutraceutical to bio-polymer. *European Journal of Lipid Science and Technology, 115*(7), 717–720.

Pires, E. B. E., et al. (2019). Production of fungal phytases from agroindustrial byproducts for pig diets. *Scientific Reports, 9*(1), 1–9.

Pokora, M., et al. (2013). Biological and functional properties of proteolytic enzyme-modified egg protein by-products. *Food Science & Nutrition, 1*(2), 184–195.

Poli, A., et al. (2018). Nutraceuticals and functional foods for the control of plasma cholesterol levels. An intersociety position paper. *Pharmacological Research, 134*, 51–60.

Posovszky, C. (2016). Congenital intestinal diarrhoeal diseases: A diagnostic and therapeutic challenge. *Best Practice and Research: Clinical Gastroenterology, 30*(2), 187–211.

Puppala, K. R., et al. (2019). Characterization of novel acidic and thermostable phytase secreting *Streptomyces* sp. (NCIM 5533) for plant growth promoting characteristics. *Biocatalysis and Agricultural Biotechnology, 18*, 101020.

Rai, A. K., Pandey, A., & Sahoo, D. (2019). Biotechnological potential of yeasts in functional food industry. *Trends in Food Science and Technology, 83*, 129–137.

Reges de Sena, A., et al. (2014). Production, characterization and application of a thermostable tannase from *Pestalotiopsis guepinii* URM 7114. *Food Technology and Biotechnology, 52*(4), 459–467.

Rittig, N., et al. (2017). Anabolic effects of leucine-rich whey protein, carbohydrate, and soy protein with and without β-hydroxy-β-methylbutyrate (HMB) during fasting-induced catabolism: A human randomized crossover trial. *Clinical Nutrition, 36*(3), 697–705.

Rizzari, C., et al. (2000). L-asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving i.m. or i.v. *Erwinia C.* or *E. coli* L-asparaginase as first exposure. *Annals of Oncology, 11*(2), 189–193.

Rizzello, C. G., De Angelis, M., Di Cagno, R., Camarca, A., Silano, M., Losito, I., De Vincenzi, M., De Bari, M. D., Palmisano, F., Maurano, F., Gianfrani, C., & Gobbetti, M. (2007). Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: New perspectives for celiac disease. *Applied and Environmental Microbiology, 73*(14), 4499–4507. https://doi.org/10.1128/AEM.00260-07

Rodriguez, E., et al. (2000). Site-directed mutagenesis improves catalytic efficiency and thermostability of *Escherichia coli* pH 2.5 acid phosphatase/phytase expressed in *Pichia pastoris*. *Archives of Biochemistry and Biophysics, 382*(1), 105–112.

Rodríguez-Mendoza, J., et al. (2019). Purification and biochemical characterization of a novel thermophilic exo-β-1,3-glucanase from the thermophile biomass-degrading fungus *Thielavia terrestris* Co3Bag1. *Electronic Journal of Biotechnology, 41*, 60–71.

Ruiz-Herrera, J., & Ortiz-Castellanos, L. (2019). Cell wall glucans of fungi. A review. *Cell Surface, 5*, 100022.

Sahib, N. G., et al. (2013). Coriander (*Coriandrum sativum* L.): A potential source of high-value components for functional foods and nutraceuticals—A review. *Phytotherapy Research, 27*(10), 1439–1456.

Sakhaei, M., & Alemzadeh, I. (2017). Enzymatic synthesis of theanine in the presence of L-glutaminase produced by *Trichoderma koningii*. *Applied Food Biotechnology, 4*(2), 113–121.

Sato, H., et al. (2012). Inhibitory effects of water-soluble low-molecular-weight β-(1,3-1,6) D-glucan isolated from *aureobasidium pullulans* 1A1 strain black yeast on mast cell degranulation and passive cutaneous anaphylaxis. *Bioscience, Biotechnology and Biochemistry, 76*(1), 84–88.

Saxena, A., et al. (2020). Characteristics of an acidic phytase from *Aspergillus aculeatus* APF1 for dephytinization of biofortified wheat genotypes. *Applied Biochemistry and Biotechnology, 191*(2), 679–694.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., & Hörtensteiner, S. (2009). *The Plant Cell, 21*(3), 767–785. https://doi.org/10.1105/tpc.108.064089

Selvaraj, S., et al. (2019). Modeling and optimization of tannase production with *Triphala* in packed bed reactor by response surface methodology, genetic algorithm, and artificial neural network. *3 Biotech, 9*(7), 259.

Selwal, M. K., & Selwal, K. K. (2012). High-level tannase production by *Penicillium atramentosum* KM using agro residues under submerged fermentation. *Annals of Microbiology, 62*(1), 139–148.

Shah, P. C., et al. (2017). Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. *AMB Express, 7*(1), 1–11.
Shao, Y., et al. (2020). Thermostable tannase from *aspergillus Niger* and its application in the enzymatic extraction of green tea. *Molecules, 25*(4), 952.
Sharafi, E., Dehestani, A., & Farmani, J. (2017). Bioinformatics evaluation of plant chlorophyllase, the key enzyme in chlorophyll degradation*, 4*(3), 167–678.
Shi, R., et al. (2017). Biochemical characterization of a novel L-asparaginase from *Paenibacillus barengoltzii* being suitable for acrylamide reduction in potato chips and mooncakes. *International Journal of Biological Macromolecules, 96*, 93–99.
Shoji, T., et al. (2006). Apple procyanidin oligomers absorption in rats after oral administration: Analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 54*(3), 884–892.
Sicherer, S. H., & Sampson, H. A. (2014). Food allergy: Epidemiology pathogenesis diagnosis and treatment. *Journal of Allergy and Clinical Immunology, 133*(2), 291–307.e5. https://doi.org/10.1016/j.jaci.2013.11.020
Sirtori, C. R., et al. (2017). Nutraceutical approaches to metabolic syndrome. *Annals of Medicine, 49*(8), 678–697.
Song, H. Y., El Sheikha, A. F., & Hu, D. M. (2019). The positive impacts of microbial phytase on its nutritional applications. *Trends in Food Science and Technology, 86*, 553–562.
Srivastava, A., & Kar, R. (2009). Characterization and application of tannase produced by *Aspergillus niger* ITCC 6514.07 on pomegranate rind. *Brazilian Journal of Microbiology, 40*(4), 782–789.
Suleria, H. A. R., et al. (2015). Marine-based nutraceuticals: An innovative trend in the food and supplement industries. *Marine Drugs, 13*(10), 6336–6351.
Suresh, S., & Radha, K. V. (2016). Statistical optimization and mutagenesis for high level of phytase production by *Rhizopus oligosporus* MTCC 556 under solid state fermentation. *Journal of Environmental Biology, 37*(2), 253–259.
Sutivisedsak, N., et al. (2013). Novel sources of β-glucanase for the enzymatic degradation of schizophyllan. *Enzyme and Microbial Technology, 52*(3), 203–210.
Takahashi, C., et al. (2012). Robust production of gamma-amino butyric acid using recombinant *Corynebacterium glutamicum* expressing glutamate decarboxylase from *Escherichia coli*. *Enzyme and Microbial Technology, 51*(3), 171–176.
Tanna, B., & Mishra, A. (2018). Metabolites unravel nutraceutical potential of edible seaweeds: An emerging source of functional food. *Comprehensive Reviews in Food Science and Food Safety, 17*(6), 1613–1624.
Tarahomjoo, S., & Alemzadeh, I. (2003). Surfactant production by an enzymatic method. *Enzyme and Microbial Technology, 33*(1), 33–37.
Tatiana, P. S. L. L., et al. (2016). Tannase production by *Aspergillus* spp. UCP1284 using cashew bagasse under solid state fermentation. *African Journal of Microbiology Research, 10*(16), 565–571.
Tavano, O. L., et al. (2018). Biotechnological applications of proteases in food technology. *Comprehensive Reviews in Food Science and Food Safety, 17*(2), 412–436.
Tian, M., & Yuan, Q. (2016). Optimization of phytase production from potato waste using *Aspergillus ficuum*. *3 Biotech, 6*(2), 256.
Torang, A., & Alemzadeh, I. (2016). Acrylamide reduction in potato crisps using: Asparaginase from *Candida utilis*, commercial asparaginase, salt immersion, and pH treatment. *International Journal of Engineering, 29*(7), 879–886.
Vafabakhsh, Z., Khosravi-Darani, K., Khajeh, K., Jahadi, M., Komeili, R., & Mortazavian, A. M. (2013). Stability and catalytic kinetics of protease loaded liposomes. *Biochemical Engineering Journal, 72*, 11–17.

Varadharajan, V., et al. (2017). Modeling and verification of process parameters for the production of tannase by *Aspergillus oryzae* under submerged fermentation using agro-wastes. *Biotechnology and Applied Biochemistry, 64*(1), 100–109.

Vaziri, A. S., Alemzadeh, I., & Vossoughi, M. (2019). Survivability and oxidative stability of co-microencapsulated *L. plantarum* PTCC 1058 and DHA as a juice carrier. *Food Bioscience, 32*, 100460.

Venkatakrishnan, K., Chiu, H. F., & Wang, C. K. (2020). Impact of functional foods and nutraceuticals on high blood pressure with a special focus on meta-analysis: Review from a public health perspective. *Food and Function, 11*(4), 2792–2804.

Vidal, A. R., et al. (2018). Effects of enzymatic hydrolysis (Flavourzyme®) assisted by ultrasound in the structural and functional properties of hydrolyzates from different bovine collagens. *Food Science and Technology, 38*, 103–108.

Wu, Q., et al. (2018). Isolation of β-1,3-glucanase-producing microorganisms from poria cocos cultivation soil via molecular biology. *Molecules, 23*(7), 1555.

Xu, F., Oruna-Concha, M. J., & Elmore, J. S. (2016). The use of asparaginase to reduce acrylamide levels in cooked food. *Food Chemistry, 210*, 163–171.

Xu, N., Wei, L., & Liu, J. (2017). Biotechnological advances and perspectives of gamma-aminobutyric acid production. *World Journal of Microbiology and Biotechnology, 33*(3), 64.

Yang, L., et al. (2002). Production of conjugated linoleic acids through KOH-catalyzed dehydration of ricinoleic acid. *Chemistry and Physics of Lipids, 119*(1–2), 23–31.

Yazdi, Z. K., & Alemzadeh, I. (2017). Kinetic mechanism of conjugated linoleic acid esterification and production of enriched glycerides as functional oil. *Canadian Journal of Chemical Engineering, 95*(11), 2078–2086.

You, S., et al. (2016). Improvement of the thermostability and catalytic efficiency of a highly active β-glucanase from *Talaromyces leycettanus* JCM12802 by optimizing residual charge-charge interactions. *Biotechnology for Biofuels, 9*(1), 1–12.

Yuan, J., Jiang, B., Li, K., Shen, W., & Tang, J. L. (2017). Beneficial effects of protein hydrolysates in exercise and sports nutrition. *Journal of Biological Regulators and Homeostatic Agents, 31*(1), 183–188.

Zhao, H. W., Lv, J. P., & Li, S. R. (2011). Production of conjugated linoleic acid by whole-cell of *Lactobacillus plantarum* A6-1F. *Biotechnology and Biotechnological Equipment, 25*(1), 2266–2272.

Zoghi, A., Khosravi-Darani, K., & Omri, H. (2018). Process variables and design of experiments in liposome and nanoliposome research. *Mini-Reviews in Medicinal Chemistry, 18*(4), 324–344.

Zuo, S., et al. (2015). Recent research progress on microbial l-asparaginases. *Applied Microbiology and Biotechnology, 99*(3), 1069–1079.

Enzymes as Active Packaging System 10

Amir Heydari and Negin Mahmoodi-Babolan

Abstract

Standard food packaging is the first step that has a vital role in protecting the product from moisture, water vapor, odors, gases, dust, microorganisms, vibrations, shocks, and pressure force, which preserved the freshness, quantity, and quality of the packaging to reach the customer. Active and smart packaging approaches enhance the communication and protective functions of food packaging to fulfill the demands of consumers for high-quality, safe, and convenient food products. Recently with the technology expansion, novel enzymes with extended applications and specificity have been advanced, and new solicitation areas are still being investigated. This chapter has focused on oxygen-reducing enzymes in food-packaging material, and intelligent packaging technologies are reviewed. The main lines are active packaging materials, enzymes as catalysts in active scavenging systems, active releasing systems, antimicrobial packaging, time–temperature indicators, removal of undesirable food components, oxygen-sensing applications, and the effect of the coating process and composition on the enzyme activity. Also, enzymatic reactions with a brief discussion investigated and explained how enzymes are used in advanced smart and food-packaging systems. Eventually, a comprehensive list of enzymes used in food smart packaging and their application are presented.

Keywords

Enzyme · Intelligent and active packaging · Antimicrobial packaging · Shelf life

A. Heydari (✉) · N. Mahmoodi-Babolan
Chemical Engineering Department, University of Mohaghegh Ardabili, Ardabil, Iran
e-mail: Heydari@uma.ac.ir

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_10

10.1 Food Packaging

Food packaging is necessary to keep the safety and quality of the foods. Excellent and well-designed packaging protects the product from external and natural agencies that may cause damage to the quantity and quality of the product, tampering resistance, contamination and spoilage, physical and chemical damage. It can maintain the freshness of the food. For making products more applicable and favorable used to reclosable openings, dispensing caps, sprays, and other features. Packaging in a suitable method assists transportation of the packaged goods with easiness and reduces marketing costs (Robertson, 2016). Good packaging has less handling losses and is easy to be handled by one person during loading; therefore, characteristics of packaging cusses that marketing costs to be less (Han, 2003).

10.2 Food Package Properties

According to the degree of protection, food can influence maximum moisture gain or oxygen uptake. Different packaging materials such as metal cans and glass containers can be regarded as nearly impermeable to the passage of odors, gases, and water vapor, although in comparison packaging materials based on the paper can be considered penetrable. Also, packaging materials based on the plastics supply varying degrees of protection, and be contingent mainly on the nature of the polymers utilized in their manufacture.

10.3 Shelf Life

For many foods and drinks in which quality declines with time, it pursues that there will be a limited length of time. Shelf life is when the product becomes undesirable, suitable, and safe to use in specified storage conditions (Labuza, 1982). Shelf life is a usual scuffle for food companies. Manufacturers and producers want foodstuffs to stay fresher to lengthen the time to sell or custom. Therefore, researchers are believed to improve shelf life to give foods and drinks a better fortuity at high quality when consumers are prepared to eat them (Labuza & Schmidl, 1985). Provisions to enhance food product safety and quality, increase, or stabilize food quickly become market demands (Labuza, 1982).

10.4 Active Packaging Systems

Active packaging, smart packaging, and intelligent packaging denote packaging systems utilized with products, such as foods (fruit and vegetables, fish, and seafood products), pharmaceuticals, and several other kinds of products (Ozdemir & Floros, 2004). Active packaging refers to the embedding of special additives into the package to expand or maintain the quality. Active packaging effectively increases

the shelf life of foods by Realini and Marcos (2014). In active packaging, different additives may be used based on the packaging film and packaged food (Lorenzo et al., 2014). Active packaging contains ingredients that can absorb carbon dioxide, releasing oxygen, ethanol emitters, ethylene scavengers antioxidants, and other preservatives (Conte et al., 2007).

Active packaging systems supply various solutions to be controlled by quality attributes that are to be protected. This theme came up, for example, in discussions of decelerating oxidation food products be considered, so the packaging must utilize an active system that includes an oxygen scavenger or antioxidants. Also, if the decay of the product is created by condensation or moisture, the packaging can include a moisture absorber. Eventually, the motivation for the organization of any novel technology is cost.

It should point out that the unique decay mechanism of different foods should be considered previously designing the packaging. The reality, when we have active packaging that it carries out some role other than supplying an inert obstacle to the exterior environment. On the other hand, active packaging is identified as a system in which the product, package, and environment interact in a positive method to expand the shelf life or to obtain some properties (Conte et al., 2007).

The shelf life of packaged food is reliant on abundant features such as the food properties or product-intrinsic (e.g., pH, product color, nutrient content, aroma, texture, water activity, viscosity, biological structure and breathing rate, the existence of antimicrobial compounds) and product-extrinsic or related to the packaging, receptacle, or external environment factors (e.g., visual, and tactile properties of food packaging or dishware, smell, surroundings lighting, temperature and aroma, storage temperature, relative humidity, and the ambient gaseous composition) (Lorenzo et al., 2014).

These features will straight affect the physical, chemical, biochemical, and microbiological deterioration of products and their shelf lives.

Active packaging aims to improve the maintenance of food in the package, and delaying shelf life involves the application of various plans like oxygen removal, temperature control, moisture control, besides chemicals, for example, sugar, salt, carbon dioxide, or natural acids or a blend of these with active packaging.

These developments in active packaging have caused developments in numerous fields, including controlled respiration rate in agricultural products, postponed oxidation in muscle foods, microbial growth, and moisture movement to dried products. Furthermore, active packaging similarly employs choosiness to modify the atmospheric concentration of gaseous combinations interior of the package by coating, lamination, micro-penetration, or polymer blending (Realini & Marcos, 2014).

10.4.1 Types of Food Active Packaging Systems

The active packaging can be utilized in various forms involved as shown in Fig. 10.1.

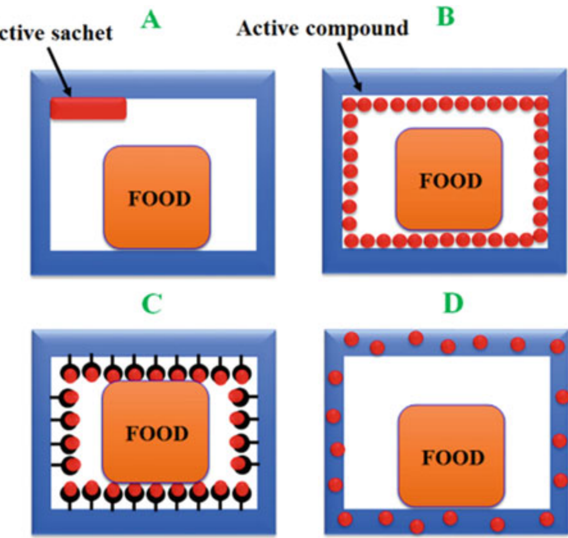


Fig. 10.1 Different types of food active packaging systems (**a**) using the active pouches and pads placed inside the packaging, (**b**) active compound coated onto the polymer, (**c**) blending of the active compound on the packaging surface, and (**d**) straight incorporated into the package structure (Almasi et al., 2020)

10.4.2 Active Compound Release Mechanisms

The direct incorporation of active combinations into the polymer matrix of the packaging system is the most regular kind of active packaging system. In this form of the active packaging system, polymer and the active agent form a composite matrix. In recent years, this type of active packaging system is the most frequently considered for food active packaging systems. There are three types of release mechanisms suggested for directly incorporated food active packaging systems.

1. Diffusion due to release: In this type of release, the active agent diffuses through the macro-porous or micro-porous polymer matrix structure and is moved from the surface of the film into the food. This mechanism is usual for releasing active composites for water-resistant and synthetic polymers. The rate of this kind of release is depended on polymer chemistry, permeability, and porosity.
2. Swelling due to release: low diffusion coefficient causes the incorporated active agent unable to penetrate the polymer matrix. When polymer swell, the diffusion coefficient of the active agent increases; therefore, it penetrates out. Since most foods have moisture and the water is the most popular penetrant, this type of release commonly happens in packing materials moisture-sensitive such as polysaccharides or protein-based films.
3. Disintegration due to release: Deformation of polymer, cleavage, and degradation are the main reasons for this kind of release. Absorption of liquids commonly from aqueous fluids through the polymer matrix influences degradation and deformation rate.

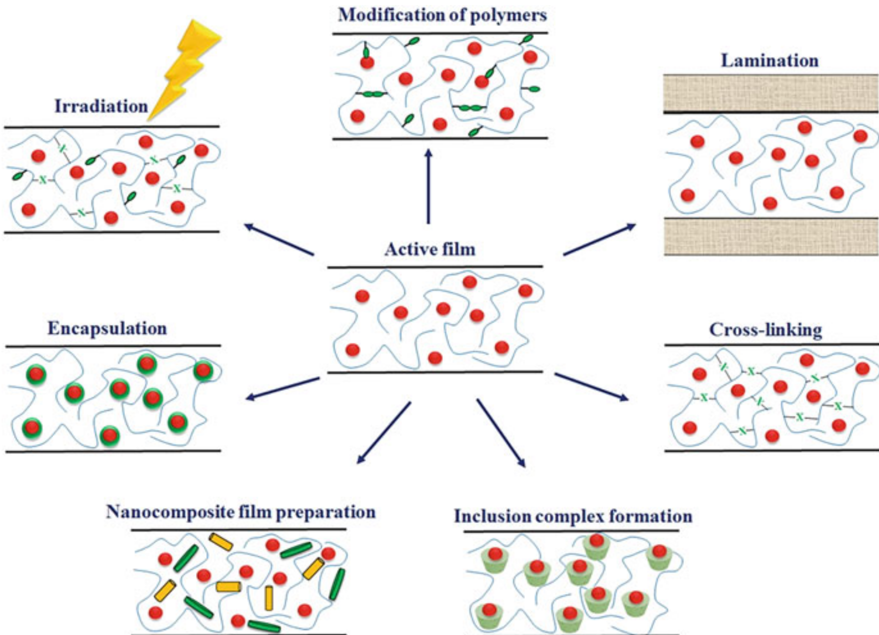


Fig. 10.2 Schematic illustration of different approaches used for release controlling from food active packaging (Almasi et al., 2020)

This kind of release happens in both nonbiodegradable and biodegradable polymers. Several approaches have been suggested to obtain a more controlled diffusion of the active agent from the packaging material. Figure 10.2 summarizes these approaches by a schematic representation.

10.5 Antimicrobial Packing

Antimicrobial packing is a type of active packaging that use some agents which kill or inhibit the growth of microorganisms that may be existing in the packaged product or packaging material itself. For control of unfavorable microorganisms on foodstuffs, antimicrobial materials can be combined and coated inside food-packaging materials (Motta et al., 2004).

For example, antimicrobial polymers provide a good antimicrobial plan for combating pathogens and have already drawn the attention of industrial and academic research (Prazak et al., 2002). Antimicrobial mechanisms demonstrate either active or passive action and polymer material categories, including bound or leaching antimicrobials used. Antimicrobial polymers are used in some industries, such as medical, food, and textile (Motta et al., 2004). Compounding polymers and

their blends with antimicrobial compounds or preservative agents reveal a new approach for designing antimicrobial packing films.

10.6 Enzyme

Enzymes are macromolecular biological catalysts (typically proteins), which significantly are available in all living organisms, which cause reactions to happen naturally, and can be seen in open self-organizing life (Liu et al., 2014). The first feature is its catalytic factor. Enzymes permit living systems to perform reactions that would happen so slowly. Enzymes accelerate (catalyze) chemical reactions; in several cases, enzymes can make a chemical reaction so much faster than it would have been without it (Wierschem et al., 2017).

Enzymes catalyze biochemical reactions with different pathways (Narayanan et al., 2009). A substrate is named chemical species observed in a chemical reaction and reacts with a reagent to create a product. It can also be called for the surface that other chemical reactions are played, or achieved a supporting role in microscopic and spectroscopic techniques. The substrate and enzyme schematic are shown in Fig. 10.3.

Nonenzymatic or chemical reactions are more slower than enzymatic reactions (Brody & Budny, 1995).

The second characteristic of enzymes that makes them significant as diagnostic and tools for research is the specificity they display regarding the reactions they catalyze (Brody & Budny, 1995). Enzymes exhibit absolute specificity, which is, they will catalyze only one particular reaction. Other enzymes will be specific for a specific type of functional group or chemical bond. Overall, there are four different kinds of specificity (Blixt & Tiru, 1976):

Absolute specificity that the enzyme will catalyze only one reaction.

Group specificity in which the enzyme will affect performance only on molecules with particular functional groups, such as phosphate, amino, and methyl groups (Budny, 1989).

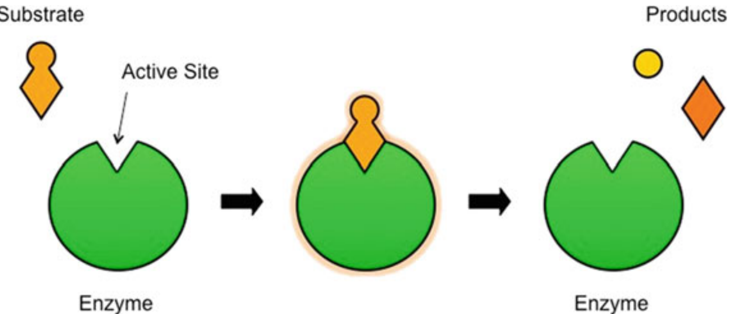


Fig. 10.3 Schematic of enzyme and substrate

Linkage specificity that the enzyme, irrespective of the rest of the molecular structure, will act on a specific type of chemical bond and the best technique to immobilize enzymes or other bioactive composites on polymers. The chemical steps included in covalent bonding are complex and timewasting. Therefore, are not presently practical for joint commercialization for food contact packaging. Enzymes are effective biological catalysts frequently utilized in the food industry to get better performance, quality, and shape of food. Using enzymes as active agents in the food packaging system is not novel technology (Adrian, 1959).

Stereochemical specificity is the enzyme performance with an optical isomer or specific esters. Enzymatic specificity requires two different forms: the first one is the type of chemical reaction and the second one is no chemical reaction, specificity for the reactant, or substrate. Therefore, for each chemical reaction in a biological system, there is a particular enzyme demand for the optimal production of reaction products. It is essential to consider many different biological reactions to pursue numerous different enzymes (Brody & Budny, 1995).

Enzymes, similar to other chemical catalysts, do not change equilibrium conditions. An enzyme enhances a forward reaction similarly, and the reverse reaction means that enzymes speed up the rate a chemical equilibrium is obtained. It considers that the enzyme does not interfere with the equilibrium concentrations ratio for the products to reactants (Nestorson et al., 2008).

In an active package system, the enzyme may be incorporated into the package structure or added straight to the packing to affect a reaction. The enzyme in packaging material should be motionless, and the substrate with a reactant role or a constituent circulated in the site to initiate a reaction. If the enzyme is placed in a static position or immobilized, it may be accomplished by making the enzyme the main part of the packaging system (Klibanov & Dordick, 1989).

Generally, active packaging commonly includes the combination of a chemical into the packaging material. Active packaging providing enzymes contains the incorporation into the packaging material of the specific enzymes in much the same way as the incorporation of a more formal chemical to make the active package. The significant differences are not changing enzyme with reaction and can continue indefinitely; the enzyme is sensitive to changes in some parameters, such as pH, temperature, etc. The range of environmental properties for the performance of the enzyme is a relatively narrow band (Kothapalli et al., 2008). Also, enzymes may be categorized according to the substrates. For example, lipases are used for lipids and proteases for proteins, etc. These are determination for the vast group to break proteins of entities that are lipid under a specific set of conditions or specific to a single protein.

Based on the several researches, the utilized enzymes incorporated in the polymers can act as oxygen scavengers (Labuza & Breene, 1989). One of the most widely investigated enzymes in active packaging is Glucose oxidase plus catalase. When the enzyme is immobilized, the surface carrying the active principle should be directly contacted with the food for activating the redox reaction, and it caused the

application to be limited (Brody et al., 2001). Scavenging reaction happens in the interface of product–packaging.

Glucose oxidase/catalase systems convert glucose into oxygen peroxide and gluconic acid, which is quickly decreased by the catalase enzyme, even though the food may become oxidized. Consequently, the scavenging reaction is suggested to be produced distinctly from the product. The liquid must also contain the reactant (ethanol, glucose). These systems are still being expanded and implemented, but, at present, there is no commercial application (Effront, 1917).

The immobilization can switch the activity of the adopted enzyme. Realty, immobilize the enzyme in the package while maintaining enzyme activities, the chemically modified polymers simplify the covalent bonding with the enzyme. To insert the enzyme into the matrix of polymer, prior chemical purification of the polymer is frequently required. The majority of enzymes are used directly in food processing, and some of them have already been utilized in active packages. For example, Soares and Hotchkiss (1998) presented naringinase in a plastic package. Hydrolysis of naringin reduced the bitterness of contained grapefruit juice (Wu et al., 2019), in Pharmacal biotechnologies industry used from immobilized lactase and cholesterol reductase in a package for liquids. Also, the reduction of lactose in milk has been used lactose content of milk or yogurt during storage. Another example is converting cholesterol reductase into coprostanol (Brody & Budny, 1995). A schematic drawing of the enzyme immobilization process in both immobilization in the dispersion phase and immobilization on the surface of the polymer film is shown in Fig.10.4.

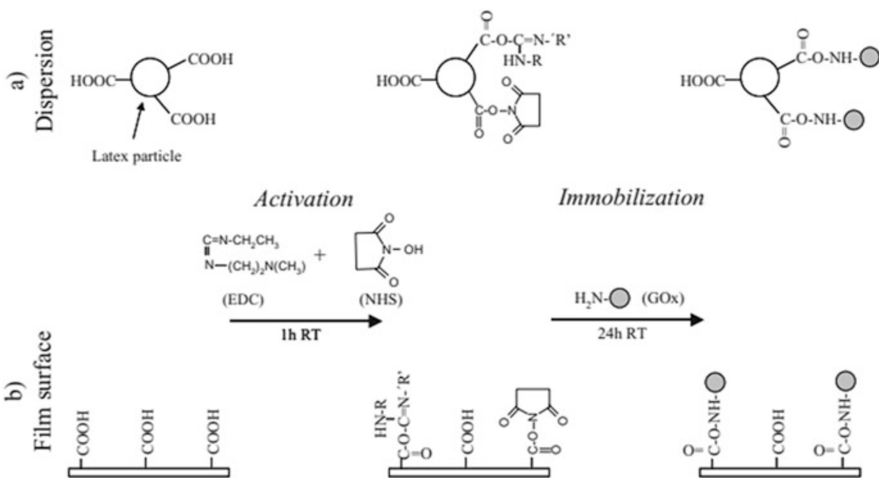


Fig. 10.4 Schematic drawing of the enzyme immobilization process. (**a**) Immobilization in the dispersion phase and (**b**) immobilization on the surface of polymer film formed from a latex dispersion (Nestorson et al., 2008)

10.6.1 Oxygen Scavenger in the Enzyme Packaging System

An oxygen scavenger is a substance that reacts enzymatically or chemically with oxygen, thus safeguarding the packaged food versus oxidative decay and quality changes owing to oxygen (Ahvenainen, 2003). Generally, oxygen scavenging systems follow the oxidation of iron powder or enzymes like glucose oxidase, with oxygen existing in the headspace of the packaging system. These reactions do some activities such as absorbing the great majority of free oxygen in the package, reducing the interaction of oxygen with food, and avoiding deterioration. Usually, oxygen scavengers are placed inside the package with a sachet too permeable to oxygen.

It should be noted that these types of sachets can be utilized for meat products, fresh pasta, and baked kinds of stuff, such as pizza dough, bread, sponge cake and cookies, nuts, coffee, and snack foods like chips. Some security issues are related to utilizing sachet types of oxygen scavenging systems in packaged foods that may ingest sachets by consumers, and the probability of sachet contents leaking into food packaging (Lopez-Rubio et al., 2004). Careful investigation and improvement should be carried out in choosing the suitable sachet material and its seal correctly before commercial application, and suitable alerting must be proclaimed on the package. The combination of catalase and glucose oxidase is a general enzymatic oxygen scavenging system that has been shown in the Fig. 10.5 (Shi et al., 2010). Glucose oxidase in the presence of oxygen reacts with glucose. This enzyme system has been embedded into acrylate matrixes of polymer for the deoxygenation of apple juice (Kothapalli et al., 2008).

10.6.2 Time–Temperature Integrator-Indicators

Time–temperature integrators (TTIs) are indicators that can display food safety and quality changes during distribution, handling, and storage (Taoukis & Labuza,

Fig. 10.5 The enzymatic reactions in the oxygen scavenging system using the combination of glucose oxidase and catalase. The mechanism complicated here is adapted from Shi et al. (2010) and Tian et al. (2013)

1989). TTIs are necessary for temperature-sensitive foods to evaluate and control the distribution chain (Giannakourou et al., 2005). TTIs must be low-priced, readable, reliable, and without any poisonous materials. Most of the TTIs are very similar to labels and can be used for food products. Several TTIs have been expanded based on different operating principles. Enzymatic TTIs utilize α-amylase (Guiavarc'h et al., 2004), lipase (Agerhem & Nilsson, 1981), or b-glucosidase (Adams & Langley, 1998). Presently, lipase is an available enzymatic TTI that can be utilized to control quality assurance. Enzymatic TTIs that have been investigated to date have utilized synthetic and then rather expensive chromogenic substrates hydrolyzed to generate a color. Consequently, if an enzyme that uses natural dyes as its substrate were available, a TTI produced using that enzyme would supply various benefits, counting developed visibility, less expensive, and extensive use of dye coding. Laccases are conventionally used for numerous industrial processes counting paper processing, detoxification of environmental pollutants, oxidation of pigments, prevention of wine discoloration, and production of chemicals from lignin (Kim et al., 2012).

10.7 Conclusion

Active packaging technologies suggest novel chances for the industries for protecting their products. Different active packaging systems are currently available based on the chemical and physical structure of the foods. Oxygen, moisture, carbon dioxide, ethylene, and ethanol are considered for the active packaging. The structure of the active packaging and the potentials of the enzymes are mentioned. The importance of this subject has produced many investigations to make more useful systems. In the following, oxygen scavenger in enzyme packaging and time–temperature integrator-indicators were investigated. Finally, investigation shows the improvement of novel indicators and enzymes that are much more precisely designed for the active packaging system.

References

Adams, J., & Langley, F. (1998). Nitrophenyl glucoside hydrolysis as a potential time-temperature integrator reaction. *Food Chemistry, 62*(1), 65–68.

Adrian, J. (1959). Le dosage microbiologique des vitamines du groupe B. *Annales de la nutrition et de l'alimentation. JSTOR, 13*, 1–175.

Agerhem, H., & Nilsson, H. J. (1981). *Substrate composition and use thereof*. Google Patents.

Ahvenainen, R. (2003). *Novel food packaging techniques*. Elsevier.

Almasi, H., Jahanbakhsh Oskouie, M., & Saleh, A. (2020). A review on techniques utilized for design of controlled release food active packaging. *Critical Reviews in Food Science and Nutrition, 61*, 1–21.

Blixt, K., & Tiru, M. (1976). An enzymatic time/temperature device for monitoring the handling of perishable commodities. *Developments in Biological Standardization, 36*, 237–241.

Brody, A., & Budny, J. (1995). Enzymes as active packaging agents. In *Active food packaging* (pp. 174–192). Springer.

Brody, A. L., Strupinsky, E., & Kline, L. R. (2001). *Active packaging for food applications*. CRC Press.

Budny, J. (1989). *A transporting storage or dispensing container with enzymatic reactor.* International Patent Application W, 89.

Conte, A., et al. (2007). Innovative active packaging systems to prolong the shelf life of mozzarella cheese. *Journal of Dairy Science, 90*(5), 2126–2131.

Effront, J. (1917). *Biochemical catalysts in life and industry: Proteolytic enzymes*. John Wiley & Sons Inc.

Giannakourou, M., et al. (2005). Field evaluation of the application of time temperature integrators for monitoring fish quality in the chill chain. *International Journal of Food Microbiology, 102*(3), 323–336.

Guiavarc'h, Y., et al. (2004). Bacillus licheniformis α-amylase immobilized on glass beads and equilibrated at low moisture content: Potentials as a time–temperature integrator for sterilisation processes. *Innovative Food Science & Emerging Technologies, 5*(3), 317–325.

Han, J. H. (2003). Antimicrobial food packaging. *Novel Food Packaging Techniques, 8*, 50–70.

Kim, K., Kim, E., & Lee, S. J. (2012). New enzymatic time–temperature integrator (TTI) that uses laccase. *Journal of Food Engineering, 113*(1), 118–123.

Klibanov, A. M., & Dordick, J. S. (1989). *Enzymatic temperature change indicator*. Google Patents.

Kothapalli, A., Morgan, M., & Sadler, G. (2008). UV polymerization-based surface modification technique for the production of bioactive packaging. *Journal of Applied Polymer Science, 107*(3), 1647–1654.

Labuza, T. P. (1982). *Shelf-life dating of foods*. Food & Nutrition Press Inc.

Labuza, T. P., & Breene, W. (1989). Applications of "active packaging" for improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods. *Journal of Food Processing and Preservation, 13*(1), 1–69.

Labuza, T. P., & Schmidl, M. K. (1985). *Accelerated shelf-life testing of foods*. Food Technology.

Liu, L., et al. (2014). Capillary electrophoresis-based immobilized enzyme reactor using particle-packing technique. *Journal of Chromatography A, 1352*, 80–86.

Lopez-Rubio, A., et al. (2004). Overview of active polymer-based packaging technologies for food applications. *Food Reviews International, 20*(4), 357–387.

Lorenzo, J. M., Batlle, R., & Gómez, M. (2014). Extension of the shelf-life of foal meat with two antioxidant active packaging systems. *LWT—Food Science and Technology, 59*(1), 181–188.

Motta, G. J., Milne, C. T., & Corbett, L. Q. (2004). Impact of antimicrobial gauze on bacterial colonies in wounds that require packing. *Ostomy/Wound Management, 50*(8), 48.

Narayanan, R., Stottrup, B. L., & Wang, P. (2009). Surface packing characterization of Langmuir monolayer-anchored enzyme. *Langmuir, 25*(18), 10660–10665.

Nestorson, A., et al. (2008). Enzyme immobilization in latex dispersion coatings for active food packaging. *Packaging Technology and Science: An International Journal, 21*(4), 193–205.

Ozdemir, M., & Floros, J. D. (2004). Active food packaging technologies. *Critical Reviews in Food Science and Nutrition, 44*(3), 185–193.

Prazak, A. M., et al. (2002). Antimicrobial resistance of Listeria monocytogenes isolated from various cabbage farms and packing sheds in Texas. *Journal of Food Protection, 65*(11), 1796–1799.

Realini, C. E., & Marcos, B. (2014). Active and intelligent packaging systems for a modern society. *Meat Science, 98*(3), 404–419.

Robertson, G. L. (2016). *Food packaging: Principles and practice*. CRC Press.

Shi, X., Lim, J., & Ha, T. (2010). Acidification of the oxygen scavenging system in single-molecule fluorescence studies: In situ sensing with a ratiometric dual-emission probe. *Analytical Chemistry, 82*(14), 6132–6138.

Soares, N., & Hotchkiss, J. (1998). Naringinase immobilization in packaging films for reducing naringin concentration in grapefruit juice. *Journal of Food Science, 63*(1), 61–65.

Taoukis, P., & Labuza, T. P. (1989). Applicability of time-temperature indicators as shelf life monitors of food products. *Journal of Food Science, 54*(4), 783–788.
Tian, F., Decker, E. A., & Goddard, J. M. (2013). Controlling lipid oxidation of food by active packaging technologies. *Food & Function, 4*(5), 669–680.
Wierschem, M., et al. (2017). Enzyme kinetics for the transesterification of ethyl butyrate with enzyme beads, coated packing and ultrasound assistance. *Chemical Engineering and Processing: Process Intensification, 111*, 25–34.
Wu, X., et al. (2019). Packaging and delivering enzymes by amorphous metal-organic frameworks. *Nature Communications, 10*(1), 1–8.

Enzymes as a Tool in Food Analysis and Foodborne Pathogen Detection

11

Preethi Sudhakara, Jerrine Joseph, S Priyadharshini, Jemmy Chirsty, Alex Anand, Davamani Christober, and Aruni Wilson Santhosh Kumar

Abstract

Enzymes are widely and progressively utilized in food processing industries. Enzymes find tremendous application in fruits and vegetable processing, dairy processing, and cereals processing. Nowadays, there is a wide application of enzymes in food analysis. Enzymes serve as an analytical tool for the detection of food-borne pathogen and allergens. These may be utilized as biosensors and are applicable in food industries. This chapter gives detailed information related to application of enzymes in food analysis and detection of food-borne pathogens.

P. Sudhakara
School of Medicine, University of Florida, Gainesville, FL, USA

J. Joseph
Center for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, India

S. Priyadharshini
Department of Food Science and Nutrition, The American College, Madurai, India

J. Chirsty · A. Anand
Center for Molecular Data Sciences and Systems Biology, Sathyabama Institute of Science and Technology, Chennai, India

D. Christober
Department of Food Science and Nutrition, The American College, Madurai, India

The American College, Madurai, India

A. Wilson Santhosh Kumar (✉)
Sathyabama Institute of Science and Technology, Chennai, India

Musculoskeletal Research Center, US Department of Veteran Affairs, Loma Linda VA, Loma Linda, CA, USA

School of Medicine, Loma Linda University, Loma Linda, CA, USA
e-mail: vcaum@mum.amity.edu

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_11

Keywords

Enzymes · Food processing · Food analysis · Food-borne pathogen

11.1 Introduction

Enzymes are defined as the biological materials responsible for fastening the reaction rate. Enzymes are the so-called multifaceted biomolecules that are indispensable for biochemical interconversions. They act as a catalyst for all the metabolic processes in the body. The advancement in rDNA technology and protein engineering has enabled us to capitalize on the various functions of enzymes from industrial to biomedical applications. Enzymes are exceptionally effective biocatalysts investigated for wide-reaching catalysis due to a few particular preferences that run from their activity in milder response conditions to their excellent component selectivity and lower ecological and physiological harmfulness (Chapman et al., 2018). Enzymes are initially involved in the dissolvable or immobilized structure for isolation, purification, and production to benefit humanity (Chaplin & Bucke, 1990). The demand for the enzyme increases by 6% yearly, especially in food and feed production (Nampoothiri et al., 2002). Enzymes are widely and progressively utilized in exploration and food processing as significant handling biocatalysts (Ermis, 2017). Enzymes are intricated in a living organism. Enzymes formed in live cells can enhance reaction rates *in vitro*. The acclimatization of enzymes in the food sector is known widely, and intense investigations consistently unravel the global food crisis. This chapter highlights the fundamentals of enzymes, sources of diverse enzymes, and their applications in the food sector.

Microbial enzymes have been catapulted to fame due to their inherent properties of the ease of their production and yield manipulation in laboratory settings. This highlights their significance and the opportunities that pave the way for microbiologists by harnessing the biomolecules' potential. The thoughtful consideration of the enzymes in the food sector has amended the primary methods to afford improved marketplaces with high quality and safety. Previously, this information about enzymes has been used in meat tenderization, baking, brewing, etc. Mainly the enzymes are produced by microbial synthesis. The microbial method for enzyme production has been favored over other methods for quick and inexpensive production.

Furthermore, microbially produced enzymes' harmful content is lesser than other sources. The first commercial application of enzymes was in the production of cheese. Enzymes in the food sector alter traditional and toxic methods, substitute them with sustainable methods, and reduce energy consumption levels. It may be worthwhile to unclose an unexplored trail for enzyme applications, which could be advantageous in the food industry.

Foodborne pathogens influence human health negatively and are comprehended to induce financial losses. Therefore, it is essential to rapidly detect foodborne pathogens and execute steps to assure their inactivation. Immunological, molecular,

and cultural approaches are often exploited to detect foodborne pathogens. High expense, lengthy analysis duration, and the need for technical personnel are some of the drawbacks of these approaches. Biosensors are known as analytical instruments. Biosensors can be used as novel techniques for detecting toxins and pathogens. These biosensors restore various signals (like biochemical signals) to electrical signals that can be measured with the help of a transducer. Various kinds of biosensors are being utilized for the detection of pathogenic bacteria. Biosensors are prompt and cost-effective devices and are employed in multiple areas such as food safety, medicine, pharmacy, measurement of environmental pollution, and military defense. Electrochemical and optical biosensors and piezoelectric immune-sensors are among the most repeatedly used biosensors in detecting foodborne pathogens.

There are many viruses and bacteria, some that cause animal disease, and others that contribute to foodborne human illness and disease that can be tested for the occurrence of foodborne pathogens. Some of the significant subjects of testing include several swine viruses, including swine influenza virus and porcine reproductive and respiratory syndrome virus. Bovine viruses include *bovine viral diarrhea virus, bovine syncytial virus, bovine rotavirus, coronavirus,* and *rhinotracheitis virus.* In addition, the *Hepatitis A virus, flaviviruses, West Nile,* and the *Usutu virus (USUV)* that have recently been on the rise in Europe and some parts of South East Asia are of much importance. The diagnosis and surveillance of bacterial pathogens encompass the full spectrum of animal and foodborne pathogens. These include *Escherichia coli* and bacteria in the *Salmonella, Klebsiella, Clostridium, Listeria, Staphylococcus, Streptococcus, Pseudomonas, Pasteurella, Actinobacillus*, and *Mycoplasma genera.*

Rapid, efficient pathogen detection and fingerprinting are crucial and usually lifesaving to control the foodborne ailment. Presently, detecting and subtyping the pathogens are distinct operations, but merging these two phases via a technique known as "metagenomics analysis," the detection is much prompter and more sensitive as many microorganisms are now employed to produce food enzymes. Enzyme technologies have been thoroughly revolutionized with the recent novel trends in biotechnology applications to fulfill the new and changing requirements of the food industry. There is an extensive tendency toward novelty in enzyme discovery and creating tailored enzymes to make them inclined for industrial food applications.

In the recent past, developments in enzyme informatics tools can simplify designing the enzymatic structure. Also, its computational dynamic simulations can produce the kinetic and energetic course of its mechanism. Enzyme informatics can also recreate an exceptional part in mutant library design and other screening platforms, but its execution of molecular approaches is still time consuming. Recent improvements in the subject will hover around novel frontier in enzyme technologies that could attribute various functional aspects to these enzyme biocatalysts. This study will consolidate the extensive characteristics of food enzymes, their diversified functions in the food industry, the destructive outcomes of foodborne pathogens, and their current detection techniques.

11.2 Enzymes as an Analytical Tool

Human health and healthcare predominately revolve around the quality of food consumed. Appealing enzymes in the food industry can manage the quality food supply problem. Hence, evaluating food quality and ensuring food safety standards are highly important. This crucial factor plays a vital role in evaluating food quality, safety, and the turnaround time to ascertain this. *Enzymes* are biomolecules that inherently possess specificity and sensitivity traits (Fig. 11.1).

Hence enzymes are a better bet than the conventional diagnostic methods that are dreary and overwhelming and require correlative modes to detect food quality and safety. Enzyme-based analytical tools to measure biological functionality are more reliable and consistent. The analytical tools should be high throughput and competent to authenticate and authorize the study of particular aspects of biology, either qualitatively or quantitatively, which is possible by enzymes. Also, a crucial part of developing such tools is to source or amalgamate the desired enzymes with the required activity. Thus, the utilization of enzymes as analytical informers is an inexpensive, simpler, and quicker manner of diagnostics. Microbes have been used from bygone days; yeast is the first reported organism to produce alcoholic beverages using barley in 6000 BC. The microbial enzymes have earned acknowledgment globally for their extensive uses in several trades. Nevertheless, recombinant DNA technology and protein engineering are assembled to confound the demand. It improves consumer goods, expense deduction, natural resources shortage, and environmental safety (Liu et al., 2013; Choi et al., 2015b).

Microbial enzymes play a crucial role (Raveendran et al., 2018). In terms of analytical procedures in the Food and Pharmaceutical industries, enzymes play a vital role. As indicated by food and regulatory agencies, food safety and quality are the censorious criteria recurrent to all food constituents, and these criteria are of substantial lucrative significance. Food safety depends on toxins, dangerous microorganisms, toxins divulged by microbes, insecticides, and pesticides (Terry et al., 2005). Processing of foods with biological enzymes is chronicled inveterate approach. Earlier in 6000 BC, bread, brewing, wine, and cheese making were done

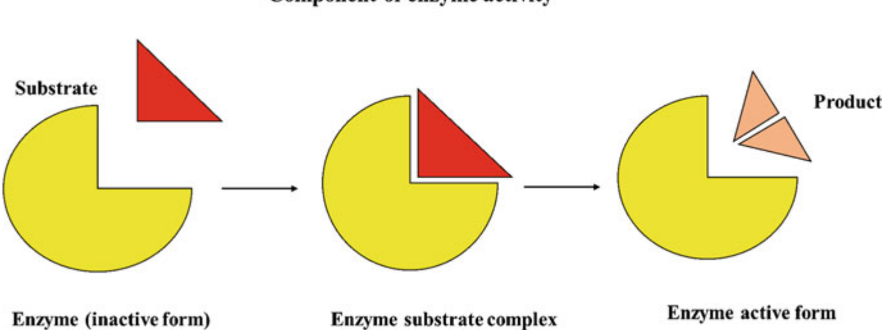


Fig. 11.1 Enzyme activity

with biological agents, but the initial microbial fermentation was from 2000 BC for vinegar production (Vasic-Racki, 2006). In 1930 for juice clarification, pectinases were used. Invertase was used during World War II for the invert sugar syrup production in the sugar industry that developed the use of an immobilized enzyme (Vasic-Racki, 2006). In 1960, the extensive use of enzymes established during the traditional acid hydrolysis was restored by slant based on utilizing amylase and glucoamylases (Panesar et al., 2010).

The pattern for the plan and execution of procedures and creation of products moored in the utilization of enzymes has consistently expanded (Norus, 2006). Enzymes from microbes play a significant part in food ventures since microbial enzymes' stability is higher than plant and animal enzymes. They can be fabricated through fermentation strategies in a lucrative way with less reality prerequisite and high consistency and process adjustment (Gurung et al., 2013). In food analytical strategies, food safety and quality control are essential, and they can be analyzed by numerous analytical procedures like HPLC, GC-MS, and LC-MS (Castillo et al., 2004). In any case, sample preparation strategies are the significant downside of this technique. Progressing diagnostic strategies in the food industry are tedious and rely upon skilled laborers, and these procedures depend on extended division techniques, costly instruments, and exceptionally unadulterated chemicals. In the field of quick screening, there is a requirement for complementary strategies to recognize food safety and quality issues. A more specific and sensitive method for screening food safety and quality issues is enzymatic analysis (Prodromidis & Karayannis, 2002). Enzymes play a crucial role in the analytical process in the food, pharmaceutical, and environmental industry. For governing the compound concentration that performs as an activator, substrate, or inhibition of specific enzymes, enzymes are used as typical analytical devices. For the analysis of modification induced physically and nutritionally and quality criterion of food, the concentration and activity of specific enzymes are used. The list of enzymes and their application, along with the microbial enzymes, are listed in Table 11.1.

11.3 Important Enzymes as Relevant Markers for Food Analysis

Enzymes are extensively used as an important marker, especially in the food and pharmaceutical industry. The broad scope of enzymes has been boon to the food analyst who investigates the different constituents of the food. Food, being more complex with increased macronutrients and very few micronutrients analyzing these food constituents, requires extremely simple and affordable, less time-consuming feasible, and specific techniques. Enzymes are used as markers for food analysis and their lustiness, adaptability, and cost-effective nature (Ashie, 2012).

Table 11.1 Microbial enzymes with their source and application

Microbial enzyme	Microbial source	Application	References
α-Amylase	B. *licheniformis*, *B. subtilis*, *B. stearothermophilus*, *B. megaterium* and *B. circulans*	Liquefaction of starch, fruit juice clarification, improvement of bread quality, brewing, and baking	Lépine et al. (2019), Luang-In et al. (2019)
Glucoamylase	*Aspergillus oryzae*, *Aspergillus niger* and *Rhizopus oryzae*	Glucose fructose syrups, production of beer	Bilal and Iqbal (2020), Karim et al. (2017)
β-galactosidase	*Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus*	Reduction of lactose intolerance	Gibson and Wang (1994), Saqib et al. (2017)
Protease	*Photobacterium, Bacillus, Vibrio*	Tenderization of meat, milk coagulation	Aruna et al. (2014), Zhang et al. (2015)
Lipase	*Bacillus sp. Pseudomonas*	Cheese production	Aravindan et al. (2007), Feng et al. (2011)
Esterase	*Bacillus subtilis*	Flavor enhancement, dietary fiber de-esterification	Faulds (2010), Sayali and Satpute (2013)
Pectinase	*Aspergillus niger, A. flavus, and Penicillium chrysogenum*	Fruit juice clarification	Pasha et al. (2013), Abdollahzadeh et al. (2020)
Phospholipase	*Listeria monocytogenes*	Development of cheese flavor	Law (2010), Titball (1993)
Cellulase	*Pseudomonas fluorescens Bacillus subtilis, E. coli, and Serratia marcescens*	Feed of animal, fruit juice clarification	Sukumaran et al. (2005), Islam and Roy (2018)
Xylanase	*Bacillus*, *Cellulomonas*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, *Arthrobacter*, *Microbacterium*, *Pseudoxanthomonas,* and *Rhodothermus*	Enhancement of beer quality	Camacho et al. (2003), Chakdar et al. (2016)
Glucose oxidase	*A. niger and Penicillium notatum*	Improvement of food flavor	Hanft and Koehler (2006), Nakamatsu et al. (1975)
Catalase	*Staphylococcus*	Hydrogen peroxide removal for production of cheese	Sîrbu (2011)

(continued)

Table 11.1 (continued)

Microbial enzyme	Microbial source	Application	References
α-Acetolactate dehydrogenase	*Enterococcus faecalis, Bacillus licheniformis, and Lactococcus lactis*	Precise beer maturation	Choi et al. (2015a)
Peroxidase	*Bacillus sphaericus, B. subtilis, Pseudomonas sp., Citrobacter sp.*	Flavor development	Regalado et al. (2004)
Asparaginase	*Escherichia coli and Erwinia chrysanthemi*	Shortening of acrylamide formation while baking	Mohan Kumar et al. (2014)

11.4 Enzyme Marker in Pasteurization of Milk

Enzymes play a vital role in detecting milk pasteurization. For increasing the shelf life of milk and milk products, heat treatment is essential. In many countries, an alkaline phosphatase test is used as a standard technique for milk ratification. Non-appearance of alkaline phosphatase in the heat-treated milk shows efficient pasteurization. It is based on the inactivation of the alkaline phosphatase enzyme using heat.

On the other hand, it reduces the pathogenic bacterial population in the milk. Not only for milk, but this rapid detection method can also be used for other dairy products like cheese production. Other primordial enzyme markers for milk include lactoperoxidase and γ-glutamyl transpeptidase (Rankin et al., 2010).

11.5 Enzyme Marker in Blanching

The main aim of blanching is to remove the enzyme polyphenol oxidase. Apart from this, another equitable goal is to decrease microbial growth and increase shelf life. Time taken for blanching is enough to destroy the indicator enzyme. Blanching of frozen foods, are done at different time intervals in order to recognize the relationship between enzyme activity and sensory scores (Williams et al., 1986).

11.6 Novel Analytical Tools

In these decades of higher technological development, the improvement in the food analysis techniques will have greater insight. Analysts and chemists will have greater value in using novel analytical tools. Traditional high-performance liquid chromatography system analyzes the hydrophobic and hydrophilic constituents in reversed phase and the normal phase. However, the conventional method is less susceptible and time consuming. So, to analyze the higher and lower molecular weight analytes,

a novel polymer-based stationary phase is introduced. Capillary electrophoresis that detects the analyte based on the molecular weight and ionic moiety is introduced as an alternative. There is a higher technique named capillary electrochromatography which is better than HPLC and capillary electrophoresis (Huck et al., 2000). The solid-phase extraction can attain a low concentration analyte. Spectroscopic strategies at this phase of the systematic technique either incorporate mass spectrometry (MS) or vibrational spectroscopy individually. Electrospray ionization is used chiefly with mass detectors like time of flight (TOF), ion trap, ion cyclotron, and quadrupoles. Laser desorption ionization methods determine higher molecular weight samples (Feuerstein et al., 2006).

11.7 Enzymes in the Food Industry

Enzymes are used in a variety of ways in the food industry. Enzyme activity aids in the safe digesting of food in various situations. The thermal stability of enzymes has been used extensively to quantify heat treatment; peroxidase activity, for example, is used to determine the sufficiency of blanched vegetables. Lactoperoxidase has also been used to test the effectiveness of the pasteurization process in milk. In some cases, residual enzyme activity affects the flavors or color of products while they are being stored. Lipoxygenase, for example, is responsible for off-flavors in frozen veggies that have not been adequately blanched. The staining of wheat flour and noodles and fruit liquids and pastes is catalyzed by polyphenol oxidase (Nielsen, 2017).

The enzyme is used to test the components of enzyme-substrate foods that are commercially accessible. The glucose content of a complex dietary matrix containing additional monosaccharides can be determined using commonly available enzymes. Enzyme activity measurement concerning food quality is also noteworthy. Milk from mastitic udders, for example, has considerably increased catalase activity. The quantity of bacteria in milk is also connected to catalase activity. Another technique to use enzyme testing to determine food quality is to estimate protein nutritional value by measuring the activity of added proteases on dietary protein samples. Enzymes can track the appearance of breakdown products such as trimethylamine in fish during storage. Enzymes are also used as preparatory tools in food analysis. In fiber analysis, amylases and proteases are used, while enzymatic hydrolysis of thiamine phosphate esters is used in vitamin analysis (Lee et al., 1991). Table 11.2 lists some of the most often used enzymes in the food business.

Food scientists must understand that the environment significantly impacts enzyme activity. At high temperatures or under other situations that are not too far off from ideal, that enzyme can deteriorate and lose its activity. As a result, therapeutic enzymes must be stored and handled with care, usually in the refrigerator or freezer. To effectively carry out enzyme analyses in foods, certain basic enzymology principles must be understood. Following a brief overview of these concepts, instances of enzymatic analysis in food systems are discussed. Under physiological conditions, enzymes are protein facilitators with high specificity and reactivity.

Table 11.2 Application of microbially produced enzyme in industries

Industry	Enzymes	Uses	Microorganisms
Baking	Maltogenic	Enhance shelf life of bread	*Bacillus stearothermophilus,*
	Amylase	Flour adjustment, bread softness	*Aspergillus sp., Bacillus sp.*
	Transglutaminase	Strengthening of dough	*Streptomyces sp., Streptoverticillium sp.*
	Xylanase	Conditioning of dough	*A. niger*
	Glucose oxidase	Strengthening of dough	*A. niger, Penicillium chrysogenum*
	Lipase	Conditioning of dough and its stability	*A. niger*
Dairy	Acid proteinase	Coagulation of milk	*Aspergillus sp.*
	Neutral proteinase	Ripening of cheese, debittering	*B. subtilis, A. oryzae*
	Lactase (beta-galactosidase)	Whey	*E. coli, Kluyveromyces sp.*
	Aminopeptidase	Ripening of cheese	*Lactobacillus sp.*
	Catalase	Cheese making	*A. niger*
	Transglutaminase	Cross-linking of protein	*Streptomyces sp.*
Beverage	Pectinase	Depectinization	*A. oryzae, P. funiculosum*
	Glucose oxidase	For the removal of oxygen	*A. niger*
	Pullulanase	Saccharification of starch	*Bacillus sp., Klebsiella sp.*
	Naringinase	Debittering	*A. niger*
	Alpha-Amylase	Hydrolysis of starch	*Bacillus, Aspergillus*
	Beta-Amylase	Hydrolysis of starch	*Bacillus, Streptomyces, Rhizopus*
	Beta-Glucanase	To inhibit haze formation	*B. subtilis, Aspergillus spp.*
	Cellulase fruit	Liquefaction	*A. niger, Trichoderma atroviride*

Enzymatic analysis determines the condition of a biological system, including meals, by measuring substances with the help of added enzymes or by measuring endogenous enzyme activity. Because enzyme catalysis occurs under relatively favorable circumstances, it is possible to measure relatively unstable molecules that would be difficult to measure using other methods. Furthermore, due to the specificity of enzyme reactions, it is possible to assess the components of complex mixtures without the time and expense of using expensive chromatographic separation procedures.

11.8 Beverage and Dairy Industry

Enzymes regulate the brewing process, ensure consistent, high-quality beer; enhance animal and vegetable proteins' functional and nutritive properties through enzymatic hydrolysis; and increase juice yield while improving color and aroma. Enzyme use

in the food business is divided into several categories, including baking, dairy, juice production, and brewing (Mojsov, 2011). Microbial enzymes are widely used in the baking business, the most prominent application market in the food industry, to increase dough stability, crumb softness and structure, and product shelf life. The usage of enzymes in the dairy industry (Qureshi et al., 2015), which is the next largest application area, is primarily due to the increased use of microbial enzymes in cheese manufacturing (Qureshi et al., 2015). The laboratory must identify appropriate enzymes with the required activity and understand the target analyte and the complexity of the matrix in which the analyte must be measured (Kieliszek & Misiewicz, 2014). Foods are typically very complex matrices, posing a significant technical barrier to the analyst tasked with determining and establishing the levels of various components. As a result, numerous analytical methods, including enzymes, have been developed to meet this issue over time. The usage of enzymes is based on specificity, which allows for the selective, sensitive, and precise measurement of macro- and micronutrients in meals. The development of biosensors with greater robustness and versatility has boosted the use of enzymes as an analytical tool in recent years. Starch is a critical ingredient in baked goods because it aids in the browning and flavoring of the crust. It is found primarily in plants in bread production.

Many enzymes are used for milk processing and cheese production in the dairy industry. Rennet is a coagulant enzyme mixture of chymosin and pepsin used in milk clotting and cheese preparation. They have been extracted from animal sources, mainly from the stomach of young calves (Theron & Divol, 2014). Plant extracts also contain many enzymes, such as leaves of Sodom apple, berries of *Solanum dubium*, *Calotropis procera*, and cardoon extracts, from microbial source *Rhizomucor miehei* and *Rhizomucor pusillus,* contain protease enzyme that helps in clotting. Protease enzyme obtained from *Cryphonectria parasitica* aggregate and transferred milk into curd and liquid part.

11.9 Necessary Enzymes as Relevant Markers for Food Analysis

Enzymes are ubiquitous, and their application has been used from the olden days in human edification. Food processing methods modify the food materials and make them appropriate for consumption (Monteiro et al., 2011). In processing, it has been used in wine, beer brewing, cheese, and bread making from 6000 BC. Based on the enzyme properties, the International Union of Biochemistry approved a class of enzymes used in the food industry, shown in the figure below (Fig. 11.2).

Food enzymes are categorized into two types: food processing and food additives. Most of them are used for food processing purposes, whereas lysozyme and invertase are used as food additives. The main aim of these food processing agents is to increase the edible foods' shelf life and preserve the health and nutritional contents of the food without affecting their taste and aroma. Uses of enzymes in the food industry process carbohydrates, proteins, and fats.

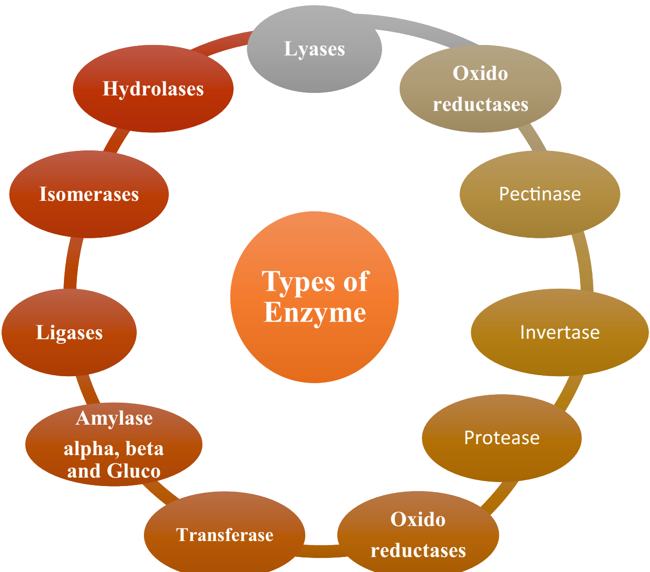


Fig. 11.2 Types of major microbial enzymes used in the food industry

11.9.1 Alpha-Amylase

The most important industrial enzymes account for around 25% of the global enzyme market, which comes from various sources, including plants, animals, and microbes (Kandra, 2003; Reddy et al., 2004). Enzymes catalyze the hydrolysis of internal 1,4-glycosidic bonds in starch to produce low molecular weight compounds like glucose, maltose, and maltotriose (Gupta et al., 2003; Rajagopalan & Krishnan, 2008). The baking sector currently employs a thermostable maltogenic amylase from *Bacillus stearothermophilus*. It is also used to clarify beer and fruit liquids and improve fiber digestion in animal feed (van der Maarel et al., 2002; Gavrilescu & Chisti, 2005; Ghorai et al., 2009).

11.9.2 Beta Amylase

Beta amylase is an enzyme with the systematic name 4-alpha-D-glucan maltohydrolase. It acts on starch, glycogen, other polysaccharides, and oligosaccharides, producing beta-maltose by an inversion, resulting in the sweet flavor of ripe fruit. This enzyme generally presents in bacteria, fungi, and plants. The molecular weight of this enzyme is 223 and 8 kDa and has various applications in the starch, sugar, brewing, and beverages industries. In bacteria, *Clostridium, Bacillus,* and *Pseudomonas* are the major groups of organisms wherein fungi, *Aspergillus*

sp. can produce these enzymes used to manufacture organic acids (Djekrif-Dakhmouche et al., 2006; Hernández et al., 2006).

11.9.3 Glucoamylase

Exoenzymes, also known as scarifying enzymes, are found in a wide range of species. It is used in the food industry for a variety of purposes, including lowering dough staling and increasing bread crust color, converting starch to maltose and other sugars, assisting in the creation of glucose in the fermentation process, and making alcoholic beverages and soy sauce (Blanco et al., 2014). Most glucoamylase enzymes lose their activity at higher temperatures and become stable at lower temperatures. The principal producers of this enzyme are *A. niger* and *A. awamori*. However, *Rhizopus oryzae* is widely used in industrial applications (Karim et al., 2017; Bilal & Iqbal, 2020).

11.9.4 Cellulase

This enzyme is generated mainly by microbes with a molecular weight of 54 kDa (bacteria and fungus). It is a complex enzyme that hydrolyzes 1,4 links in cellulose chains and contains endo-1,4-D-glucanase, exo-1,4-glucanase, and D-glucosidase. Fungi produce more cellulase than bacteria (Gaur & Tiwari, 2015). *Trichoderma reesei, Penicillium*, and *Aspergillus* are the three species. *Bacillus cereus, Bacillus megaterium, Bacillus mycoides*, *Clostridium straminisolvens, Flavobacterium balustinum*, and *Acetobacter xylinum* (Wachinger et al., 1989; Tomme et al., 1995; Gaur & Tiwari, 2015; Sharma et al., 2015; Tabssum et al., 2018). In the food industry, cellulase increases the nutritional characteristics of consumable food products. Macerating enzymes, in addition to the combination of pectinase and hemicellulase (Bhat, 2000), are essential in extracting olive oils to generate superior quality with less waste (Galante et al., 1998). It is also utilized to break up roughage in the dough (Chandrasekaran, 2013).

11.9.5 Pectinase

Pectin is a branching heteropolysaccharide with a high molecular weight. Pectinase has a molecular weight of up to 31 kDa. Fruit juice is made with enzymes that rapidly operate on hard pectin. It splits pectin molecules by making juice less viscous, producing pectin-free starch, refining vegetable fibers, and degumming natural fibers (Christopher & Kumbalwar, 2015). It can be found in higher plants and microbes in their natural state. Pectinase, employed in tea fermentation, eliminates pectin’s foam-forming ability and removes the mucilaginous layer from coffee beans in tea and coffee (Oumer & Abate, 2018). Commercial pectinase can be

found in microbial strains such as *Moniliella* SB9, *Penicillium* spp., and *Aspergillus* spp. (Priya & Sashi, 2014).

11.9.6 Invertase

Invertase is a non-pathogenic and non-toxic enzyme also known as beta-fructofuranosidase, and the molecular weight of this enzyme is 205 kDa (Romero Gomez et al., 2000). The primary source of this enzyme is plants and microorganisms. Where *Saccharomyces cerevisiae*, *Candida utilis*, *A. niger* are the primary source for the production of invertase commercially. It is used in alcoholic beverages, lactic acid, and glycerol production. It is also used in the chocolate preparation of infants' food to improve the taste of the food (Christopher & Kumbalwar, 2015).

11.9.7 Protease

Proteases represent one of the major groups of industrial enzymes, with a molecular weight of 126 kDa. It breaks the peptide bonds present in the protein and separates them as amino acids. *Bacillus* and *Aspergillus* spp. is the primary microorganism that produces protease enzymes. It is used for several purposes such as brewing, meat tenderization, baking, hydrolyzed animal proteins, functional meat proteins, etc.

11.9.8 Lipase

Lipase enzymes deconstruct lipids into fatty acids and glycerol. This is something that can be used in the baking sector. It separates milk fat and gives terrific flavors to the cheese. The flavor of the cheeses improves the creamy texture and taste of the products. Excessive lipid lysis leads to unavoidable odors. Most commercial lipases develop flavor in dairy products, meat, vegetables, fruit, baked goods, milk, beer, and others (Nagodawithana & Reed, 1993). The major lipase enzyme producers are *Pseudomonas* sp., *Bacillus* sp., and *Penicillium* sp.

11.10 Enzymes as Biosensors

11.10.1 Enzymatic Biosensors Role in Food Technology

Food quality and safety-related assessment technologies need a necessary improvement since they directly impact the consumers' health. Reported microbial contamination, pesticides occurrence, food adulteration, mycotoxin presence, freshness analysis, presence of claimed antioxidants, health hazards caused by foodborne

microorganisms demonstrated the need for improved and faster technology to ensure the safety and quality of the food products. Conventional techniques for food quality assessments were replaced with fast and easy techniques like biosensors. Biosensors are more extraordinary than other demonstrative instruments due to different food quality and security innovations. Enzymatic biosensors are commonly utilized in the food business (Table 11.3). Different properties of biosensors, especially optical, mechanical, and electrochemical, can be adjusted by utilizing nanomaterials. Biosensors are an appropriate detecting tool for quality control and revealing the contamination in food. It can be a systematic and feasible detecting method in the food chain.

The most important part of the food industry is maintaining food safety and quality control. Biosensors have numerous food safety and quality control applications. Enzyme biosensors are an essential technique as it has a notable usage compared to the conventional method. It is effortless and less time consuming, and it does not involve skilled labor and costly equipment (Santos et al., 2006; Hooda et al., 2018). There are two main biosensors' main constituents: the bioreceptor and the transducer (Viswanathan et al., 2009). Biosensors work on three principles based on the device used to create biosensors, segments of molecular remembrance, and transducer. There are few enzymes for specific foods to act as a biosensor. There are numerous applications in the food industry in which biosensors are involved in quantifying different food constituents to determine their shelf life.

Biosensor technologies have the insightful potential to screen adverse substances in foods on-site and in-situ phases. Biological agents like enzymes usage in biosensors improved the specificity due to their binding affinity to a specific substrate and related catalytic effect. In addition, they lack interference with the compounds present in a complex sample. Due to sensitivity and specificity, enzymatic biosensors are considered an inevitable technology for food quality screening.

Table 11.3 Enzymes utilized in biosensors (Modified from Akyilmaz et al., 2010)

Enzymatic biosensors	Food constituents
Glucose oxidase, glucose dehydrogenase	Glucose
Oxalate oxidase	Oxalate
Tyrosinase	Tyrosine
Malate dehydrogenase	Malate
Galactose oxidase	Galactose
Cholesterol oxidase	Cholesterol
Alcohol oxidase	Alcohol
Peptidase, glutamate oxidase	Aspartame
Tyrosinase	Phenols
Lipoxygenase	Essential fatty acids
Fructose-5-dehydrogenase	Fructose
Glucose oxidase, mutarotase, invertase	Sucrose
Galactose oxidase, peroxidase	Lactose
Glutaminase	Glutamine

This book chapter briefly highlights the recent advancements of enzymatic biosensors' role in food quality analysis.

The food industry participates in the mandated food quality and safety assessment since they are essential for consumer health and nutritional value claims. The need for reliable and straightforward analytical procedures to ensure the quality of the food product from the production stage to consumption has recently been highlighted by an increase in demand for food preservation (Antiochia et al., 2013). The food manufacturing process is divided into primary and secondary processing. During main and secondary processing, toxicants from food additives may infiltrate food items. Toxicants such as drug residues, pesticides, and disease microbes may interact with the chemical compounds in food products during storage, causing food poisoning and rendering them unfit for human consumption. Enzymes are biological molecules that can sense the substrates and catalyze specific biochemical reactions. Like biosensors, devices can identify the specific material or analyte in the food samples. In a biosensor, bioreceptor and the transducer are considered essential components. Bioreceptor is used to screen the biomolecule, and they may be enzymes, antibodies, DNA, or cell. Enzymes were the most used bioreceptor molecules in biosensor applications. Enzymes enhance the reliability of biosensors since their catalytic effects are more specific and sensitive (Monosik et al., 2012). An enzymatic biosensor contains an enzyme to recognize the target analyte and mediate the reaction to produce the chemical signal. This transducer results in a physical signal from the chemical signal and an electronic amplifier. In addition, enzymes are considered promising bioreceptor molecules due to the broader role in the various analytical techniques, the flexibility in terms of transducers types like electrochemical, optical, and thermal modes, required in a low quantity, and the availability of high-quality commercial enzymes. However, enzymes were also associated with certain limitations like size, due to which they were not accessible to the relevant substrates, which in turn diminished the enzymatic activity. Enzymes reported with a limited lifespan may be deactivated by the enzyme-specific components in the sample. Various physicochemical parameters also influenced the enzymatic activity and cost of the commercially available enzymes (Marazuela & Moreno-Bondi, 2002; Borgmann et al., 2012). According to Vargas-Bernal et al.'s studies, enzymes used in the biosensor were classified into six major classes, and the oxidoreductases class was the most used enzyme in biosensor design (Vargas-Bernal et al., 2012). The first enzymatic biosensor was launched in 1962, and glucose oxidase was the first enzyme incorporated in the biosensor (Clark & Lyons, 1962).

Biosensors comprise biological receptors that can recognize a target, such as enzymes, aptamers, cells, antibodies, and so on. Electrochemical, optical, mass, and thermal methods are used in the binding process. Chemical biosensors based on enzymes are based on biological recognition. The enzymes must catalyze a particular biological reaction and be stable under the biosensor's usual working settings to function. The target analyte and matrix complexity in which the analyte must be quantified are used to build biosensors. Combining MBs (magnetic particles) with screen-printed electrodes (SPEs) is the best electrochemical biosensing compound

for food safety (Vasilescu et al., 2016). They are cheaper and can be obtained from various materials with a minimal level of sample volumes (Power et al., 2018). The enzyme-based sensor detects food contamination in various sectors of the food industry as shown in Fig. 11.3.

11.10.2 First-Generation Enzyme Sensors

In 1956, Leland Charles Clark Jr published research on the electrode used to measure oxygen levels in the blood (Clark et al., 1958). Electrochemical sensors and enzyme transducers were described as membranes at a 1962 Academy of Sciences symposium. Clark described the functioning enzyme electrode with glucose oxidase attached to the oxygen sensor later in 1967. Guilbault and Montalvo first described the potentiometric enzyme electrode in 1970. It works by immobilizing the enzyme urease on an ammonium-selective liquid membrane electrode. In 1973, they described a hydrogen peroxide-based glucose and lactate enzyme sensor using a platinum electrode. Klaus Mosbach invented a thermistor (heat-sensitive sensor) in 1974. In 1975, Clark introduced the glucose analyzer from Yellow Springs Instrument Company, which used an amperometric sensor to detect hydrogen peroxide. Lubbers and Opitz described a fiber-optic sensor with an immobilized indication to monitor carbon dioxide or oxygen levels. Later, by immobilizing alcohol oxidase, the optical biosensor concept was expanded to detect alcohol levels.

11.10.3 Second-Generation Enzyme Sensors

With the help of an artificial pancreas, Clemens and his colleagues built an electrochemical glucose biosensor. Miles later marketed it in 1975 as the Bio-stator (Gouda et al., 2002). Bio-stator was not accessible on the market. VIA Medical introduced a new semi-continuous catheter-based blood glucose analyzer. The lactate analyzer LA 640 was introduced by La Roche in 1976 to transport electrons from lactate dehydrogenase to an electrode.

11.10.4 Third-Generation Enzyme Sensors

Advanced biosensors have both immobilized enzymes and mediators in the same electrode. Liedberg used the surface plasmon resonance (SPR) method to track affinity interactions in 1983. In 1984, ferrocene and its derivatives were employed as immobilized mediators with oxidoreductases in the manufacture of low-cost enzyme electrodes based on screen-printed enzyme electrodes, according to Turner and his coworkers.

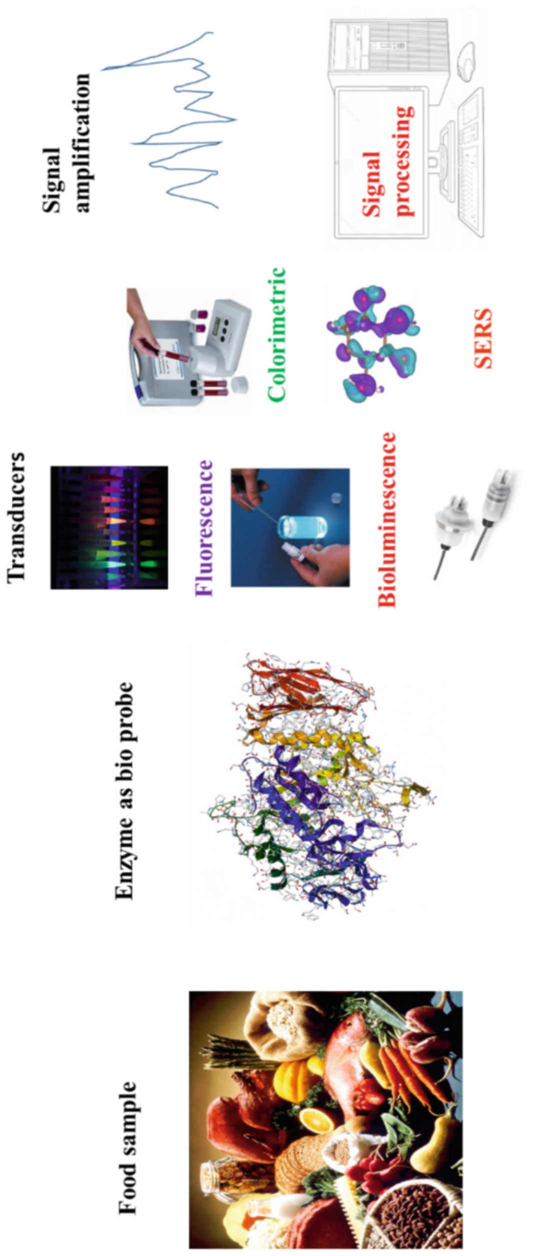


Fig. 11.3 Schematic representation of enzyme-based biosensor

11.11 Types of Biosensor

11.11.1 Electrochemical Biosensors

This biosensor employs an electrochemical transducer, in which metabolic reactions generate electrochemical signals, which are then captured using potentiometric, amperometric, or conductometric systems. It is a chemically distinct electrode with a biological coating covering the conducting substance. These sensors work with electrochemical sensors, which produce an electrochemical signal detected by an electrochemical detector when the electroactive analyte is oxidized or reduced. Voltammetric and potentiometric biosensors are two of the subgroups.

11.11.2 Voltammetric Sensors

Voltammetric sensors are subdivided into amperometric sensors. It measures the current potential features of a specific reaction's reduction or oxidation. Amperometric sensors work on the premise of applying a fixed voltage to an electrochemical cell, which results in a current due to an oxidation or reduction reaction. This current aided in the identification of the reaction's species. Unlike amperometry, which detects current using redox reaction products, this sensor employs glucose, lactate, and sialic acid analyzers. Electrochemical activity is seen in ion channel sensors. The cationic marker of electrostatic attraction or repulsion is improved or suppressed when these analytes are bound to a receptor molecule on an immobilized electrode surface. Voltammetric biosensors such as fumaric, maleic, and adenine nucleotides are employed with these sensors (Fig. 11.4).

The enzyme biosensor is characterized based on the substrate used for the food and drug samples (Amine et al., 2006; Tudorache & Bala, 2007). The hypoxanthine-sensitive sensors are used to detect fish freshness. Similarly, the xanthine oxidase-based biosensors are used to disclose the levels of hypoxanthine and xanthine, mainly involved in meat spoilage. To test the quality of the food products, oxygen-based biosensor has been used. Other than that, enzyme-based biosensors are used in food to maintain the freshness of edible products, detection of glucose level in beverages, analysis of cholesterol in butter, food components of sugars, and the detection of pathogenic organisms (Luong et al., 1991; Mello & Kubota, 2002). Some commercial biosensors used in the food industry are listed in Table 11.4 (Chapman et al., 2018).

11.11.3 Novel Analytical Tools

The nanotechnological interventions for food sensors provide significant benefits in identifying contaminants, especially in the food sector. Chemical nanosensors (chemical nanosensors) and biosensors (nano-biosensors) based on nanomaterials (nanosensors) can be utilized online and incorporated into existing production

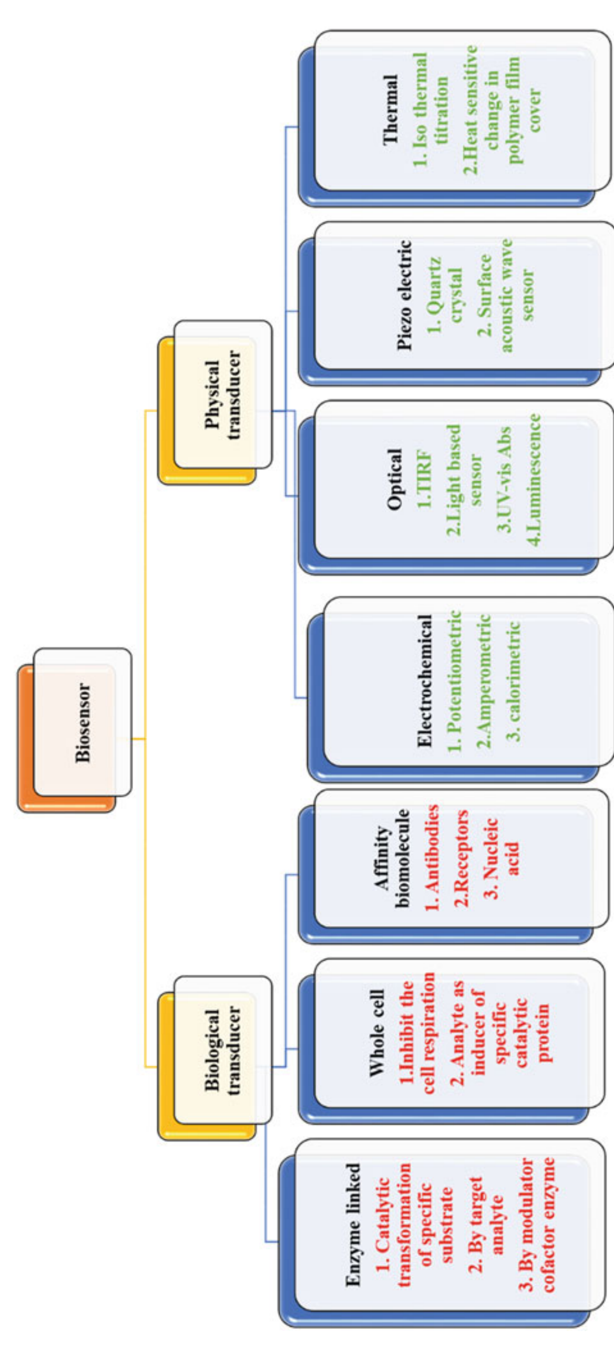


Fig. 11.4 Biosensors and their physical and biological elements.

Table 11.4 Commercial biosensors used in the food industry

S. N.	Company	Biosensor used to detect the materials
1	Affinity sensors	Toxin's detection
2	Ambri Ltd	Detection of *Salmonella*
3	Biacore AB	Detection of mycotoxins
4	Biosensor systems design	Detection of toxins
5	Biosensores S. L.	Detection of toxins
6	Chemel AB	Detection of lactose and saccharose
7	Georgia Research Tech Institute	*Campylobacter* and *Salmonella* detection in pork
8	IVA Co. Ltd	Detection of heavy metals
9	Massachusetts Institute of Technology	E. *coli* O157:H7 detection in lettuce
10	Michigan State University's electrochemical biosensor	*Salmonella* and E. *coli* O157:H7 detection in meat
11	Oriental Electric	Fish deterioration
12	Texas Instruments, Inc.	Detection of allergens
13	Universitat Autonoma de Barcelona in collaboration with CSIC	Detection of atrazine traces

processes and distribution lines or offline as quick, simple, and disposable sensors for food contamination. Food contaminants may be challenging to detect in this environment of chemicals. Nanosensors are an emerging technology that could be used to detect a variety of food pollutants, including mycotoxins and food allergies, due to their unique applications. Nanosensors are thus more cost-effective, quick, and sensitive than instrumental and traditional methods. Recent advancements in nanosensor technology may increase demand for its use in food contamination detection. As the food laboratory faces increased pressure to minimize cost, time, and complexity, the role of nanosensors will become increasingly more crucial. This chapter seeks to provide a broad overview of the potential applications of nanosensors in the detection and analysis of food contaminants. In recent years, the traditional PCR technique has been superseded by a simplified application in POC devices using multienzyme bioreceptors to determine DNA fragments' properties from allergens or adulterants. Surface platform resonance (SPR) characteristics and high conductivity are attributes of nanomaterials used in these sensor systems, such as Au NPs and AgNPs. This will make it easier to incorporate optical and electrochemical sensors. Magnetic nanoparticles, such as Fe_2O_3 NPs, will aid in the separation of analytes and will boost their performance.

However, carbon nanotubes and graphene-based nanomaterials enhance electrochemical signals due to their outstanding electrical conductivity. In 2017, electrochemical affinity biosensing was utilized to test food allergies, adulterants, and gluten. Andrei et al. (2016) used Nanoceria, a nanosensor device with oxidase-like activity, to covert phenolic antioxidants caffeic acid, gallic acid, and quercetin to quinones to measure wine's antioxidant content. He et al. (2018) developed manganese dioxide nanosheets to detect ascorbic acid levels in orange fruit and juice. The

hazardous chemical biphenol A (BPA) is released from the polycarbonate and epoxy resin during the food packaging process. Tyrosinase converts BPA to quinine to detect BPA NPs such as Au, Ni, or Fe_2O_3 (Alkasir et al., 2010).

11.12 Food Packaging

Biosensors play a vital role in detecting foodborne pathogens, and it plays a crucial role in food packaging as a part of smart packaging, which can be used in packaging materials to check their freshness and shelf life. As a part of smart packaging, the freshness indicator, time temperature indicators, integrity indicator, and radio frequency identification are used (Park et al., 2015).

11.13 Enzymes for Detecting Food Adulterants

Food is required for the survival of life. We all eat food to obtain energy for various metabolic functions. Food is required for all living species to grow, function, heal, and maintain life processes. There are many different types of food on the market today, and we all rely on diverse food sources daily, such as vegetables, fruits, grains, pulses, legumes, etc. Small stones in cereals and grains, darkly stained vegetables like cabbage and broccoli, fruits, dark red meat, and other items may have been encountered while shopping for fresh vegetables and other foods. Food adulteration is described as polluting food or food materials by adding a few substances, collectively known as adulterants. Adulterants are substances or products of lower quality that are added to food for economic or technical reasons. The inclusion of these adulterants lowers the nutritional value of food and contaminates it, rendering it unfit for ingestion. These adulterants can be found in various foods that we regularly consume, including dairy, cereals, legumes, grains, meat, vegetables, fruits, oils, and beverages. In underdeveloped countries, contamination or adding to food components is typical. Milk, for example, can be diluted with water to increase its volume, and starch powder is frequently added to increase its solid content.

The urease microbial enzyme from Arthrobacter creatinolyticus was immobilized on a poly (acrylonitrile-methyl methacrylate-sodium vinyl sulfonate) (PAN) membrane to create a potentiometric urea biosensor. Glutaraldehyde was used as a cross-linking agent in the biosensor. The following reaction is catalyzed by urease. The production of ammonium ions produced by the breakdown of urea by urease was used to detect urea in samples. A commercially accessible electrochemical workstation was used to perform potentiometric experiments. Milk samples were spiked with known urea concentrations and evaluated using the biosensor for real-world analysis. The measured potentiometric measurements were in good agreement with the spiking concentrations. The biosensor had a detection limit of 0.3 mM and linearity over the range of 1–100 mM. It was able to maintain appropriate sensitivity for 13 cycles in a row. It could be kept for 70 days at 4 °C.

Adulteration of food items is caused mainly by food firms' marketing strategies, mimicked food goods, a lack of awareness of proper food intake, and gaining profit with fewer inputs.

11.14 Methods of Food Adulteration

Adulteration is the illicit practice of increasing the number of high-quality products by introducing raw and other low-cost materials. This tainted food is highly hazardous and causes various health problems, including nutrition deficiency diseases, kidney disorders, and organ failure, including heart, kidney, and liver failure.

11.14.1 Melamine

Melamine is a commercially manufactured chemical molecule (1,3,5-triazine-2,4,6-triamine). Inflating nitrogen content and proteinaceous substances in food is a significant food adulteration. Melamine's specificity and ability can be determined using two approaches. First, commercial enzyme-linked immunosorbent assay (ELISA) kits were tested using ammeline, ammelide, or cyanuric acid, revealing that 4,6-diamino-1,3,5-triazine structure, or 1,3,5-triazine-4,6-diones were required. Melamine detection limits in infant powder ranged from 0.2 to 3 mg/g per sample (Kim et al., 2010). This melamine produces significant toxicity and kidney stone formation (Hau et al., 2009). Brown et al. (2007) labeled it a hazardous substance because it causes renal failure by forming insoluble melamine cyanurate crystals in the kidneys. Nanosensor to detect melamine in various feed matrices quickly, Au nanoparticles, cadmium telluride (CdTe) quantum dots, SWCNTs, and other sensors are used (Li et al., 2010). Zhou et al. (2011) employed an Au nanoparticle biosensor to detect melamine in various milk and dairy products. Milk powder, infant formula, lactose, whey protein, wheat bran, and wheat gluten are all examples of foods that include it (Mecker et al., 2012). Melamine was also detected using water-dispensable cadmium telluride quantum dots coated with thioglycolic acid.

11.14.2 Meat Adulteration

The demand for ready-to-eat halal foods such as burgers, pizzas, hot dogs, sandwiches, soups, cookies, sweets, and creams has risen recently (Jeddah, 2011; Mascini, 2015). Pork is being used more frequently in Halal meals, including beef meatballs, to maximize profits (Mascini, 2015). It was identified utilizing NPs as a colorimetric nanobiosensor, which changed the color of the meat from pinkish-red to purple with a 525 nm absorption peak. Ali et al. (2011) used hybrid biomaterials with functionalized nanoparticles that are noncovalently or covalently coupled to biomolecules such as peptides, proteins, and DNA to detect pork adulteration in processed meat.

Several adulterants such as food coloring compounds and preservatives have been found with modern technology. Olive oil, milk, honey, and saffron are some of the most commonly reported contaminated foods, which can be discovered using HPLC and infrared spectroscopy (Moore et al., 2012). The adulterants included in butter, pork, and beef meatballs were detected using Raman spectroscopy and FTIR spectroscopy. Adulterants such as sunflower and corn oils commonly replace olive oil and can be detected using FTIR spectroscopy.

11.14.3 Enzymes for Detecting Contaminants

Food adulteration can arise from numerous sources, such as natural toxic compounds, bacterial contamination, pesticides, veterinary drugs, and chemicals used in food dispensation. Identifying this substance is essential for people affected by diseases (Viswanathan et al., 2009). It has been mostly separated into two classes: biological and chemical contaminants. Some critical assays analyze the phytocompound such as sugars, alcohols, amino acids, flavors, and sweeteners. Chromatography and spectroscopy have been used to detect contaminants from the olden days. Currently, many sensors have been developed to detect the presence of these contaminants in food without affecting their taste and flavor.

11.14.4 Biological Contaminants

Toxic and pathogenic biological substances created during food preparation, packing, and storage are biological pollutants. Toxins such as aflatoxins, ochratoxins, and other foodborne microorganisms are the most common pollutants that cause foodborne illnesses. Pathogenic bacteria *include Campylobacter, Salmonella, Listeria monocytogenes, E. coli, Staphylococcus aureus*, and *Bacillus cereus*. Toxicology can be detected using a variety of approaches. The most prevalent mycotoxins generated by Aspergillus spp. are aflatoxin and ochratoxins. It is cancer-causing, teratogenic, mutagenic, and immunosuppressive. A nanosensor has been created to detect this mycotoxin using the immunosensor approach. (Sharma et al., 2010). Using antibody-conjugated MNPs and a magnetic field, Salmonella in milk was detected. Sack et al. (2004) defined Vibrio cholera as an acute intestine infection induced by eating tainted food or water. Cholera toxin, a colorimetric nanobiosensor detected with glycol nanoparticles, is secreted by this bacteria (Schofield et al., 2007).

11.14.5 Chemical Contaminants

Unwanted dangerous chemicals are introduced into foods directly or indirectly from natural sources, pollution, or formation during food processing, distribution, and storage. Pesticides, heavy metals, and melamine are just a few chemical pollutants

Table 11.5 Nanobiosensors used in the detection of chemical and biological contaminants

Nanomaterial	Contaminants	Biosensor
Biological		
Gold nanoparticle	Aflatoxin	Immunoelectrode
Silver core and a gold shell	AF B1	Immuno-dipstick assay
Antigen-modified and antibody-functionalized DCNPs	AF B1 and OTA	Immunosensing probes
Nanostructured zinc oxide	Mycotoxin	ITO glass plate
Magnetic nanoparticles and TiOz nanocrystals	*Salmonella*	Optical nanocrystal probes
Oligonucleotide-functionalized Au nanoparticles	*Escherichia coli*	Piezoelectric biosensor
Glyconanoparticles	Cholera toxin	Colorimetric bioassay
Chemical contaminants		
Gold nanoparticle	Carbofuran	Electroimmunosensor
Antibody competitive nanoparticle	Chloramphenicol	Immunosensor IPR
MTT-stabilized gold nanoparticle	Melamine	Colorimetric sensor
Quantum dots	Melamine	Colorimetric sensor
CNTs	Penicillin	Enzyme-based biosensor

that harm human and animal health. Nanosensors have the potential to be a helpful instrument for detecting chemical pollutants. Table 11.5 shows a variety of nanosensors and nanobiosensors for detecting chemical and biological pollutants.

11.14.6 Biosensors and Bioamines to Quantify the Freshness of Food Products

Chemical compounds produced in surrounding storage due to the decomposition of food components served as a vital signal for food freshness. The concentrations of xanthine, hypoxanthine, and biogenic amines, for example, have altered over time. The breakdown of inosine monophosphate in seafood produces chemicals like xanthine and hypoxanthine, which act as biomarkers for fresh food. Biogenic amines (BA), such as histamine, putrescine, and cadaverine, are also generated by the decarboxylation of amino acids and are used as freshness markers in meat, drinks, and cheese (Calvo-Pérez et al., 2013). Bioamines are essential for numerous physiological activities at low concentrations, but they can cause health problems in consumers. BAs were produced in plants, animals, and microbial populations by pathogenic microbial decarboxylation of specific amino acids, such as histidine, or by the amination and transamination of ketones and aldehydes.

In contrast, others have aromatic structures, such as Phenylethylamine (PEA) and Tyramine (Tyr) (Tyr). Then there are a few BAs that have heterocyclic structures, such as Tryptamine (Tryp) and Histamine (Hist) (His). The presence of BAs in dietary samples was determined using the time-consuming high-performance liquid chromatography (HPLC) method (Hernández et al., 2006). BA's biosensors were

designed as an effective strategy for facilitating quick and exact estimation in the search for a steady approach to accurate discoveries. Many amine oxidase enzyme designs have been used to construct biosensors, to increase precise biorecognition and signal transduction.

11.14.7 Biosensors and Histamine Oxidase Applications

Substances like histamine were biogenic amines. Their presence varies with degrees in several food products, and their increased concentration is considered an indicator of the depleted level of freshness. Histamine intoxication is the most prevalent foodborne infection among humans who consume fish products. If histamine concentration exceeds 500 ppm in fish products induces histamine poisoning (Gonzaga et al., 2009). Bacterial contamination in fish gut and skin induces histidine decarboxylase activity and results in histamine production. Histamine levels were screened by immobilized histidine decarboxylase and horseradish peroxide loaded on the electrodes using the bovine serum albumin and glutaraldehyde complex. Another study used the histamine oxidase enzyme from the bacterial sp. *Arthrobacter crystallopoietes* (HOD) to build a biosensor. HOD biosensor was effectively employed to measure histamine levels in fish and meat products, comparatively yielding a good agreement with the results obtained from HPLC analyses (Rosini et al., 2014).

11.14.8 Biosensors to Quantify D and L Amino Acid

Amino acids have been extensively researched due to their nutritional importance and speed up metabolic processes. D-amino acids (D-AAs) are, on the other hand, primarily found in microbiological, aqueous, soil, and other environmental pollutants. In general, the presence of D-AAs reduces protein digestion in the body, resulting in decreased bioavailability of essential amino acids and nutritional value. D-AAs are also utilized to evaluate the adverse effects of heat and alkaline treatments on dairy products. D-AA biosensors based on the D-amino acid oxidase (DAAO) enzyme were frequently employed to evaluate milk samples (Sarkar et al., 1999; Rosini et al., 2008). The FAD domain is a cofactor expressed in humans, not bacteria, in this peroxisomal enzyme.

11.15 Biosensors and Antioxidants' Concentration

The antioxidant activity of phenolic chemicals found in berries has been shown to help prevent cardiovascular disease and aging and have anti-cancer properties. Since the TYN enzyme converts hydroquinone in foods like red wine to p-quinone, it then reduces to hydroquinone at the electrode and generates a current. The TYR enzyme from Agaricus bisporus is an oxidase enzyme that catalyzes the conversion of

phenolic substances to quinone derivatives, with amperometric techniques detecting the end products. The TYR biosensor can detect the "total phenolic content" and "total antioxidant capacity" of samples with low non-polyphenolic antioxidant levels in general. The reduced lifespan of the Tyr enzyme reduces its stability. The tert-butyl hydroquinone chemical quantities in standard salad dressing items were also measured using the Tyr biosensor.

11.15.1 Biosensors for Heavy Metals

In recent days, heavy metal-induced toxicity among the food has been observed and studied extensively. Heavy metals like Fe, Zn, Cd, Ni, Mn, Cr, Co, Pb, Cu, and Al were leached from the eating utensils as well as cookware made up of iron, old stainless steel, new stainless steel, old and new aluminum, and clay pots lead to metallic contamination of food and water (Ghorai et al., 2009). In practice, the usage of enzyme-based biosensors is used to detect these toxic substances like heavy metals. Since these heavy metals react with the thiol groups in the enzymes like glucose oxidase, urease, glutathione S-transferase, alkaline phosphatase, lactate dehydrogenase, acid phosphatase, and invertase and biosensors, the function is based on enzymatic inhibition. Nevertheless, limited enzymes have an affinity to heavy metals.

11.15.2 Biosensors and Ethanol Concentration

Assessing the ethanol concentration and control is vital in brewing and winemaking and also needs the exact data about the ethanol content in the beverages. Immobilized alcohol oxidase catalyzes the reaction on the ethanol biosensor. Earlier studies on ethanol biosensors used the combination of enzymes of alcohol oxidase (AOx) along with horseradish peroxidase (HRP) and a mediator ferrocene immobilized on an electrode. Later, Shkotova et al. established an amperometric transducer and AOx-based biosensor immobilized on resydrol polymer and used to detect ethanol concentration in alcoholic beverages. Another enzyme, pyrroloquinoline quinone alcohol dehydrogenase (PQQ-ADH), is used in amperometric biosensors to detect ethanol. PQQ-ADH has advantages like oxygen independence and does not require a soluble cofactor. Certain studies employed the bi-enzymatic biosensor to detect the ethanol concentration in alcoholic beverages.

11.15.3 Fructose Dehydrogenase-Based Biosensors

Because fructose is harmless, it is commonly employed in artificial sweeteners. Fructose dehydrogenase (FDH) is a human enzyme that produces fructose in the intestinal mucosa. This enzymatic process is also used to make corn syrup in corn plants (Siepenkoetter et al., 2017). Fructose corn syrup was thought to be a better

substitute for natural sugars. As a result, fructose has been used to manufacture beverages and foods (Antiochia et al., 2013).

On the other hand, overuse of fructose has been associated with high blood pressure, decreased glucose tolerance, insulin resistance, and hepatic steatosis. For many years, biosensors based on FDH have been used to detect fructose content in food and beverages. To immobilize the NDFDH-modified nanoporous gold electrodes, FDH was coupled with a thiol and diazonium-bound carboxylic acid functional group. The fructose level in beverages may be accurately measured using this manufactured electrode.

11.15.4 Xanthine Oxidase-Based Biosensors on Assessing the Fish Freshness

Xanthine is a thoroughly studied fish freshness indicator. Adenosine triphosphate (ATP) concentration in stored fish steadily degraded to generate xanthine as the storage period extended beyond the permissible interval (Venugopal, 2002). On the other hand, manufacturing needs fresh fish for human consumption and fish products. Thus, a few decades ago, investigations began using electrochemical xanthine biosensors as a biomarker to assess the freshness of fish. However, the stability of this biosensor is restricted; thus, Devi et al. devised a xanthine oxidase–chitosan–gold-coated nano-iron modified pencil graphite electrode with a detection limit of 0.1 μM to increase the stability. This biosensor's xanthine detection and storage precision have been increased. Dervisevic et al. developed a CHIT–polypyrrole–AuNP-modified glassy carbon electrode (GCE) with XOD as an electrocatalyst that converts xanthine to uric acid in a follow-up study. With a detection limit of 0.25 μM, this modified amperometric sensor could efficiently detect xanthine.

11.15.5 Lactate Dehydrogenase-Based Biosensors on Quantifying the Lactate Concentration

Lactate concentrations in red wine, white wine, beer, yogurt, curd, and milk samples were used as biomarkers to determine the food quality. The food industry utilized lactate biosensors to detect the desired level of lactate content in fermentation. Lactic acid fermentation is induced by *Lactobacillus* spp. in fermented food products, and the lactate content suggests an enhanced *Lactobacillus* population. Batra et al. created an electrochemical lactate sensor using a PGE modified with graphene oxide (GO) nanoparticles and LDH in 2016. This upgraded LDH–GO–PGE lactate biosensor is currently being utilized to assess lactate levels in fermentation industries (Batra et al., 2016). Lactose-related biosensors were also frequently employed, as lactose intolerance affects roughly 15% of Northern Europeans, 80% of black and Latino persons, and almost 100% of American Indians and Asians. These individuals lack the digestive enzyme galactosidase, which converts lactose to

galactose and simple glucose. Electrochemical biosensors containing co-immobilized-galactosidase and glucose oxidase enzymes were created to assess the trace amount of lactose in lactose-free dairy products.

11.16 Biosensors in Mycotoxins Detection

Fungal species *Aspergillus, Fusarium, and Penicillium* produce specific secondary metabolites that cause food poisoning among humans and animals after ingesting contaminated food products (Turner et al., 2009). Contaminated fruits and cereals induce mycotoxin in certain beverages like beer and wine. Sometimes contamination of livestock animals' feed during the processing also introduced the mycotoxin in food products (Becker-Algeri et al., 2016; Iqbal & Selamat, 2016). Thus, residual mycotoxin presence needs to be evaluated to ensure the food product quality. Mycotoxins were categorized into distinct groups, namely aflatoxins, ochratoxin A (OTA), zearalenone (ZEN), fumonisins, ergopeptine alkaloids, and trichothecenes based on their impact on the economy and associated health risk. Enzymatic electrochemical sensors were developed by "Grupo de Electroanalítica" GEANA (Argentina) using enzyme horseradish peroxidase to assess the CIT in rice.

11.16.1 Epidemiology of Foodborne Illnesses

Foodborne infections that are infectious and toxigenic have been known for more than a century. *Salmonella, Staphylococcus aureus,* and *Clostridium perfringens* were the most common pathogens of concern worldwide by the mid-1900s. Botulism was a fatal disease rarely linked to commercially canned foods, home canning of vegetables, or traditional Arctic marine mammal products.

There was little interest in or research on acute foodborne illness agents during those days. According to what was learned during outbreaks, most outbreaks might have been averted if adequate time and temperature handling and storage protocols had been followed, especially with poultry and beef. *Clostridium, Salmonella, and Staphylococcus* issues were mainly resolved after the population was educated. However, additional agents like *Campylobacter, E. coli* O157:H7, *Listeria*, and *Yersinia* species emerged in the 1980s. It took many more years to realize that these disorders might lead to life-threatening complications or even death and that several goods could transmit them. Only a few of these diseases are detectable by existing monitoring systems (Kothary & Babu, 2001; Wang et al., 2010; Fukushima et al., 2011; Lindström et al., 2011). However, more and more international outbreaks are being recognized thanks to DNA typing and next-generation sequencing technology.

11.16.2 Worldwide Status of Foodborne Illnesses

Despite the adoption of new regulatory concepts, know-how, and initiatives on a national and international level, there has been a broad plateau in the number of instances of foodborne disease. The fundamental reason for this is that surveillance of waterborne and foodborne illnesses has been limited in detecting instances other than small clusters of patients. For decades, the traditional system of making outbreak reports available to a central authority in a small number of countries has been the source of our knowledge, but it is far from adequate.

Only significant outbreaks are reported, whereas home infections are never reported. As a result, epidemics in mass catering and restaurants are widely highlighted. Another issue that raises the risk of foodborne disease is the global food market. Changes in lifestyle and activities, more extensive operations, and higher throughput lead to increased profit, increasing the risk of contaminated animal products. Even if the livestock is not ill, larger-scale animal rearing allows for more microbial diseases. Antibiotics are mainly used to aid in the growth of animals. However, widespread use has led to growing antibiotic resistance and inappropriate use for self-treatment of human illnesses, posing a more significant threat to the global population. Other contamination sources, including rats and rodents in warehouses, have been identified as critical connections in the transmission chains of zoonoses.

Newer strains in humans are derived from environmental sources, where intergeneric gene transfer may occur. Larger markets result in a more significant geographic dispersion worldwide, resulting in massive outbreaks with millions of patients if problems arise. This is an example of how tracing a product's origins can be challenging, mainly if the originating company is located in another country. People are introduced to new meals by immigrants, and the public requires a diverse range of products to consume. This can lead to new goods or modifying current ones, risk introducing a new pathogen at each stage (Alvarez-Ordóñez et al., 2013).

11.17 Bacterial Pathogens

11.17.1 Staphylococcus aureus

Because of contamination from humans, cattle, and the environment, several *Staphylococcus* genus species and subspecies are potentially harmful to foods. Both coagulase-negative and coagulase-positive strains can produce enterotoxins that cause human gastroenteritis. *S. aureus* is most commonly associated with food poisoning. Enterotoxins are the most dangerous of the several toxins produced by staphylococci. Enterotoxins are proteins generated by other Staphylococci strains that, if allowed to flourish in foods, form enterotoxin. *S. aureus* is the primary producer of structurally related, toxicologically comparable proteins (Lancette & Bennett, 2001).

Meat and beef products, poultry and egg products, salads such as bacon, fish, rice, potato, and macaroni, baking products such as cream-filled pastries, and cream pies are the most common foods associated with staphylococcal food poisoning. Some of these products are polluted and mismanaged as a result. Chemical, animal, or environmental contaminants can contaminate pre-processed foods. At the same time, foods exposed to temperatures favorable to *S. aureus* growth have a significantly greater capability to form enterotoxin. This is also true of meat and milk products that have been fermented. Secreted enterotoxins are a structurally and functionally related family of virulence factors with critical super-antigenic qualities that disrupt adaptive immunity. Standard enterotoxins (SEA-SEE) and modern enterotoxins are two types of enterotoxins (SEG-SElY).

The pathophysiology of several human diseases, such as toxic shock syndrome (TSS), sepsis-related infections, and pneumonia, is determined mainly by members of these classes. These have emetic properties and are frequently linked to food poisoning outbreaks. The enterotoxins are primarily responsible for the disease's consequences when the patient lacks denaturation resistance. The enterotoxin genes are frequently found on many mobile genetic elements. The prevalence of enterotoxins varies greatly among *S. aureus* isolates, and it is regulated by numerous overlapping regulatory mechanisms (Lancette & Bennett, 2001).

11.17.2 Listeria monocytogenes

Listeria monocytogenes causes occasional outbreaks and ingestion associated with infected food products as another significant cause of death due to foodborne disease. Over the last few decades, *Listeria monocytogenes* have emerged as a foodborne pathogen of considerable importance. Most of our knowledge about foodborne *Listeria* transmission routes has been acquired through the study of worldwide epidemiological evidence. Such outbreaks were related to food from hospitals patients who had consumed spinach, carrots, and radishes.

The use of manure from infected sheep has been blamed for several outbreaks. Most listeriosis infections in pregnant women have resulted in miscarriages, stillbirths, and sporadic or live deliveries of poorly infants. Meningitis, aspiration pneumonia, and sepsis symptoms were seen in many non-pregnant people who showed no evidence of immunosuppression. These outbreaks accounted for around 41% of the overall death rate. Serotype 4b was assigned to some of these isolates, and *L. monocytogenes* isolates from coleslaw packets were known as Serotype 4b.

Over the past few years, whole-genome sequencing has proven to be a valuable tool in *L. monocytogenes* surveillance and outbreak investigations. WGS has allowed rapid and reliable identification of infection clusters, helped trackback to sources of infection, and ultimately removed the origin of infection. Various projects have been initiated to encourage the typing of *L. monocytogenes* based on WGS and many other foodborne pathogens. Nevertheless, there remains a need to create a transition to an international standard. (Camargo et al., 2017; Colagiorgi et al., 2017).

11.17.3 Bacillus cereus

Many extracellular compounds involved in pathogenesis are found in *Bacillus cereus,* and many strains are employed as probiotics in humans. On the other hand, others are highly poisonous and linked to dietary deaths. There are two forms of foodborne sickness caused by these genera: diarrheal and emetic. Enterotoxins produced in the small intestine during pathogen vegetative growth cause the former, while the emetic toxin is pre-formed in the meal before ingestion causes the latter. The disease is spread via food because of the surviving spores in both forms. *B. cereus,* in addition to being an opportunistic pathogen, can cause a variety of systemic and local illnesses, including septicemia and endophthalmitis. The diarrheal disease is typically self-limiting, and the number of *B. cereus* outbreaks is widely underestimated due to the relatively short duration of the disease (typically less than 24 h). The total recovery is swift after the symptoms resolve.

The *B. cereus* community comprises many species whose members are capable of causing foodborne emetic or diarrheal diseases. With the lack of modernity in this pathogen's diagnostic and characterization aspects, tens of thousands of diseases have been documented in the world each year. Still, the issues are far from conquered. Today, methods such as whole-genome sequencing are not widely used to classify *B. cereus* isolates from foodborne diseases. In addition to using state-of-the-art genomic methodologies, it is possible to use WGS techniques to address future microbiological and epidemiological problems raised by *B. cereus* outbreaks in the group. One specific obstacle is that, unlike other pathogens, cases of disease caused by *B. cereus* are usually not reportable, while foodborne diseases are widely documented, regardless of pathology. When mixing this with a usually moderate course of *B. cereus* disease, human isolates of the *B. cereus* are rarely available for techniques such as WGS.

In addition, even if clinical isolates of the *B. cereus* group are obtainable, WGS should not be used to describe the isolate in cases where the disease or infection is mild. The data and methods available from laboratories around the world not just enable further detection of other outbreaks of *B. cereus.*

11.17.4 Clostridium perfringens

Clostridium perfringens produces two diseases transmitted through food: a common foodborne illness and necrotic enteritis (pig-bel). Ingestion of infected foods causes *C. perfringens,* one of the most frequent foodborne infections. While the link between *C. perfringens* and foodborne sickness was first proposed over a century ago, definitive evidence demonstrating that an enterotoxin is linked to the organism's sporulation in diseased intestines did not develop until the 1960s and 1970s. The bacteria have many characteristics that contribute to its capacity to cause foodborne illness: (1) an omnipresent distribution throughout the natural environment, allowing it to contaminate food; (2) the ability to develop rapidly in food, causing high levels

of food poisoning; and (3) the ability to produce an intestinally active enterotoxin, which is responsible for *C. perfringens'* characteristic gastrointestinal symptoms.

C. perfringens-related food poisoning is one of the most common causes of food contamination. The association between *C. perfringens* and foodborne sickness was originally postulated around 100 years ago, it was not until the 1960s and 1970s that conclusive evidence emerged demonstrating that an enterotoxin is linked to the organism's sporulation in the intestines. The bacteria have several characteristics that contribute to its capacity to cause foodborne illness:

1. It has a wide distribution in the natural environment, allowing plenty of opportunities to contaminate food.
2. Its ability to generate heat-resistant spores allows it to survive incomplete food cooking or insufficient sterilization.
3. The ability to produce an intestinally active enterotoxin is responsible for *C. perfringens* food poisoning's typical gastrointestinal symptoms.

However, unlike other foodborne illness agents, it is frequently underreported, owing to the anaerobic conditions required for its development and the sickness's apparent mildness. Meat and poultry-based products, as previously stated, are the most prevalent carriers via which intestinal contents spread after slaughter. The presence of this organism in the spore condition on herbs and spices (as well as meat and poultry) poses a hazard since it can survive the chilling cycle and restart growth in temperature-abused or inadequately cooled items, resulting in the enormous number of cells needed for an outbreak. The organism's specific development requirements are easily met in meat and poultry products.

Temperature exploitation of cooked meals is often linked to outbreaks, showing that the organism's optimum development temperature is relatively high [43–46 °C]. This, together with its ability to form spores, are two remarkable traits contributing to its etiological role in foodborne disease. The sickness is more likely to be purchased in restaurants, hospitals, jails, schools, and caterers (Lindström et al., 2011).

11.17.5 Clostridium botulinum

Botulism comes in a variety of types. Symptoms of foodborne botulism can appear within 12–36 h and can take up to a week or longer after consuming botulinum neurotoxin-containing food. Initial symptoms include nausea and vomiting, though it is unclear whether the neurotoxin or other *Clostridium botulinum* compounds are to blame.

The organism may get established in the intestines of young infants, most likely because the normal intestinal flora has not yet developed sufficiently to prevent pathogen colonization. Chronic constipation, severe exhaustion, and increasing paralysis, as well as other neurological symptoms, may be present in the patient. In some instances, spores of *C. botulinum* have been discovered in specimens from

children who died of sudden infant death syndrome (SIDS) (Health Protection Agency, 2011).

11.17.6 *Salmonella* Species

Serotypes of *Salmonella* can cause localized gastroenteritis in humans, but the severity of Salmonella infections in different hosts is determined by pathogen virulence, host resistance, and immunity. Many serotypes have limited host ranges, such as *Salmonella typhi* and *Salmonella paratyphi* A, linked to human infections, and *Salmonella gallinarum,* linked to poultry infections. Many serotypes have limited host ranges, such as *S. typhi* and *S. paratyphi A,* linked with human infections, and *S. gallinarum,* associated with poultry infections.

Salmonella serotypes Typhimurium and Heidelberg have a broad host range and can colonize and infect various animal hosts. Human infections of *S. typhimurium* are caused primarily by transmission-contaminated foods such as poultry items. Chicken eggs and egg products have been closely connected to *Salmonella enteritidis*. This could be linked to its apparent proclivity for infection and reproductive tissue invasion in sensitive chickens, as well as contamination of internal shell eggs.

Nonetheless, because there are so many cases of salmonellosis from non-poultry and non-food sources, such as domestic mammalian and reptile pets, any attempt to assign quantifiable rates to specific sources is futile. Bacteria may colonize and develop in the small intestine, penetrate and dwell in intestinal tissues, and cause an inflammatory response in the host when people eat a Salmonella infectious dose. *Salmonella* can enter the body through macrophages or other immune cells and go to lymph nodes in the liver, spleen, or mesenteric arteries.

11.17.7 *Shigella* Species

Shigella is spread through fecal–oral, feces, direct touch with people (fingers), food, flies, and inanimate items (fomites). More than two-thirds of all shigellosis cases occur in youngsters aged one to five who appear to use their lips to explore their surroundings. Intake of contaminated water is frequently cited as a method of shigella transmission. When people are crammed together, disease transmission and bacillary dysentery epidemics are intensified. Inadequate sanitation creates an environment for direct fecal–oral contamination, such as in daycare centers, custodial institutions, psychiatric hospitals, nursing homes, and mass displacement and gathering, such as in wartime refugee camps or political unrest.

In addition, those infected with the human immunodeficiency virus (HIV) might develop more severe and chronic types of shigellosis. Secondary attack rates among household contacts could be as high as 40% following primary case exposure. Shigellosis outbreaks appear to follow seasonal patterns in some areas, with

transmission peaking in arid regions like Egypt's hot, dry season. During times of water scarcity, it is primarily ascribed to dirty water use and poor personal cleanliness.

On the other hand, peak events occur in China and Thailand during the rainy season because of water-washed-based transmission after heavy rains. These represent the link between shigellosis and the unclean settings that encourage fecal transmission. Shigellosis is most common in developing countries during the summer months, when raw foods, fresh fruits and vegetables, and recreational facilities are most plentiful. Years later, the proportion of *S. flexneri, S. sunny, S. boydi,* and *S. dysenteriae* cases in developing countries was estimated to be 60% (mainly serotype 2a), 15%, 6%, and 6% (30% of *S. dysenteriae* cases were type 1), respectively; and 16% (predominantly serotype 3a), 77%, 2%, and 1% in developed countries, with nearly half of travelers registering incidents and returning travelers from (Kothary & Babu, 2001).

11.18 The Foodborne Vibrios

11.18.1 Vibrio vulnificus

Vibrio vulnificus is the opportunistic bacteria that cause foodborne illness and devastating infections in humans. Ingestion of raw or undercooked seafood, contamination of an open wound, or a sore contaminated with seawater or seafood drippings are all possible entrance points. *V. vulnificus* produces wound infections by entering existing or parallel wounds, ulcers, and insect bites, in addition to being foodborne. Exposure to seawater or shellfish leaking from a cut is nearly always the cause of these wounds. Puncture wounds from a bite from a marine animal (e.g., crabs, stingrays), stabbing or laceration wounds from the use of shellfish/fish cleaning equipment, lacerations from commercial or recreational use of fishing gear, rocks, or debris in or near seawater, or contamination of an existing wound or insect bite with seawater or shellfish drippings are all reported wound scenarios.

Puncture wounds from a bite from a marine animal (e.g., crabs, stingrays), stabbing or laceration wounds from the use of shellfish/fish cleaning equipment, lacerations from commercial or recreational use of fishing gear, rocks, or debris in or near seawater, or contamination of an existing wound or insect bite with seawater or shellfish drippings are all reported wound scenarios. Because *V. vulnificus* is associated with various seafood species, the health status is unknown. The human sickness produced by this organism and other marine vibrios could become a growing food safety concern. This problem is exacerbated by the fact that demand for seafood is increasing, and it can now only be fulfilled by aquaculture and marine fisheries sources harvesting seafood. These issues highlight the importance of monitoring systems to track the safety of our seafood (Kothary & Babu, 2001).

11.18.2 Vibrio parahaemolyticus

Most foods implicated with *V. parahaemolyticus* infections are seafood or similar foods, such as raw seafood, cooked seafood, fine seafood, and seafood. Tuna, shellfish (oysters, clams, etc.), crab, lobster, and shrimp were among the seafood used. In Japan, a majority of cases are caused by eating raw fish. However, this is not often the case in other countries, where cases are frequently caused by poor preparation, recontamination, or storage. In cases of food poisoning, foods that do not include marine elements may be contaminated by secondary contamination.

In Japan and the United States, *V. parahaemolyticus* food poisoning incidents are only reported during the summer. However, in Thailand, cases are reported all year. The seasonal change in the prevalence of infection may be linked to the epidemic strain's seasonal occurrence in the marine environment. While several reports demonstrate *V. parahaemolyticus* distribution in the aquatic environment in various world regions, epidemiologic research assessing the occurrence of virulent strains in both clinical specimens and the marine environment in an endemic context is rare (Parveen et al., 2008).

11.18.3 Vibrio cholerae

According to epidemiological data, fecally infected water is the predominant mode of cholera transmission. Food has recently been recognized as a life-threatening factor in cholera transmission. Seafood, particularly mussels, oysters, and shrimp, has been implicated when consumed uncooked, undercooked, or prepared with dirty water. While *V. cholerae* can be found in rice, fish, eggs, and milk, environmental studies in Bangladesh have found that most foods are not infected with the bacteria.

Cholera expanded through seaports and river ports during the seventh pandemic, particularly in Africa during the 1970s epidemics. Patients with active disease are regarded to be the predominant source of infection. Asymptomatic carriers may play a role in cholera transmission. However, this is not required. El Tor vibrios appear to live longer in the carrier form than Classical vibrios, but this difference is not epidemiologically significant. It has been demonstrated that *V. cholerae* O1 can survive in the aquatic environment without the help of zooplanktons and that *V. cholerae* is connected with a variety of aquatic flora, including water hyacinth duckweed and green algae. The blue–green algae have been discovered. *V. cholerae* is found in Anabaena variabilis, which serves as a reservoir. It has been demonstrated that *V. cholerae* O1 can survive in the aquatic environment without the help of zooplanktons and that V. cholerae is connected with a variety of aquatic flora, including water hyacinth duckweed and green algae. The blue–green algae have been discovered. *V. cholerae* is found in *Anabaena variabilis*, which serves as a reservoir.

Similarly, the recent isolation of toxigenic *V. cholerae* O1 from coastal waters in the United States and Australia, where cholera cases were unknown, suggests that these species have adapted to the aquatic environment as a natural microhabitat.

Although cholera is a watery illness, new evidence suggests that food, particularly seafood, plays an essential role in cholera transmission. Foods are likely to be fecally contaminated during preparation, primarily if the diseased food handlers work in an unsanitary setting. High moisture content, neutral or alkaline pH, low temperature, high organic content, and lack of other competing bacteria are physicochemical features of foods that promote *V. cholerae* O1 survival and development. Seafood, such as fish, shrimp, crabs, oysters, and clams, have been blamed for cholera epidemics in various nations, including the United States and Australia.

Dirty rice, millet gruel, and vegetables have been linked to epidemics. Cholera can be transmitted through various foods, including fruit (excluding sour fruit), chicken, meat, and dairy products. Food must be made and consumed in a sanitary environment free of feces to prevent foodborne cholera transmission hazards. To reduce foodborne cholera transmission, proper food preparation, storage, and reheating before eating and hand washing with clean water before and after defecation are beneficial health interventions (Sack et al., 2004; Parveen et al., 2008).

11.18.4 Yersinia enterocolitica

Effective health practices preventing foodborne cholera transmission include adequate food preparation, storage, reheating before eating, and hand washing with clean water before and after defecation. According to studies, the potential of *Yersinia enterocolitica* to thrive and develop in chemically contaminated foods under varied storage circumstances has been proven to be better at room temperature and refrigeration than at moderate temperatures. Cooked meals allow *Y. enterocolitica* to survive longer than raw foods, probably due to better nutrient availability and the presence of certain psychrotrophic bacteria, including non-pathogenic *Y. enterocolitica* strains. Bacterial growth in uncooked food can be inhibited. Within 24 h or 10 days, a viable number of *Y. enterocolitica* will increase by more than one million on cooked beef or pork at 25 °C, or 7 °C. Raw beef and pork grow at a slower rate. *Y. enterocolitica* can grow at refrigerator temperatures (soybean curd). Pork, cattle, lamb, chicken, and dairy products, particularly milk, butter, and ice cream, are all potential hosts for *Y. enterocolitica. Y. enterocolitica* can be found in various terrestrial and freshwater habitats, including soil, wetlands, lakes, rivers, wells, and streams, and can persist for long periods in soil, vegetation, streams, reservoirs, wells, and spring water. Infections with *Yersinia pseudotuberculosis* have been observed in various domesticated or caged animals, including horses, goats, deer, and monkeys. In two well-known *Yersinia* outbreaks, infection with human pseudotuberculosis has no known etiology. Untreated drinking water infected with wild animal feces was illegal (Fukushima et al., 2011).

11.18.5 Campylobacter

C. jejuni, C. colli, and *C. lari* have also polluted natural water surfaces. Consuming unchlorinated, contaminated water or eating food prepared with untreated or inadequately treated water might lead to infection.

In every corner of the globe, *Campylobacter* is one of the most common human bacterial causes of diarrhea. Approximately 80% of *Campylobacter* infections are thought to be caused by diet. Infection among young children is hyperendemic in developed countries. Domesticated animals (chickens, pigs, goats, horses, dogs, cats, and birds) are likely to get infected. Ingesting undercooked poultry, pork, and beef, unpasteurized milk, contaminated drinking water, and infected animal excrement can spread this virus to humans.

Campylobacter jejuni and *C. coli* invade domestic fowl with incredible ease. At least 60% of chickens sold in supermarkets are contaminated with *Campylobacter*, and broiler chickens are responsible for 50–70% of human infections in underdeveloped nations. The most common way to contract *Campylobacter jejuni* is by eating undercooked chicken. Red meat, on the other hand, can be contaminated. Infection can also be spread by eating barbecued pork or sausages. Infection can also be spread by eating raw or undercooked fish, shellfish, or mushrooms.

In the recent past, raw and unpasteurized milk was a common source of *Campylobacter* and was responsible for large foodborne outbreaks. However, outbreaks caused by raw milk have dropped significantly in developing nations due to the regular pasteurization of milk and improved public awareness (Acheson & Allos, 2001).

11.18.6 Escherichia coli

Foodborne infections, or infectious diseases spread by food, are a bothersome and sometimes life-threatening concern for millions of individuals. Drinking infected foods or beverages causes foodborne illness. Several different microorganisms, or disease-causing pathogens, can contaminate foods, resulting in various foodborne diseases. More than 250 distinct foodborne diseases have been documented, with the majority of them being infections caused by bacteria, viruses, and parasites.

The epidemiology of foodborne microbial illnesses has improved in the previous 10 years. It is ascribed not only to an increase in the human population's vulnerability to diseases or changes in lifestyle, such as more adventurous eating, fast food, and less time spent preparing food, but also to the introduction of newly known foodborne pathogens. Bowel infections are among the most prevalent infectious disorders in humans (Kaper et al., 2004). The gastrointestinal tract is a specific target for many foodborne viruses.

11.19 Aflatoxins and Aspergillus flavus

11.19.1 Aflatoxins

The aflatoxins are a collection of poisonous chemicals produced by structurally related *Aspergillus flavus, A. parasiticus*, and *A. nomius*. Some strains of these fungi can thrive on specific crops in the field and during storage under favorable temperature and humidity circumstances, resulting in aflatoxins formation.

Approximately 18 aflatoxins: aflatoxins (AF) B1, B2, G1, and G2 are naturally occurring aflatoxins, whereas the other 14 are animal-system metabolic products. Naturally occurring aflatoxins are found in various foods; AFB1 is usually the most prevalent in quantity and toxicity. *A. flavus* is the most frequent corn contaminant in temperate zones around the world, and it produces both AFB1 and AFB2. *A. parasiticus* is the most common peanut contaminant in tropical and subtropical areas, producing all four naturally occurring poisons. Aflatoxin M1 (AFM1) is a poisonous metabolite of AFB1 formed in mammals' livers. Because aflatoxins are very stable compounds, they can be detected in varying levels in finished foods depending on the manufacturing methods or procedures utilized. Aflatoxin levels in foods have been examined concerning various processing methods.

The occurrence of aflatoxins in food and feed is extensively documented in the literature, with the most widespread exposure happening in humidified and hot environments. Aflatoxins (except AFM1) have been discovered in corn and maize products, peanuts and peanut products, cotton, various tree nuts (e.g., Brazil, pistachio, almonds, pecans, and walnuts), copra and its products, edible seeds (e.g., watermelon, sunflower), wheat, sorghum, spices, rice, unrefined vegetable oils, figs, and dried fruits). They were sporadically detected in other materials at very low or negligible rates (Richard et al., 2003).

11.20 *Fusarium* and Fumonisins: Toxigenic *Fusarium* Species

11.20.1 Toxins Produced by *Fusarium* Species

Fusarium is a fungus that causes plant disease and is commonly found in soil. Because they infect plants in the field rather than in grain storage, *Fusarium* species infect cereal grains, known as "field fungus." As of 1996, more than 60 species of *Fusarium* have been identified in raw and cooked foods worldwide, with more than 100 secondary toxigenic metabolites characterized. Variations in mycotoxin-producing potential have been identified between strains within the same species. Detailed assessments of the various presence of the numerous mycotoxins produced by the *Fusarium* species have recently appeared in the literature. The most potent poisons to *Fusarium* include deoxynivalenol (vomitoxin), fumonisins, and zearalenone from human exposure and regulatory concern.

11.20.2 Other Molds and Mycotoxins

Most fungal species found in soil are susceptible to invasion by field crops. Many fungal species can infect growing plants and produce secondary poisonous compounds termed mycotoxins under ideal temperature and humidity conditions. Some fungal species can multiply as plants and grain kernels age, producing poisonous compounds that are not eliminated or destroyed during harvesting. Assume that harvested grains (crops) are not stored properly. In that instance, some fungus on the crops brought in from the field may continue to thrive and create mycotoxins, or they may eventually be replaced by fungi that may multiply under different storage circumstances and produce new mycotoxins. As a result, mycotoxins can infiltrate the food supply throughout preharvest and postharvest periods. Small amounts of these compounds in foods and feeds can be permissible, as long as the amounts are not considered detrimental to human and animal health.

Part of the goal of this chapter is to provide an overview of the significant mycotoxins that are causing significant public health problems at both the national and worldwide levels. Aflatoxin A, deoxynivalenol, fumonisins, patulin, and zearalenone are the most commonly regulated mycotoxins (Zinedine et al., 2007).

11.21 Foodborne Viruses

11.21.1 Caliciviruses

Caliciviruses are an enteric virus family that infects a variety of marine and terrestrial organisms. While there are three genogroups of human caliciviruses, there are two distinct physical and biological forms of human viruses: classic human caliciviruses and Norwalk-like viruses. These viruses are known as small round standardized viruses (SRSVs) in the literature. While classic calicivirus strains have been shown to induce gastroenteritis in adults, classical caliciviruses are primarily associated with self-limiting gastroenteritis in newborns and babies. Traditional caliciviruses account for just a small percentage of instances of infantile gastroenteritis. As a result, traditional caliciviruses are generally considered less medical and foodborne concern than the NLV.

Multiple genotypes are occasionally discovered during outbreak investigations. This common group infection goes undiscovered until an infected person contaminates a typical food or water source or fosters rapid human-to-human transmission through intimate contact with people in a closed or institutional setting. Contact with dirty linens, vomitus, excrement, aerosols, or fomites can direct person-to-person transmission in these conditions. NL disease outbreaks have been reported at banquets, geriatric institutions, mental units, emergency rooms, cafeterias, recreational lakes, swimming pools, dormitories, campgrounds, hotels, schools, restaurants, and cruise and navy ships (Scharff, 2012).

Although mussels are well known for spreading NL illness, other foods have also played a role in the disease's transmission. Viruses are commonly transmitted

through raw fruits and salads. Surface contamination of fruits and vegetables can occur due to irrigation or fertilization, harvesters or transporters, or food preparation contamination. Sick food handlers have contributed significantly to the spread of NL illness by contaminating foods with unwashed hands or exposing objects to unsanitary surfaces. One illness outbreak in Newfoundland was caused by mixing potato salad in a sink where a restaurant worker had vomited the day before. According to epidemiological studies, many NL disease outbreaks are caused by a general lack of awareness among some food handlers due to inadequate sanitation and hygiene training.

11.21.2 Hepatitis

Hepatitis A and E are the only hepatitis viruses spread through the feces–oral route; the others are primarily transmitted through parenteral routes. Hepatitis A virus (HAV) is transmitted chiefly through fecal–oral, direct person-to-person contact, and contaminated food and drink. For example, HAV is sexually transmitted, particularly among homosexuals, and has been documented on multiple occasions via blood products, particularly factor VIII. The hepatitis E virus is spread through contaminated food and infected water. Except for vertical transmission, there is no known parenteral transmission of the hepatitis E virus.

11.21.3 Hepatitis A Virus

Virus-related disease burden varies substantially around the globe and is directly influenced by socioeconomic growth and sanitation rates. The disease is more prevalent in small and high endemic countries; in the former, infection and disease are uncommon due to low virus circulation in the population due to adequate cleanliness and sanitation. In countries with high endemicity, on the other hand, the virus circulates widely. As a result, infection is common in young children, often asymptomatic and unnoticed, contributing to long-term safety. This is true in areas of intermediate endemicity, or countries with the highest disease rates, where developed and developing people coexist (Mesquita et al., 2011).

11.21.4 Hepatitis E Virus

The development of hepatitis A and B viral serological tests revealed that neither virus was responsible for most hepatitis cases. This resulted in the coining of the term non-A, non-B hepatitis, which had two epidemiologically distinct variants: a parenteral type with transmission routes similar to parenterally transmitted hepatitis B virus infection and an enterically distributed non-A, non-B hepatitis with transmission routes similar to hepatitis A virus infection.

On the Asian continent, enterically non-A transmitted non-B hepatitis was responsible for enormous epidemics of watery hepatitis. Serological investigations revealed that the etiologic culprit was a new virus unrelated to hepatitis A. The infection was significantly more widespread than the geographic location of the significant waterborne epidemics suggested. In addition to outbreaks, the virus, commonly known as the hepatitis E virus (HEV), caused sporadic instances of hepatitis among indigenous populations and imported cases from travelers in developing countries.

The virus is present at lower concentrations in the blood, up to 100,000 particles per mL, and the viraemic stage is mainly associated with the time when the virus is expelled in the feces. It can now explain why the number of blood-borne hepatitis A infections is increasing. It has not been identified whether the virus secreted in saliva has a function in transmitting the hepatitis A virus. No evidence is found that sharing dining utensils, cigarettes, or kissing can spread hepatitis A infection.

The fecal–oral route is the most common hepatitis A virus transmission, with person-to-person transmission being the most fundamental mode of virus transmission. The findings of infection rates among patient household contacts and children in the surroundings of daycare centers exemplified this point.

The virus is hardy, and it can survive in water and food for up to 10 months. As a result, foodborne outbreaks (common-source epidemics) are becoming more widespread. Food handler contamination—generally a food handler in the disease's asymptomatic pre-icteric stage—can cause foodborne outbreaks. It will mostly show up in those who do not wash their hands properly and handle raw foods like salads, sandwiches, and cold meats. Foods may be polluted, particularly shellfish from water near sewage outlets or vegetables fertilized with untreated human night soil. Drinking or bathing in filthy fecal water can potentially result in severe waterborne outbreaks.

There is no doubt that improvements in clean water and sanitation and personal and public hygiene have considerably impacted the global epidemiology of hepatitis A infection. Endemic transmission has ceased in several highly developed countries, such as Scandinavia, Germany, Switzerland, and Japan. Imports or travelers returning from underdeveloped nations account for most illnesses in these countries. HAV antibodies are present in less than 10% of blood donors in these countries. In most other affluent countries, endemic viral transmission is also growing, resulting in increased vulnerability in adults and higher morbidity and mortality (Scharff, 2012).

11.22 New Technologies for Microbial Detection

Some of the new techniques for microbial detection are ELISA, Lateral Flow Immunoassay (LFIA), colloidal gold label-paper-based dipstick, and quantum dots labels. ELISA has better sensitivity and lesser time consuming compared to culture-based technique. The LFIA technique is a simple, rapid, feasible, and potent foodborne pathogen detector. Other novel techniques to detect the antibodies are

optical strategies—gold nanoparticles combined with Raman spectrum molecules, label-free method for pathogen detection, and electrochemical strategies—high sensitivity, feasible, and fast compared to optical strategies (Jyoti et al., 2011).

11.23 Microbial Biofilms and Food Safety

Biofilm is formed by surplus microbial communities based on food fabricating conditions and the colonizing species. Combined biofilms possess higher resistance to the anti-biofilm compounds like disinfectants and biocides (Giaouris et al., 2015). Biofilms are made of extracellular matrices, and those matrices are formed of polysaccharides. These matrices can attach to hard surfaces like equipment in food industries. Overall, biofilm formation helps the microorganism associated with the food industry resists chemical protection and physical and mechanical resistance (Wingender et al., 2016). The most crucial part of biofilm in the food industry is that most microbes that form biofilms in the food industry are human pathogens, and they get attached to the surfaces of food industry equipment (Colagiorgi et al., 2017).

11.24 Biofilm in the Food Industry and Health Defects

Foodborne illnesses depend on the biofilm formation on the surface of the food industry. The occurrence of biofilm changes the type of the food industry, such as pipes, water, animal carcasses, dispensing tubes, and packing materials (Camargo et al., 2017). The five critical foodborne pathogens that can form biofilm are *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella enterica, and Staphylococcus aureus*.

11.25 Techniques for Controlling the Formation of Biofilm in the Food Industry

Biofilm detection is incompatible with the classic culture-based approach. It takes time, and few bacteria are viable but not culturable (VBNC) because they have limited metabolic activity. The biofilm produced by VBNC-PCR amplification is used to detect it. Biofilms can also be detected via metagenomics and meta-transcriptomics. The previous approach of biofilm detection is being replaced with an alternate method for improved efficiency due to the time consuming and higher reactive equipment.

Furthermore, these old technologies are unsuitable for use in the food business. As a result, online monitoring is employed. Electrochemical detection and vibrational detection are two different approaches. Controlling biofilm in the food business can be done in various ways. These approaches include cleaning and disinfection, clean-in-place, chemical-based controls, ultrasonication, enzymatic disruption, phage, and hurdle technology.

11.26 Food Chain—Safety and Quality

As the distance between the producer and the consumer has grown due to globalization, one of the significant issues is supplying quality food without contamination. Food traceability is an essential criterion in ensuring food safety and quality. Globalization has posed a challenge not only to food safety but also to food quality. Consumers want to know everything there is to know about retailing history. Traceability is crucial in this case. Traceability is based on unit identification, product tracking, and data storage principles. The importance of food in the country cannot be overstated. Food production is a global phenomenon that influences social, economic, and environmental factors. Assume that any food-related difficulties will impact the country's socioeconomic condition. It has an impact on the environment because of increased transportation carbon labeling. Food providers have recently relied on two techniques to assure global food safety: managing the food chain by adhering to regulations and standards and regulating logistics through traceability. FAO and WHO have a significant role in food safety, FDA legislation, and International Standardization Organizations (ISO) participating in the international food trade. Analyzing the risk Hazard Analysis and Critical Control Points (HACCP) is essential for maintaining food safety standards. After HACCP, good manufacturing procedures are implemented. Many rules and regulations were in place to assure food quality and safety across the food supply chain (Aung & Chang, 2014).

11.27 Methodologies for Improved Quality Control Assessment of Food Products

In these decades of higher technological development, the improvement in the food analysis techniques will have greater insight. Analysts and chemists will have greater value in using novel analytical tools. Traditional tools are more time consuming and require skilled labor, so the demand for novel analytical tools has increased. Traditional high-performance liquid chromatography system analyzes the hydrophobic and hydrophilic constituents in reversed phase and the normal phase. However, the conventional method is less susceptible and time consuming. So, to analyze the higher and lower molecular weight analytes, novel polymer-based stationary phase is introduced. Capillary electrophoresis that discrete the analyte based on the molecular weight and ionic moiety is introduced as an alternative. There is a higher technique named capillary electrochromatography which is better than HPLC and capillary electrophoresis (Huck et al., 2000). Spectroscopic strategies at this phase of the systematic technique either incorporate mass spectrometry (MS) or vibrational spectroscopy individually. Laser desorption ionization determines higher molecular weight samples (Feuerstein et al., 2006). Vibrational spectroscopy is used for detection.

11.28 New Developments in Food Quality Assurance and Safety Control

The essential aspects of food are maintaining food safety and food quality. There were numerous techniques to detect food safety primarily. We rely upon the traditional method, which is time consuming and high cost. Then after several findings in the 1960s, an electronic nose was introduced, which will mimic the human olfactory system with its sensors. There were many factors for accepting and rejecting the food and one among the important factor is the aroma, the smell of the food. Aroma plays a crucial role in food. Aroma acts as a biomarker to test food contamination. Few biosensors are listed in Table 11.6.

The food industry is benefiting from significant progress in developing enzymatic biosensors with various transduction systems that can be used in food safety, quality, and process control; studies are primarily focused on determining the composition and contamination of raw materials and processed foods. The food industry is benefiting from significant progress in developing enzymatic biosensors with various transduction systems that can be used in food safety, quality, and process control; studies are primarily focused on determining the composition and contamination of raw materials and processed foods.

11.29 Conclusion

Intense research is ongoing to find a solution to the global food crisis. Enzyme assimilation is well known in the food processing industry. This chapter addresses the fundamentals of enzymes and the sources of various enzymes and their applications in the food industry. The intrinsic features of microbial enzymes have rocketed them to renown due to their simplicity of synthesis and yield manipulation in laboratory settings. Enzymes in food processing convert physiochemical processes to environmentally friendly processes, improve product biodegradability, and reduce energy usage. The food processing industry could benefit from a new approach for enzyme uses. Foodborne infections also harm human health and create

Table 11.6 E-nose in accordance with food (Rayappan et al., 2017)

Food	E-nose
Meat	NST 3210 emission analyzer, along with the ANN prediction algorithm, Heracles II electronic nose, Artificial Olfactory System (AOS) ISE Nose 2000, Total Volatile Basic Nitrogen (TVB-N), Cyranose-320
Milk	BH-114: Bloodhound Sensors Ltd., Applied Sensor 3320
Fish and seafood	Colorimetric array of sensors
Fruits and vegetables	FOX 4000
Adulterants	Pen 2

financial losses. As a result, rapid diagnosis of foodborne pathogens and the application of countermeasures are critical. Preventing foodborne illness requires quick detection of the pathogen. Enzymes can be used as biosensors to detect food adulteration and contamination for faster food product screening. The dietary substrate determines the enzyme biosensor's characteristics. Molecular diagnostics and serological methods are two new technologies for food sector monitoring.

While there are various methods for detecting food safety, the most common is the time consuming and expensive method. The electronic nose stimulates the olfactory system of humans using sensors, which is a breakthrough. Many aspects influence whether or not a person accepts or rejects food. One of the most crucial factors is the aroma of the dish. In food, the aroma is significant. The aroma as a biomarker can detect the contamination in food. With the expanding dimensions of the food industry, several critical procedures such as food preservation, monitoring, adulteration, and foodborne diseases are critical to the industry's overall performance. Furthermore, enzymes perform multiple roles in all processes, one of which is the constant monitoring of food and food products.

References

Abdollahzadeh, R., et al. (2020). Screening of pectinase-producing bacteria from farmlands and optimization of enzyme production from selected strain by RSM. *Folia Microbiologica, 65*(4), 705–719. https://doi.org/10.1007/s12223-020-00776-7

Acheson, D., & Allos, B. M. (2001). *Campylobacter jejuni* infections: Update on emerging issues and trends. *Clinical Infectious Diseases, 32*(8), 1201–1206. https://doi.org/10.1086/319760

Akyilmaz, E., Yorganci, E., & Asav, E. (2010). Do copper ions activate tyrosinase enzyme? A biosensor model for the solution. *Bioelectrochemistry, 78*(2), 155–160. https://doi.org/10.1016/j.bioelechem.2009.09.007

Ali, M., et al. (2011). Nanobiosensor for detection and quantification of DNA sequences in degraded mixed meats. *Journal of Nanomaterials, 2011*. https://doi.org/10.1155/2011/781098

Alkasir, R. S. J., et al. (2010). Enzyme functionalized nanoparticles for electrochemical biosensors: A comparative study with applications for the detection of bisphenol A. *Biosensors and Bioelectronics, 26*(1), 43–49. https://doi.org/10.1016/j.bios.2010.05.001

Alvarez-Ordóñez, A., et al. (2013). Heterogeneity in resistance to food-related stresses and biofilm formation ability among verocytotoxigenic *Escherichia coli* strains. *International Journal of Food Microbiology, 161*(3), 220–230. https://doi.org/10.1016/j.ijfoodmicro.2012.12.008

Amine, A., et al. (2006). Enzyme inhibition-based biosensors for food safety and environmental monitoring. *Biosensors and Bioelectronics, 21*(8), 1405–1423. https://doi.org/10.1016/j.bios.2005.07.012

Andrei, V., et al. (2016). A single use electrochemical sensor based on biomimetic nanoceria for the detection of wine antioxidants. *Talanta, 156–157*, 112–118. https://doi.org/10.1016/j.talanta.2016.04.067

Antiochia, R., Vinci, G., & Gorton, L. (2013). Rapid and direct determination of fructose in food: A new osmium-polymer mediated biosensor. *Food Chemistry, 140*(4), 742–747. https://doi.org/10.1016/j.foodchem.2012.11.023

Aravindan, R., Anbumathi, P., & Viruthagiri, T. (2007). *Lipase applications in food industry*. CSIR.

Aruna, K., Shah, J., & Birmole, R. (2014). Production and partial characterization of alkaline protease from *Bacillus tequilensis* strains Csgab0139 isolated from spoilt cottage cheese. *International journal of applied biology an pharmaceutical technology, 5*(3), 201–221.

Ashie, I. N. A. (2012). Enzymes in food analysis. In *Food biochemistry and food processing* (pp. 39–55). Wiley Online Books. https://doi.org/10.1002/9781118308035.ch3

Aung, M. M., & Chang, Y. S. (2014). Traceability in a food supply chain: Safety and quality perspectives. *Food Control*. https://doi.org/10.1016/j.foodcont.2013.11.007

Batra, B., Narwal, V., & Pundir, C. S. (2016). An amperometric lactate biosensor based on lactate dehydrogenase immobilized onto graphene oxide nanoparticles-modified pencil graphite electrode. *Engineering in Life Sciences, 16*(8), 786–794. https://doi.org/10.1002/elsc.201600082

Becker-Algeri, T. A., et al. (2016). Mycotoxins in bovine milk and dairy products: A review. *Journal of Food Science, 81*(3), R544–R552. https://doi.org/10.1111/1750-3841.13204

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances, 18*(5), 355–383. https://doi.org/10.1016/S0734-9750(00)00041-0

Bilal, M., & Iqbal, H. M. N. (2020). State-of-the-art strategies and applied perspectives of enzyme biocatalysis in food sector—Current status and future trends. *Critical Reviews in Food Science and Nutrition, 60*(12), 2052–2066. https://doi.org/10.1080/10408398.2019.1627284

Blanco, C. A., et al. (2014). Innovations in the brewing industry: Light beer. *International Journal of Food Sciences and Nutrition, 65*(6), 655–660. https://doi.org/10.3109/09637486.2014.893285

Borgmann, S., et al. (2012). Amperometric biosensors. In *Advances in electrochemical science and engineering* (pp. 1–83). https://doi.org/10.1002/9783527644117.ch1

Health Protection Agency. (2011). Botulism, *Epidemiological data*.

Brown, C. A., et al. (2007). Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. *Journal of Veterinary Diagnostic Investigation, 19*(5), 525–531. https://doi.org/10.1177/104063870701900510

Calvo-Pérez, A., et al. (2013). Disposable amperometric biosensor for the determination of tyramine using plasma amino oxidase. *Microchimica Acta, 180*(3), 253–259. https://doi.org/10.1007/s00604-012-0926-y

Camacho, N. A., Aguilar, O., & G. (2003). Production, purification, and characterization of a low-molecular-mass xylanase from Aspergillus sp. and its application in baking. *Applied Biochemistry and Biotechnology, 104*(3), 159–171. https://doi.org/10.1385/ABAB:104:3:159

Camargo, A. C., et al. (2017). *Listeria monocytogenes* in food-processing facilities, food contamination, and human listeriosis: The Brazilian scenario. *Foodborne Pathogens and Disease, 14*(11), 623–636. https://doi.org/10.1089/fpd.2016.2274

Castillo, J., et al. (2004). Biosensors for life quality: Design, development and applications. *Sensors and Actuators B: Chemical, 102*(2), 179–194. https://doi.org/10.1016/j.snb.2004.04.084

Chakdar, H., et al. (2016). Bacterial xylanases: Biology to biotechnology. *3 Biotech, 6*(2), 150. https://doi.org/10.1007/s13205-016-0457-z

Chandrasekaran, M. (2013). *Valorization of food processing by-products*. Retrieved from http://www.crcnetbase.com/isbn/9781439848876.

Chaplin, M. F., & Bucke, C. (1990). *Enzyme technology*.

Chapman, J., Ismail, A. E., & Dinu, C. Z. (2018). Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts, 8*(6), 20–29. https://doi.org/10.3390/catal8060238

Choi, E. J., Ahn, H. W., & Kim, W. J. (2015a). Effect of α-acetolactate decarboxylase on diacetyl content of beer. *Food Science and Biotechnology, 24*(4), 1373–1380.

Choi, J.-M., Han, S.-S., & Kim, H.-S. (2015b). Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnology Advances, 33*(7), 1443–1454. https://doi.org/10.1016/j.biotechadv.2015.02.014

Christopher, N., & Kumbalwar, M. (2015). Enzymes used in food industry a systematic review. *International Journal of Inovative Research in Science Engineering and Technology, 4*(10), 9830–9836. https://doi.org/10.15680/IJIRSET.2015.0410073

Clark, L. C., et al. (1958). Monitor and control of blood oxygen tension and pH during total body perfusion. *Journal of Thoracic Surgery, 36*(4), 488–496. https://doi.org/10.1016/S0096-5588(20)30126-4

Clark, L. C., & Lyons, C. (1962). Electrode systems for continuous monitoring in cardiovascular surgery. *Annals of the New York Academy of Sciences, 102*, 29–45. https://doi.org/10.1111/j.1749-6632.1962.tb13623.x

Colagiorgi, A., et al. (2017). Listeria monocytogenes biofilms in the wonderland of food industry. *Pathogens. Multidisciplinary Digital Publishing Institute, 6*(3), 41.

Djekrif-Dakhmouche, S., et al. (2006). Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering, 73*(2), 190–197. https://doi.org/10.1016/j.jfoodeng.2005.01.021

Ermis, E. (2017). Halal status of enzymes used in food industry. *Trends in Food Science & Technology, 64*, 69–73. https://doi.org/10.1016/j.tifs.2017.04.008

Faulds, C. B. (2010). What can feruloyl esterases do for us? *Phytochemistry Reviews, 9*(1), 121–132. https://doi.org/10.1007/s11101-009-9156-2

Feng, W., et al. (2011). Isolation and characterization of lipase-producing bacteria in the intestine of the silkworm, *Bombyx mori*, reared on different forage. *Journal of Insect Science, 11*, 135. https://doi.org/10.1673/031.011.13501

Feuerstein, I., et al. (2006). Material-Enhanced Laser Desorption/Ionization (MELDI)—A new protein profiling tool utilizing specific carrier materials for time of flight mass spectrometric analysis. *Journal of the American Society for Mass Spectrometry, 17*(9), 1203–1208. https://doi.org/10.1016/j.jasms.2006.04.032

Fukushima, H., Shimizu, S., & Inatsu, Y. (2011). *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* detection in foods. *Journal of Pathogens, 2011*, 735308. https://doi.org/10.4061/2011/735308

Galante, Y. M., De Conti, A., & Monteverdi, R. (1998). Application of trichoderma enzymes in textile industry. In G. F. Harman & C. P. Kubicek (Eds.), *Trichoderma and gliocladium* (Enzymes, biological control and commercial applications) (Vol. 2, pp. 311–326). Taylor and Francis.

Gaur, R., & Tiwari, S. (2015). Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnology, 15*(1), 19. https://doi.org/10.1186/s12896-015-0129-9

Gavrilescu, M., & Chisti, Y. (2005). Biotechnology-a sustainable alternative for chemical industry. *Biotechnology Advances, 23*(7–8), 471–499. https://doi.org/10.1016/j.biotechadv.2005.03.004

Ghorai, S., et al. (2009). Fungal biotechnology in food and feed processing. *Food Research International (Ottawa, Ont.), 42*(5–6), 577–587. https://doi.org/10.1016/j.foodres.2009.02.019

Giaouris, E., et al. (2015). Intra-and inter-species interactions within biofilms of important foodborne bacterial pathogens. *Frontiers in Microbiology, 6*, 841.

Gibson, G. R., & Wang, X. (1994). Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *Journal of Applied bacteriology., 77*(4), 412–420.

Gonzaga, V. E., et al. (2009). Histamine levels in fish from markets in Lima, Perú. *Journal of Food Protection, 72*(5), 1112–1115. https://doi.org/10.4315/0362-028x-72.5.1112

Gouda, M. D., et al. (2002). Enhancement of operational stability of an enzyme biosensor for glucose and sucrose using protein based stabilizing agents. *Biosensors and Bioelectronics, 17*(6), 503–507. https://doi.org/10.1016/S0956-5663(02)00021-0

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial amylase: A biotechnological perspective. *Process Biochemistry, 38*, 1599–1616.

Gurung, N., et al. (2013). A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Research International, 2013*. https://doi.org/10.1155/2013/329121

Hanft, F. and Koehler, P. (2006) 'Studies on the effect of glucose oxidase in bread making', Journal of the Science of Food and Agriculture. John Wiley & Sons, Ltd, 86(11), pp. 1699–1704. doi: https://doi.org/10.1002/jsfa.2455.

Hau, A. K., Kwan, T. H., & Li, P. K. (2009). Melamine Toxicity and the Kidney. *Journal of the American Society of Nephrology, 20*(2), 245–250. https://doi.org/10.1681/ASN.2008101065

He, L., et al. (2018). Rapid and sensitive colorimetric detection of ascorbic acid in food based on the intrinsic oxidase-like activity of MnO2 nanosheets. *Luminescence, 33*(1), 145–152. https://doi.org/10.1002/bio.3384

Hernández, M. S., et al. (2006). Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. *Journal of Food Engineering, 73*(1), 93–100. https://doi.org/10.1016/j.jfoodeng.2005.01.009

Hooda, V., et al. (2018). Alcohol quantification: Recent insights into amperometric enzyme biosensors. *Artificial Cells, Nanomedicine, and Biotechnology, 46*(2), 398–410. https://doi.org/10.1080/21691401.2017.1315426

Huck, C. W., et al. (2000). Development and evaluation of a new method for the determination of the carotenoid content in selected vegetables by HPLC and HPLC—MS—MS. *Journal of Chromatographic Science, 38*(10), 441–449.

Iqbal, S., & Selamat, J. (2016). *Food safety; Basic concepts, recent issues, and future challenges*. https://doi.org/10.1007/978-3-319-39253-0.

Islam, F., & Roy, N. (2018). Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. *BMC Research Notes, 11*(1), 445. https://doi.org/10.1186/s13104-018-3558-4

Jeddah. (2011). Opinion: Halal food products: Lucrative business amid strong demand. *The Saudi Gazette - Business Section*.

Jyoti, A., et al. (2011). Identification of environmental reservoirs of nontyphoidal salmonellosis: Aptamer-assisted bioconcentration and subsequent detection of *Salmonella typhimurium* by quantitative polymerase chain reaction. *Environmental Science & Technology, 45*(20), 8996–9002. https://doi.org/10.1021/es2018994

Kandra, L. (2003). α-Amylases of medical and industrial importance. *Journal of Molecular Structure: THEOCHEM, 666–667*, 487–498. https://doi.org/10.1016/j.theochem.2003.08.073

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic Escherichia coli. *Nature Reviews Microbiology, 2*(2), 123–140. https://doi.org/10.1038/nrmicro818

Karim, K. M. R., Husaini, A., & Tasnim, T. (2017). Production and characterization of crude glucoamylase from newly isolated *Aspergillus flavus* NSH9 in liquid culture. *American Journal of Biochemistry and Molecular Biology, 7*(3), 118–126.

Kieliszek, M., & Misiewicz, A. (2014). Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiologica, 59*(3), 241–250. https://doi.org/10.1007/s12223-013-0287-x

Kim, C.-W., et al. (2010). Determination of spatial distribution of melamine−cyanuric acid crystals in rat kidney tissue by histology and imaging matrix-assisted laser desorption/ionization quadrupole time-of-flight mass spectrometry. *Chemical Research in Toxicology, 23*(1), 220–227. https://doi.org/10.1021/tx900354z

Kothary, M. H., & Babu, U. M. A. S. (2001). Infective dose of foodborne pathogens in volunteers: A Review. *Journal of Food Safety, 21*(1), 49–68. https://doi.org/10.1111/j.1745-4565.2001.tb00307.x

Lancette, G., & Bennett, R. W. (2001). *Staphylococcus aureus* and *Staphylococcal enterotoxins*. In K. Ito (Ed.), *Compendium of methods for the microbiological examination of foods* (pp. 387–403). American Public Health Association.

Law, B. A. (2010). Enzymes in dairy product manufacture. In *Enzymes in food technology* (pp. 92–93). Wiley Online Library.

Lee, B. L., Ong, H. Y., & Ong, C. N. (1991). Determination of thiamine and its phosphate esters by gradient-elution high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications, 567*(1), 71–80. https://doi.org/10.1016/0378-4347(91)80311-Y

Lépine, A. F. P., et al. (2019). Higher chain length distribution in debranched type-3 resistant starches (RS3) increases TLR signaling and supports dendritic cell cytokine production. *Molecular Nutrition & Food Research, 63*(2), e1801007. https://doi.org/10.1002/mnfr.201801007

Li, L., et al. (2010). Visual detection of melamine in raw milk using gold nanoparticles as colorimetric probe. *Food Chemistry, 122*(3), 895–900. https://doi.org/10.1016/j.foodchem.2010.03.032

Lindström, M., et al. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology, 28*(2), 192–198. https://doi.org/10.1016/j.fm.2010.03.020

Liu, L., et al. (2013). How to achieve high-level expression of microbial enzymes: Strategies and perspectives. *Bioengineered, 4*(4), 212–223. https://doi.org/10.4161/bioe.24761

Luang-In, V., et al. (2019). Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical and Pharmacology Journal, 12*(3), 1061–1068. https://doi.org/10.13005/bpj/1735

Luong, J. H. T., Groom, C. A., & Male, K. B. (1991). The potential role of biosensors in the food and drink industries. *Biosensors and Bioelectronics, 6*(7), 547–554. https://doi.org/10.1016/0956-5663(91)80018-S

van der Maarel, M. J. E. C., et al. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology, 94*(2), 137–155. https://doi.org/10.1016/S0168-1656(01)00407-2

Marazuela, M. D., & Moreno-Bondi, M. C. (2002). Fiber-optic biosensors—An overview. *Analytical and Bioanalytical Chemistry, 372*(5–6), 664–682. https://doi.org/10.1007/s00216-002-1235-9

Mascini, B. K. (2015) 'Enzyme inhibition based biosensors for environmental monitoring', in Current enzyme inhibition. Bentham Science Publisher, p. 1–207. doi:10.2174/157340805774580484.

Mecker, L. C., et al. (2012). Selective melamine detection in multiple sample matrices with a portable Raman instrument using surface enhanced Raman spectroscopy-active gold nanoparticles. *Analytica Chimica Acta, 733*, 48–55. https://doi.org/10.1016/j.aca.2012.05.001

Mello, L. D., & Kubota, L. T. (2002). Review of the use of biosensors as analytical tools in the food and drink industries. *Food Chemistry, 77*(2), 237–256. https://doi.org/10.1016/S0308-8146(02)00104-8

Mesquita, J. R., et al. (2011). Norovirus, hepatitis A virus and enterovirus presence in shellfish from high quality harvesting areas in Portugal. *Food Microbiology, 28*(5), 936–941. https://doi.org/10.1016/j.fm.2011.01.005

Mohan Kumar, N. S., et al. (2014). Reduction of acrylamide formation in sweet bread with l-asparaginase treatment. *Food and Bioprocess Technology, 7*(3), 741–748. https://doi.org/10.1007/s11947-013-1108-6

Mojsov. (2011) Applications of enzymes in the textile industry: A review. In: *2nd international congress: Engineering, Ecology and Materials in the Processing Industry: Jahorina, Bosnia and Herzegovina; Tehnoloski Fakultet Zvornik*, pp. 230–239.

Monosik, R., et al. (2012). Application of enzyme biosensors in analysis of food and beverages. *Food Analytical Methods, 5*(1), 40–53. https://doi.org/10.1007/s12161-011-9222-4

Monteiro, C. A., et al. (2011). Increasing consumption of ultra-processed foods and likely impact on human health: Evidence from Brazil. *Public Health Nutrition, 14*(1), 5–13. https://doi.org/10.1017/S1368980010003241

Moore, J. C., Spink, J., & Lipp, M. (2012). Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *Journal of Food Science, 77*(4), R118–R126. https://doi.org/10.1111/j.1750-3841.2012.02657.x

Nagodawithana, T. W., & Reed, G. (1993). *Enzymes in food processing*. Academic Press.

Nakamatsu, T., et al. (1975). Microbial production of glucose oxidase. *Agricultural and Biological Chemistry, 39*(9), 1803–1811. https://doi.org/10.1080/00021369.1975.10861864

Nampoothiri, K. M., et al. (2002). Advances in fermentation technology. *International Sugar Journal, 104*, 493–499.

Nielsen, S. S. (2017). *Food analysis*. Retrieved from http://rave.ohiolink.edu/ebooks/ebc/9783319457765.

Norus, J. (2006). Building sustainable competitive advantage from knowledge in the region: The industrial enzymes industry. *European Planning Studies, 14*(5), 681–696. https://doi.org/10.1080/09654310500500239

Oumer, O. J., & Abate, D. (2018). Screening and molecular identification of pectinase producing microbes from coffee pulp. *BioMed Research International, 2018*, 2961767. https://doi.org/10.1155/2018/2961767

Panesar, P., Marwaha, S., & Chopra, H. (2010). *Enzymes in food processing: Fundamentals and potential applications*.

Park, Y. W., et al. (2015). Application of biosensors in smart packaging. *Molecular & Cellular Toxicology, 11*(3), 277–285. https://doi.org/10.1007/s13273-015-0027-1

Parveen, S., et al. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology, 128*(2), 354–361. https://doi.org/10.1016/j.ijfoodmicro.2008.09.019

Pasha, K. M., Anuradha, P., & Subbarao, D. (2013). Applications of pectinases in industrial sector. *International Journal of Pure and Applied Sciences and Technology, 16*(1), 89.

Power, A. C., et al. (2018). Carbon nanomaterials and their application to electrochemical sensors: A review. *Nanotechnology Reviews, 7*(1), 19–41. https://doi.org/10.1515/ntrev-2017-0160

Priya, V., & Sashi, V. (2014). Pectinase enzyme producing Microorganisms. *International Journal of Scientific and Research Publications, 4*(1), 2250–3153. Available at: www.ijsrp.org

Prodromidis, M. I., & Karayannis, M. I. (2002). Enzyme based amperometric biosensors for food analysis. *Electroanalysis, 14*(4), 241–261.

Qureshi, M. A., Khare, A. K., & Pervez, A. (2015). Enzymes used in dairy industries. *International Journal of Applied Research, 1*(110), 523–527. Available at: www.allresearchjournal.com

Rajagopalan, G., & Krishnan, C. (2008). Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Bioresource Technology, 99*(8), 3044–3050. https://doi.org/10.1016/j.biortech.2007.06.001

Rankin, S. A., et al. (2010). Invited review: The application of alkaline phosphatase assays for the validation of milk product pasteurization. *Journal of Dairy Science, 93*(12), 5538–5551. https://doi.org/10.3168/jds.2010-3400

Raveendran, S., et al. (2018). Applications of microbial enzymes in food industry. *Food Technology and Biotechnology, 56*(1), 16–30. https://doi.org/10.17113/ftb.56.01.18.5491

Rayappan, J. B. B., et al. (2017). Developments in electronic noses for quality and safety control. *Advances in Food Diagnostics*, 63–96. https://doi.org/10.1002/9781119105916.ch3

Reddy, N., Nimmagadda, A., & Sambasiva Rao, K. R. S. (2004). An overview of the microbial -amylase family. *African Journal of Biotechnology, 2*. https://doi.org/10.5897/AJB2003.000-1119

Regalado, C., García-Almendárez, B. E., & Duarte-Vázquez, M. A. (2004). Biotechnological applications of peroxidases. *Phytochemistry Reviews, 3*(1), 243–256. https://doi.org/10.1023/B:PHYT.0000047797.81958.69

Richard, J., et al. (2003). Mycotoxins: Risks in plant, animal and human systems. *CAST Task Force Report, 139*, 101–103.

Romero Gomez, S., Augur, C., & Viniegra-González, G. (2000). Invertase production by *Aspergillus niger* in submerged solid-state fermentation. *Biotechnology Letters, 22*, 1255–1258. https://doi.org/10.1023/A:1005659217932

Rosini, E., et al. (2008). A biosensor for all d-amino acids using evolved d-amino acid oxidase. *Journal of Biotechnology, 135*(4), 377–384. https://doi.org/10.1016/j.jbiotec.2008.06.001

Rosini, E., et al. (2014). Evolution of histamine oxidase activity for biotechnological applications. *Applied Microbiology and Biotechnology, 98*(2), 739–748. https://doi.org/10.1007/s00253-013-5159-4

Sack, D. A., et al. (2004). Cholera. *Lancet (London, England), 363*(9404), 223–233. https://doi.org/10.1016/s0140-6736(03)15328-7

Santos, A. S., et al. (2006). Amperometric biosensor for ethanol based on co-immobilization of alcohol dehydrogenase and Meldola's Blue on multi-wall carbon nanotube. *Electrochimica Acta, 52*(1), 215–220. https://doi.org/10.1016/j.electacta.2006.04.060
Saqib, S., et al. (2017). Sources of β-galactosidase and its applications in food industry. *3 Biotech, 7*(1), 79.
Sarkar, P., et al. (1999). Screen-printed amperometric biosensors for the rapid measurement of L- and D-amino acids. *The Analyst, 124*(6), 865–870. https://doi.org/10.1039/a901404g
Sayali, P., & Satpute, S. (2013). Microbial esterases: An overview. *International Journal of Current Microbiology and Applied Sciences, 2*, 135–146.
Scharff, R. L. (2012). Economic burden from health losses due to foodborne illness in the United States. *Journal of Food Protection, 75*(1), 123–131. https://doi.org/10.4315/0362-028X.JFP-11-058
Schofield, C. L., Field, R. A., & Russell, D. A. (2007). Glyconanoparticles for the colorimetric detection of cholera toxin. *Analytical Chemistry, 79*(4), 1356–1361. https://doi.org/10.1021/ac061462j
Sharma, A., et al. (2010). Antibody immobilized cysteamine functionalized-gold nanoparticles for aflatoxin detection. *Thin Solid Films, 519*(3), 1213–1218. https://doi.org/10.1016/j.tsf.2010.08.071
Sharma, S., et al. (2015). Bioprocessing of crop residues using fibrolytic enzymes and *Flavobacterium bolustinum* for enriching animal feed. *International Journal of Biotechnology for Wellness Industries, 4*(1), 12–17. https://doi.org/10.6000/1927-3037.2015.04.01.2
Siepenkoetter, T., Salaj-Kosla, U., & Magner, E. (2017). The immobilization of fructose dehydrogenase on nanoporous gold electrodes for the detection of fructose. *ChemElectroChem, 4*(4), 905–912. https://doi.org/10.1002/celc.201600842
Sîrbu, T. (2011). The searching of active catalase producers among the microscopic fungi. *Analele Universitatii Din Oradea, Fascicula Biologie, 18*(2), 164–167.
Sukumaran, R., Singhania, R., & Pandey, A. (2005). Microbial cellulases-production, applications and challenges. *Journal of Scientific & Industrial Research, 64*, 832–844.
Tabssum, F., et al. (2018). RSM based optimization of nutritional conditions for cellulase mediated Saccharification by *Bacillus cereus*. *Journal of Biological Engineering, 12*, 7. https://doi.org/10.1186/s13036-018-0097-4
Terry, L. A., White, S. F., & Tigwell, L. J. (2005). The application of biosensors to fresh produce and the wider food industry. *Journal of Agricultural and Food Chemistry, 53*(5), 1309–1316. https://doi.org/10.1021/jf040319t
Theron, L. W., & Divol, B. (2014). Microbial aspartic proteases: Current and potential applications in industry. *Applied Microbiology and Biotechnology, 98*(21), 8853–8868. https://doi.org/10.1007/s00253-014-6035-6
Titball, R. W. (1993). Bacterial phospholipases C. *Microbiology and Molecular Biology Reviews, 57*(2), 347–366.
Tomme, P., Warren, R. A. J., & Gilkes, N. R. (1995). *Cellulose hydrolysis by bacteria and fungi* (pp. 1–81). Academic Press. https://doi.org/10.1016/S0065-2911(08)60143-5
Tudorache, M., & Bala, C. (2007). Biosensors based on screen-printing technology, and their applications in environmental and food analysis. *Analytical and Bioanalytical Chemistry, 388*(3), 565–578. https://doi.org/10.1007/s00216-007-1293-0
Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta, 632*(2), 168–180. https://doi.org/10.1016/j.aca.2008.11.010
Vargas-Bernal, R., Rodríguez-Miranda, E., & Herrera-Pérez, G. (2012). *Evolution and expectations of enzymatic biosensors for pesticides* (pp. 329–356). https://doi.org/10.5772/46227
Vasic-Racki, D. (2006). History of industrial biotransformations—Dreams and realities. *Industrial biotransformations, 40*, 1–36.

Vasilescu, A., et al. (2016). Electrochemical affinity biosensors based on disposable screen-printed electrodes for detection of food allergens. *Sensors (Basel, Switzerland), 16*(11). https://doi.org/10.3390/s16111863

Venugopal, V. (2002). Biosensors in fish production and quality control. *Biosensors and Bioelectronics, 17*(3), 147–157. https://doi.org/10.1016/S0956-5663(01)00180-4

Viswanathan, S., Radecka, H., & Radecki, J. (2009). Electrochemical biosensors for food analysis. *Monatshefte für Chemie - Chemical Monthly, 140*(8), 891. https://doi.org/10.1007/s00706-009-0143-5

Wachinger, G., et al. (1989). Identification of mycelium-associated cellulase from *Streptomyces reticuli*. *Applied and Environmental Microbiology, 55*(10), 2653–2657. https://doi.org/10.1128/AEM.55.10.2653-2657.1989

Wang, Q., Chang, B. J., & Riley, T. V. (2010). Erysipelothrix rhusiopathiae. *Veterinary Microbiology, 140*(3–4), 405–417. https://doi.org/10.1016/j.vetmic.2009.08.012

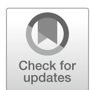
Williams, D. C., Lim, M. H., Chen, A. O., Pangborn, R. M., & Whitaker, J. R. (1986). Blanching of vegetables for freezing: Which indicator enzyme to choose. *Food Technology, 40*, 130–140.

Wingender, J., et al. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews. Microbiology, 14*(9), 563–575.

Zhang, X.-Y., et al. (2015). Diversity of cultivable protease-producing bacteria in sediments of Jiaozhou Bay, China. *Frontiers in Microbiology, 6*, 1021. https://doi.org/10.3389/fmicb.2015.01021

Zhou, Q., et al. (2011). Development of gold nanoparticle-based rapid detection kit for melamine in milk products. *Journal of agricultural and food chemistry., 59*(22), 12006–12011. https://doi.org/10.1021/jf202919a

Zinedine, A., et al. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology, 45*(1), 1–18. https://doi.org/10.1016/j.fct.2006.07.030



Enzymes in Flavor Development and Food Preservation

12

Fataneh Hashempour-Baltork and Parastou Farshi

Abstract

Flavors and fragrances have extensive application in the pharmaceutical, cosmetic, feed, chemical, and food sectors. Most of the flavor compounds on the market are formed from animal and plant sources, through chemical synthesis or extraction; however, a prompt alteration for the bio-production and use of flavor compounds with (micro) biological origin—bioflavors—is occurring. Enzymatic flavor production is commonly preferred over fermentation due to their high yields of production. On the other hand, it is undeniable that food is important for mankind and there is not any way of living without eating. Although the need to feed has not changed during the years, the way of food preservation has seen a lot of changes in today's global market which is highly competitive, and it is desirable to use the cheapest method of food preservation, such as food additives which can be a good choice. Among food additives, enzymatic process for longitude shelf life is one of the suitable choices. Using of bioprocesses involving enzymes in food processing can decrease the antinutritive factors and toxins of the by-products, besides solving the environmental issues, and increasing their consumer acceptance by enhancing their nutritive value. Although, it is important to select the proper fermentative processing technologies.

F. Hashempour-Baltork
Halal Research Center of IRI, Iran Food and Drug Administration, Ministry of Health and Medical Education, Tehran, Iran

P. Farshi (✉)
Food Science Institute, Kansas State University, Manhattan, KS, USA
e-mail: pfarshi@ksu.edu

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_12

Keywords

Enzyme · Flavor · Food preservation · Shelf life · Fermentation · Bioflavor · Ecofriendly

12.1 Enzyme in Flavor Development

12.1.1 Introduction

In the modern food industry, different types of enzymes have been applied for the synthesis of important food additives, food properties, and development of food production processes. Flavor and fragrances have found wide application in the food, feed, cosmetic, chemical and pharmaceutical sectors. Most of the flavor compounds in the market are formed from animal and plant sources, which takes place through chemical synthesis or extraction; however, there is a quick alteration for the bio-production and use of flavor compounds with (micro) biological origin, namely, bioflavors. Chemical synthesis usually results in an unfavorable and environmentally unfriendly production process of racemic mixture compounds. Moreover, the consumer has established a "chemophobia"-approach for synthetic or chemical compounds, specifically for food and products being used in the home. The definition of natural flavors by EC Flavor Directive (88/388/EEC), is "flavoring ingredients or formulations which are achieved by suitable enzymatic, microbiological, or physical processes from plant or animal origin materials" (Vandamme & Soetaert, 2002). Until now, animal and plant sources were considered to be essential sources of bioflavors; however, the amount of these compounds is usually very low in these sources or they are only present in exotic (plant) species, which results in their expensive formulation and isolation (Carocho et al., 2015). However, nowadays biotechnology is considered as a preferred method with the advantage of "natural" labeling that can draw consumers' attention. De novo microbial production (fermentation) and enzymatic synthesis are two biotechnological ways to obtain bioflavors. Enzymatic flavor production is commonly preferred over fermentation due to their high yields of production (Asunción Longo & Sanromán, 2005; Vandamme & Soetaert, 2002).

Enzyme immobilization and coenzyme regeneration techniques can lead to an effective and exclusive biocatalytic processes for flavor synthesis (Kragl et al., 1996). The benzaldehyde with almond and cherry taste can be made from the cyanogenic glycoside amygdalin, which is present in almond meal and cherry kernels, by mandelonitrile lyase and b-glucosidase enzymes (Fig. 12.1). An industrial example for this is L-menthol production which is the major ingredient present in peppermint oil (Fig. 12.2) (Menzel & Schreier, 2007).

Recently, synthesis of flavor compounds by biotechnological processes has gained growing importance in the food industry. By considering the growing market, and the growing public concern for the total wholesomeness and chemical safety of food ingredients, researches on bioprocesses and inventing new products are also

Fig. 12.1 Enzymatic route to benzaldehyde

Fig. 12.2 Stereoselective bioconversion of DL-methyl acetate into L-menthol

increasing (Basset, 1990). In 2017, the total global flavors and fragrances market got to US$28.2 billion, which means an increase of 4.6% rather than the previous year, and it is estimated to increase at 4.9% average annual rate to get to approximately US$36 billion in 2022 (Consultants, 2018). A wide range of options are provided by enzymes, for food flavor production. The specificity of the enzymes and whether they are being used through whole-cell or cell-free systems support the production of chemicals that are hard to synthesize; also their stereoselectivity is considered as a principal advantage for the food industry where a particular optical conformation can affect flavor properties. Application of enzymes as food additives can produce or liberate flavor from precursors, as well as correcting off-flavors produced by specific naturally occurring compounds, or those that are produced during processing (Bigelis, 1992). Besides intentional addition of enzymes to the food, they can also be inherent to the food or may originate from microbial sources, or may come from contamination.

In this chapter, several ways that enzymes can have effect on flavor are reviewed, showing examples of actual research, their potential applications, as well as examples of the fundamentals of biosynthesis and the market potential of some products. In some instances, processes and reactions known for two decades are stated and they are updated with recent developments, such as reaction in organic media or whole-cell biocatalysts.

12.1.2 Enzymatic Synthesis of Flavor Compounds

12.1.2.1 Lyases

Alliinases

Alliinases [S-alk(en)yl-L-cysteine sulfoxide lyase; alliin lyase; EC 4.4.1.4] remove S-alk(en)yl-L-cysteine sulfoxides, and in this way they produce pyruvate, ammonia, and thiosulfinates, (Fig. 12.3). Pyridoxal-5′-phosphate (PALP) is needed by this enzyme as a cofactor.

The strong aroma and flavor of the most of the species of the Amaryllidaceae family are because of the activity of this enzyme. Among other lyases, this enzyme is detached from the substrate in the intact tissue, and the reaction occurs upon breakdown of cells. The first alliinase to be explained was from garlic bulbs (Tishel & Mazelis, 1966). Until now alliinases have been purified from onion, Welsh onion, Chinese chive, garlic, and leek (Whitaker et al., 2002). Despite that they are from the same genus, their specific alliinases are different in physicochemical properties. It is many years that garlic and onion are being used by several cultures for different purposes. In spite of having therapeutic and nutritional importance in diets, they are being consumed around the world due to their unique flavor and their properties, such as enhancing the flavors of other foods. Diallylthiosulfinate (allicin) is the major sulfur compound in garlic that provides its distinctive aroma. This thiosulfinate results from the elimination reaction by alliinase which is done on S-2-propenyl-L-cysteine sulfoxide (alliin), via the intermediate 2-propenesulfenic acid. There are three flavor precursors in onions, such as S-methyl-L-cysteine sulfoxide, S-propyl-L-cysteine sulfoxide, and S-1-propenyl-L-cysteine sulfoxide. Different thiosulfinates made by the activity of alliinase on the mentioned substrates impart distinctive onion flavors, based on their concentrations (Masuda et al., 2012). Other *Allium spp.* have diverse combinations of these substrates in addition to S-2-propenyl-L-cysteine sulfoxide; this compound is not present in onion (Masuda et al., 2012). It is stated that the lachrymatory factor in onion (syn-propanethial-S-oxide) is originated from S-1-propenyl-L-cysteine sulfoxide (Jayathilaka et al., 2014).

Alliinases do the primary reaction which is responsible of producing distinctive aromas and flavors in vegetables of the *Allium* genus. The maintenance of full enzymatic flavor is the principal challenge of food processors who deals with

S-alk(en)yl-L-cysteine sulfoxide — Cysteine sulfoxide lyase, H2O → Thiosulfinate + $2\,[CH_3C(=O)COOH]$ + $2NH_3$

Fig. 12.3 Alliinase general reaction

onion and garlic. Some preharvest and postharvest practices, such as environmental factors influencing the flavor strength, including growing temperature, water supply, and sulfur content of soil, are monitored in alliaceous foods to enhance their flavor potential (Kamenetsky, 2007). Flavor potential of onions goes up with storage of its bulb for a few months (Petropoulos et al., 2017). Nearly, half of the S-1-propenyl-L-cysteine sulfoxide is bound as a peptide, L-glutamyl-S-allyl-L-cysteine sulfoxide, in nature. It has been stated that the substrate is liberated by the activity of glutamyl transferase. The most part of the flavor potential of the onion is lost during dehydration, which is due to the loss of the substrate more than the enzyme. In the other word 30% of the alliinase substrate is changed to cyclo alliin during the primary phases of dehydration.

Cyclo alliin and the S-alkyl-L-cysteines are not substrates for alliinase. The altered onion powder includes only one-third of the onion aroma of fresh onion juice (Ramírez & Whitaker, 1999). Meticulous consideration of the speed of drying is critical throughout the design of the dehydration process to diminish the substrate loss; process with longer time normally leads to better yields. The activity of alliinase can also lead to the development of unnecessary pigments in alliaceous vegetables, such as pink pigments in onions and leeks, and green pigment in garlics (Whitaker et al., 2002). Despite the ability of alliinase to catalyze the preliminary reaction and thiopropanal-S-oxide formation (in onion), the formation of pigment is dependent on the concentration of carbonyl and may differ from pink to red based on the composition.

Cystine Lyases in Plants

Cystine lyases (cystathionine L-homocysteine lyase; EC 4.4.1.8) is able to cleave L-cystine through generating pyruvate, ammonia, and thiocysteine or cysteine persulfide (Fig. 12.4). The substrate for this enzyme is not exactly determined. Cysteine lyase and cystathionase are both found in fungi and bacteria. However, cystine lyase does not consider cystathionine as a substrate, in plants. The activity of this enzyme was first identified in plants by Tishel and Mazelis (1966). They studied the capability of homogenates of cabbage leaves for degrading L-cystine (not L-cystathionine), to pyruvate. Also it is stated that this enzyme needs pyridoxal-5′ phosphate (PALP) as a cofactor.

Cystine lyases have importance for food quality. These enzymes catalyze the preliminary reaction in important vegetables of the Brassica genus, producing distinctive aromas and flavors. Cruciferous vegetables, such as cabbage, cauliflower, Brussels sprouts, and broccoli, have specific sulfurous flavors and aromas which

Fig. 12.4 General reaction of cysteine lyase

results from the activity of the mentioned enzymes and subsequently tissue softening happens in these vegetables. Cystine lyase was identified to be the key enzyme in broccoli, and its activity results in off-aroma decay (Barrett et al., 2000).

In a study, addition of the purified cystine to blanched broccoli was observed and the enzyme-added broccoli could produce the aroma characteristic of the unblanched broccoli. Chin and Lindsay (1993) determined the sulfur compounds produced in damaged tissues of cabbage as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and methanethiol (MT). Although these compounds are specific to brassica vegetables, they are unfavorable for the consumer. These unpleasant sulfurous odors make people to avoid consuming them, especially in the use of modified atmospheres. The mechanisms of the formation of these compounds in cabbage, which results from the activity of cystine or cystine sulfoxide lyase, were clarified (Chin & Lindsay, 1994a). Researchers could not find significant differences between the DMDS formation in anaerobic and aerobic circumstances; however, DMTS was only identified in anaerobic conditions. Different treatments, such as soaking the vegetable in caraway (Carum carvi) seed extract, ascorbic acid (500 ppm), phosphoric acid (0.1 M), sodium hydroxide (0.01 M), and tertiary butylhydroquinone (TBHQ), have been done to decrease the levels of these undesirable volatiles in broccoli stored in modified atmosphere condition (Chin & Lindsay, 1994b). All of the mentioned treatments could decrease the intensity of sulfurous aromas, in which alkaline treatment had the highest effect that could suppress the formation of DMDS. Obenland and Aung (1996) discovered that the formation of MT and DMDS in an intact broccoli flowerets can be nearly removed by infiltration of hydroxylamine which is an inhibitor of cystine lyase, and can be increased 2.8 times by infiltration of S-methyl cysteine sulfoxide before the anaerobiosis.

12.1.2.2 Isomerase

Xylose (Glucose) Isomerase

High-Fructose Corn Syrup (HFCS) is a combination of fructose and glucose made from cornstarch (Parker et al., 2010). HFCSs have extensive use in food industry and the most substantial application is their addition to soft drinks as a sweetener where they substitute cane and/or beet sugar. HFCs have 10–20% lower price than sucrose, in the same levels, and have less calorie due to the lower absorption of fructose. Glucose and fructose in HFCS have better solubility rather than sucrose and this accounts for their higher advantage to sucrose. Therefore, they have lesser affinity for crystallization in a broad range of food products. This feature has made them suitable compounds for application in different products, such as meat products, ice cream, canned products, confectionery, bakery, jam and jellies, sauces, and pickles (Parker et al., 2010).

The process for producing HFCSs includes three continuous enzymatic steps. Starch which is primarily originated from corn is used as the raw material in this process. Starch is consisted of amylose (linear) and amylopectin (branched) which are both polymers of D-glucose. The glucose moieties are linked by 1,4-glycosidic

bonds in amylose and the main chain of amylopectin. However, the branches are linked by 1,6-glycosidic bonds.

The starch which is produced from corn is exposed to liquefaction, in the first step of enzymatic process, by application of a thermostable bacterial amylase (EC 3.2.1.1) from *Bacillus spp,* such as *B. stearothermophilus* and *B. licheniformis*. In this step, enzyme is able to produce a dextrin solution with a dextrose equivalent (DE) of 10 by cleaving the 1,4 linkages in an endo fashion.

In the second step, other enzyme named as glucoamylase (amyloglucosidase; EC 3.2.1.3) releases single glucose moieties from the nonreducing end by hydrolyzing the dextrins in an exo fashion.

Glucoamylase shows a higher activity for the 1,4-glycosidic bonds rather than the 1,6-branch bonds. Moreover, in some situations a debranching enzyme, such as pullulanase (EC 3.2.1.41), is also used and the produced glucose solution has a DE of 95.

In the next step of enzymatic process, other enzyme named as glucose isomerase catalyzes the conversion of glucose into fructose until a concentration of 42% (molar fraction based on total sugars). This reaction is exclusive because it is taking place in a column reactor using immobilized form of the enzyme. Glucose isomerase was one of the earliest enzymes used for this purpose on an industrial scale. This process is done by encapsulating the enzyme in a packed-bed column reactor with 1- to 2-mm particles (Ruiz-Matute et al., 2010). The diameter and height of the columns may be 1.5 m in and 4–5 m, respectively. This system has a high yield which produces 500,000 kg fructose (dry weight) per day by 4000 kg enzyme. High-glucose syrup with DE ¼ 95, resulted from previous glucoamylase reaction with 45% solids and pH adjusted to 7.5, is continuously pumped through the column. Several factors have effect on the temperature and flow rate of the system. The flow rate should be suitable to provide enough residence time for production of 42% fructose. Low flow rates give rise to the risk of microbial contamination, also extremely high rates do not provide enough conversion and result in channeling through the support material. Temperatures below 55–60 °C increase the syrup's viscosity and increase the risk of microbial contamination. However, higher temperatures decrease the stability of the enzyme and results in the production of unfavorable by-products via chemical reactions such as Maillard reactions.

Practically, temperatures of 55–60 °C are being used. Regulation of the glucose stream's flow rate can compensate for the enzyme's inactivation to maintain the required conversion. These processes can continue for several weeks, under the mentioned circumstances until the activity of the enzyme has become too low. After getting to that point, the columns are disassembled and packed again with a batch of fresh biocatalyst. The final HFCS is consisted of sugars, such as oligosaccharides (up to 4%), fructose (42%), and glucose (54%) (Parker et al., 2010; Ruiz-Matute et al., 2010).

12.1.2.3 Oxidoreductases

Oxidative and reductive processes play a significant role in foods. They affect many aspects of food stuffs, such as taste, shelf life, texture, nutritional value, and

appearance. Both nonenzymatic and enzymatic redox processes are included. Sometimes processes lead to detrimental effects, such as textural problems, off-flavor, and decreased shelf life. In some cases, also, they provide positive effects to the final aroma, a better texture, a more desired appearance, or an improved shelf life. It is very important to control redox behavior throughout all stages of processing and storage, in food systems. Redox reactions in foods were controlled primarily by addition of antioxidants or chemicals, designing air-tight packaging materials, cautiously choosing raw materials, or by adjusting process situations, until now. However, there is not much paid attention to redox reactions in foods by the addition of oxidoreductases or by modifying the profile or changing the content of oxidoreductases in raw materials of foods by genetic implements. Apparently, the main reason for this little attention for using oxidoreductases in foods is that it is still not possible to produce most of the enzymes, cost-effectively. Moreover, general concerns about the application of recombinant enzymes in foodstuffs are indicating their commercial introduction. Various oxidoreductases are included in the in situ or in vivo biogenesis of appropriate aroma compounds, in vitro production of flavors and top notes, and in the endogenous development of off-flavors. The present applications of oxidoreductases for controlling the taste of food products are provided below**:**

Lipoxygenases

Lipoxygenase (LOX), which was previously known as carotene oxidase or lipoxidase, is a dioxygenase containing iron, which catalyzes the oxidation of polyunsaturated fatty acids comprising of cis,cis-1,4-pentadiene groups (arachidonic, linolenic, and linoleic acids) to the equivalent conjugated cis-, trans-dienoic monohydroperoxides. Moreover, LOX has a broad range of substrates which are phenolic compounds (Markus et al., 1991); also it is able to oxidize other substrates rather than the actual substrate. This process is identified as co-oxidation and involves compounds, such as polyphenols and carotenoids. LOX is one of the endogenous enzymes that plays a significant role in off-flavor formation and flavor generation and in almost all food products it is originated from raw materials of plant. Based on the type of food product and final concentration, a flavor compound can be a desirable aroma component or an off-flavor at higher concentrations. For instance, alcohols and C6-aldehydes derived from oxidation of (poly)unsaturated fatty acids, which are catalyzed by the LOX, mostly have a positive impact on the aroma profile (such as in wines and juices), but in other products, such as beverages (e.g., beer), does not have desirable effect on flavor. Similarly, endogenous LOX is known to have impact on the bread flavor due to generating carbonyl compounds in dough systems (Pico et al., 2015).

The C6 compounds' formation involves the consecutive action of four enzymes, including two redox enzymes, such as a LOX, and an acylhydrolase, a yeast-derived alcohol dehydrogenase, and a hydroperoxide lyase (Whitaker et al., 2002).

Normally, the formation of off-flavor is inhibited by the means of LOX isoenzyme-deficient crop variants, by genetic tools or by screening or controlling the oxygen levels during processing, in addition to eliminating the putrid oxidation

products subsequently (Mandal, 2012). Physical techniques, such as adsorption, or enzymatically altering the undesired alcohols or aldehydes to their equivalent carboxylic acid which are less flavorful, can be good ways to deodorize the off-flavors. This can be done by aldehyde dehydrogenase/oxidase or alcohol oxidase. The application of redox enzymes for this purpose has been proved for different food products, such as fish oil, cream, noodles, soybean products, cooked rice, and margarine (Raghuvanshi & Bisht, 2010; Antrim & Taylor, 1990).

Additionally, LOX is mostly applied to dairy products and beverages to modify their flavor profile for in vitro production of numerous natural top note flavors. Examples of these applications can be the conversion of polyunsaturated fatty acids to delta-decalactone (butter flavor) (Tunick, 2007) or to several short to medium-chain aldehydes/alcohols (Schwab et al., 2008; Scrimgeour et al., 2005). A well-known flavoring derived from fatty acid (FA), amdaldehydes/alcohols, comprises the above-mentioned C5 and C6 compounds, (E3, Z5, Z8)-undecatetraene (seaweed), 1-octen-3-one (field mushroom), (E3, E5)-undecatriene (balsamic), (E2, E6)-nonadienal (cucumber), and (Z5)-octadien-3-one (geranium leaves).

The regioselectivity and unsaturation degree of the LOX determines the formation of various hydroperoxy compounds, from which consequently the mentioned compounds can be derived by enzymatic reactions. Linolenic acid is being used as a substrate for (Z3)-hexenol; however, for most of the other alcohols/aldehydes, higher unsaturated fatty acids are needed. (Z3)-hexenol production has lately been industrialized by means of plant homogenates, such as green peppers and alfalfa sprouts, which are pretty rich in hydroperoxide lyase enzyme which is needed to cleave the hydroperoxide fatty acid to more little fragments.

Reductive enzymatic power of baker's yeast was responsible to transform (Z3)-hexenal to (Z3)-hexenol (Ricard, 1991). After the linoleic acid hydroperoxide formation, a unique strategy is needed to be followed for the production of lactones. It includes the fermentative oxidation of the hydroperoxide intermediate by the yeast *Pichia etchellsii*, and the consequent cyclization of 5-hydroxydecanoic acid to the equivalent S(-)-decalactone (Laane et al., 1997).

Moreover, there are relatively extensive spectrum of phenolic compounds that LOXs accept as substrates. Conversions of coniferyl benzoate and isoeugenol from Siam resin into vanillin is within the interest of flavor industry. Presently, the industrialization of these biotransformations is hindered due to the fact that isoeugenol is not easily accessible and that coniferyl benzoate is hard to work within a reactor (Priefert et al., 2001). Other reactions of LOX which are well known involve the co-oxidation reaction of carotenoids to ionone. Also it is stated that LOX and alpha-amylase combination can generate a toasted or cookie-like flavor in starchy materials (Desjardins et al., 1997).

Alcohol Oxidases and Dehydrogenases

Generally, aldehydes are considered as stronger flavor compounds rather than their alcoholic equivalents. Thus, alcohol oxidases are the enzymes which are of interest for the in vitro production of flavoring compounds in beverages. Enzymes, such as methanol oxidase from *Candida, Pichia,* and *Hansenula,* are good examples of

alcohol oxidases that are used for the formation of natural acetaldehyde from ethanol (Lopez-Gallego et al., 2007). This enzyme is produced throughout growth on methanol. Phase cells are collected and incubated with ethanol, at the final stage of the logarithmic growth. Therefore, 1.5% natural acetaldehyde concentrations can be reached, that can be further concentrated to the preferred application level. The substrate specificity of the alcohol oxidase is different, in various yeasts. Hence, this method can also be used to transform other alcohols, to their equivalent aldehyde. Therefore, dehydrogenases can be used as substitutes to alcohol oxidases. However, there is a limitation to use these dehydrogenases, because they need the costly cofactor $NAD(P)^{+}$instead of oxygen which is cheap, as an electron acceptor. Although there are several designed complex NAD(P) cofactor stimulating systems and significant cost reductions, it is obvious that oxidases are preferred in industrial applications to their dehydrogenase equivalents. The application of dehydrogenases for food purposes is limited to whole-cell transformations. There is a special kind of alcohol oxidase, namely, vanillyl alcohol oxidase (VAO) which is derived from *Penicillium simplicissimum*. Recently, it has been indicated that this enzyme, which contains flavin and is relatively stable, has an extremely wide substrate specificity and can convert para-substituted phenols into flavoring compounds or flavor precursors (Ewing et al., 2018). In addition to the natural coniferyl alcohol and vanillin, allylphenols and various vinylphenols, such as para-vinylguaiacol, can be generated from cheap raw materials and oxygen (as an electron acceptor). VAO can also be used for the production of building blocks of flavor. VAO can also be applied to a natural mixture of phenolic compounds to enrich foods or flavor preparations with a variety of aldehydic compounds and vinylic/allylic.

Sulfhydryl Oxidases

Formation of disulfide bonds from (protein) thiols is catalyzed by sulfhydryl oxidase. This enzyme is a glycoprotein which contains Cu/Fe and has been detected in goat, pig, human rabbit, bovine, and rat milks (Thorpe et al., 2002). The enzyme is able to oxidize the cysteine and sulfhydryl groups of glutathione, and milk proteins using molecular oxygen as electron acceptor, and transform them to their equivalent disulfides (Faccio et al., 2011). SOX of bovine milk might be added to UHT milk for reducing the cooked flavors. The enzyme has been immobilized on porous glass, and its efficacy in improving the cooked flavor has been showed on a pilot scale through the immobilized enzyme columns.

Peroxidases

Peroxidase (POX) happens extensively in nature and is the name which is generally applied to a group of both nonspecific and specific enzymes which use hydrogen peroxide as an electron acceptor. POXs, particularly the enzymes containing heme, are able to catalyze a majority of various reactions, including N-demethylation, oxidation, hydroxylation, and sulfoxidation; therefore, there is a special interest for these enzymes in the production of specific flavoring top notes. Demethylation of methyl N-methylanthranilate (ex Citrus) to mono-methylanthranilate is an example of the application of these enzymes (Van Haandel et al., 2000). The mentioned

produced compound is the main top note flavor in Concord grapes. Horseradish, microperoxidase, and soybean were found to be appropriate catalysts for this reaction. Moreover, POX increases the fresh flavor profile when it is used in tomato paste (Wilding & Woolner, 1997).

Polyphenol Oxidases

Polyphenol oxidase (PPO) enzymes are a group, including various enzymes with different activities. Tyrosinase, a monophenol monooxygenase, is able to transform a phenol into a catechol group. The other enzymes of this group, catechol oxidase or 1,2-diphenol oxidase (EC 1.10.3.1), converts catechol into an **O**-quinone, and laccase is able to produce **p-**quinone from 1,4-diphenol. Generally, the first two activities are related, since catechol is much more oxidized rather than a phenol. Also most of the enzymes, that are able to catalyze the oxidation of 1,4-diphenol, can act on 1,2-diphenols. In addition, guaiacols which are polyphenolic compounds impart the bitter taste in several food products. PPOs are able to oxidize these compounds; therefore, they can be used to decrease bitterness. There are several studies about the application of these enzymes for this purpose, such as debittering of cacao beans (Takemori et al., 1992), Adzuki beans (Okazawa et al., 1993), and coffee beans (Small & Asquith, 1989). The other example of their application is the use of laccase for debittering of olives (Charoenprasert & Mitchell, 2012).

12.1.2.4 Hydrolayses

Lipase and Esterases

Lipases and esterases are lipolytic enzymes that are able to catalyze the hydrolysis of triglycerides which are primarily different in the activities on substrates. These enzymes are different in substrates which can be in emulsion or solution forms. Lipases show extremely greater activity on emulsified substrates and prefer long-chain fatty acids; however, esterases indicate their activity on aqueous solutions of short acyl chain esters (Arpigny & Jaeger, 1999). Ester synthesis is generally catalyzed by enzymes, such as esterases and lipases, which can be expressed in many food microorganisms. These enzymes are serine hydrolases and are able of hydrolyzing or synthesizing esters based on the environmental conditions. Microbial lipases are commercially important due to their better stability, availability, and affordability rather than lipases from other sources (Molimard & Spinnler, 1996). Generally there are following common types of lipases according to (Waldmann & Parhofer, 2019): milk lipases; lipoprotein lipase; pancreatic lipases; hepatic lipases; hormone-sensitive botanical lipases; and microbial lipases, including *Candida* and *Torulopsis* yeast species, *Geotrichum*, *Aspergillus, Rhizopus, Penicillium,* and *Mucor* mold species, *Staphylococcus*, *Pseudomonas*, and *Achromobacter* bacterial species. Esters synthesized by lipase play a significant role in food industry as flavor and aroma constituents. Synthesizing esters by lipase can be conducted in vessels in batch or continuous condition and esters achieved from these sprouts have more purity and low cost, rather than other chemical methods which require special conditions and lead to redundant by-products production. However, low conversion

rate of these systems is their main drawback (Aravindan et al., 2007). Commercial lipases are mostly used in dairy products, meat industry, baked foods, fruit, vegetables, beer, and wine (Nagodawithana & Reed, 1993). Immobilized lipase on silica and microemulsion-based organogels are extensively being applied for synthesis of ester (Ghosh et al., 1996; Sharma et al., 2001). Lipases are typically highly specific. For instance, in a study Larios et al. (2004) in which the synthesis of esters in n-hexane was considered, it was found that lipase (fraction B) from *Candida antarctica* indicates substrate specificity. This specificity involves both acids and alcohols. It was shown that it has activity on unsaturated fatty acids and short-chain fatty acids with linear and branched chain structures, and 2-phenylethyl, n-butyl, isopentyl, and geraniol as alcohols. Generally enantiomeric selectivity of enzymes is among their important properties for food aroma synthesizing. As an example, only (S)-form of 2-methylbutanoic acid methyl ester has the fruity flavor which is the main flavor compound of apple and strawberry. It is found that lipases from *Aspergillus niger, Rhizomucor miehei,* and *Aspergillus javanicus* have the selectivity for this flavor compound (Kwon et al., 2000). Moreover, in a medium without solvent, lipase from *Candida rugosa* was selected for L-menthol esterification with long-chain unsaturated fatty acids to regulate the strong flavor related to it, whereas it was acted weakly on D-menthol (Shimada et al., 1999). However, some lipases indicate only restrained enantioselectivity, like *Candida antarctica* lipase (fraction B) selectivity for chiral short-chain carboxylic acids (Larios et al., 2004). Generally, lower water activity medium is preferred for lipase activity, so solvents with high hydrophobic activity are more favorable due to their insolubility in water layer around the enzyme surface which is important for protection of their active stereoconfiguration (Huang et al., 1998; Langrand et al., 1990). Additionally, enzymatic synthesis of esters can take place in systems without solvent. In these systems, reactant itself (i.e., an alcohol) acts as a solvent. This method is desirable in food industry, but because of the heterogeneity of the reaction, there are limitations, such as mass transfer and consequently low reaction rate in these systems. Researchers could find a new thermostable esterase derived from a marine mud metagenomic library, with potential use in industry, which is named as EST4. It is reported that this enzyme can efficiently produce several short-chain flavor esters under high concentrations of substrate and in media which is nonaqueous (Gao et al., 2016).

Researchers found that thermo-tolerant strain, such as *Bacillus licheniformis* (cloned in E. coli), has good ability to produce thermophilic lipolytic enzymes. These enzymes can tolerate high temperatures and organic solvents, which indicates their promising feature to use them for ester synthesis (Alvarez-Macarie & Baratti, 2000; Dominguez et al., 2004; Fuciños et al., 2005). In a study by Chang et al. (2001) the capability of mutant and wild-type lipases from *Staphylococcus epidermidis* to catalyze the formation of flavor esters was investigated in aqueous phase. This lipase was shown to have strong specificity for geranyl esters, unsaturated esters, and medium-chain esters. Seitz has stated that *Debaryomyces klockeri* and *Candida mycoderma* yeasts can produce lipases that have the best activity at pH 4.5 (Seitz, 1974). In another study it is shown that lipases from *Penicillium, Aspergillus*, and *Paecilomyces*, which are responsible for the fatty acid profile of the

cocoa bean, show different activities and specificities which are useful when they are applied to milk fat (Hansen et al., 1973).

Applications of lipolytic enzymes require good interdependence between the total substrate composition and the enzyme activity. For instance, the activity of milk lipase was as a result of the effect of the both stimulatory and inhibitory activity of several milk components on the enzyme (Hansen et al., 1973). In a study by Baianu et al. (2003), the free lipase derived from *M. miehei* could conduct the direct esterification of geraniol and citronellol with short-chain fatty acids, which had high yields in n-hexane. According to Sánchez et al. (2002), flavor esters, such as hexyl esters, produced by lipases are green note flavor compounds that are widely used as flavor and fragrance in different industries, including food industry. Recently, there is an increasing requirement for natural flavors, including "green note" characterized by hexanol derivatives. For instance, hexyl butyrate is of particular significance as it characterizes a model of flavor ester. Shieh and Chang (2001) have investigated the capability of immobilized lipase from *R. miehei* to catalyze the transesterification of tributyrin and hexanol. They determined that decreasing the amounts of tributyrin as a co-substrate would decrease the cost for the production of the hexyl butyrate. However, for transesterification of hexanol with triacetin with the same lipase, results indicated that substrate molar ratio and temperature of the reaction were the factors with the paramount importance and the added water content had less impact on the reaction.

In dairy industry lipases and esterases are regularly used to enhance the buttery flavor of the final product. Lipases are mostly used to hydrolyze milk fat. By modifying fatty acid chain lengths, lipases can enhance flavor of different kinds of cheeses. Short-chain fatty acids, specifically butanoic acid, play an essential role in dairy products' flavor and different commercial enzymes which have different specificities towards short-chain fatty acids are considered as flavoring tools (Saerens et al., 2008). There are several studies about the cheese ripening acceleration and fat, cream, and butter lipolysis by lipases (Ghosh et al., 1996; Sharma et al., 2001). Lipase from pregastric glands, such as bovine and porcine, and pregastric tissues of ruminants were initially used for enhancing cheese flavor.

Various kinds of cheeses can be produced by using a specific kind of microbial source or mixture of them. To produce enzyme-modified cheese (EMC) usually cheese is incubated with enzymes at an increased temperature and in this way a concentrated flavor is produced which can be used in sauces, snacks, soups, and dips. In these cheeses the concentration of fat is tenfold higher rather than normal cheeses (Ghosh et al., 1996; Sharma et al., 2001). Indigenous or exogenous milk enzymes can contribute to lipolysis. Exogenous enzymes can be either microbial lipases and/or mammalian pregastric esterase lipases. This process is an essential factor in flavor development of cheese. From different reviews on lipolysis in cheese, it can be derived that lipolysis results in the FFAs formation which are considered as major flavor compounds of cheeses and can be transformed to other flavor compounds, such as acetoacetate, aldehydes, β-keto acids, lactones, methyl ketones, esters, and secondary alcohols which are more effective and can directly affect flavor in different kinds of cheeses (Collins et al., 2003). Lactic acid bacteria (LAB) are

used in cheese fermentation as starter adjuncts, starter cultures, or as secondary microbial flora (Beresford et al., 2001; Crow et al., 2001). Moreover, Custry et al. (1987) reported that methyl ketones are main group of compounds contributing to cheese flavor and are originated from the FAs released by lipolysis reaction. A secondary flora is needed for enzymatically oxidization of these FAs into methyl ketones via β-oxidation. Additionally, the oxy fatty acids in milk fat can also be the source of methyl ketones.

Development of cheese flavor is a complex process in which enzymes from different sources are responsible for the degradation of proteins, carbohydrates, and milk fat. Straight-chain carbons of C2 to C18 are the main FFAs in cheese which have quite high flavor perception thresholds, leading to different flavor notes, such as pungent, goaty, rancid, soapy, waxy, or cheesy notes (Collins et al., 2003). However, the flavor perception thresholds of esters which are resulted from FFAs with carbon chain of less than C10 is much lower, revealing a fruity flavor notes which are pleasant for some people. Esters are also able to mask effect of off-flavors, such as pungent and sharp off-flavors. On the other hand, high levels of ethyl esters of short-chain FFAs can cause a fruity flavor defect in cheeses, such as Cheddar (Bills et al., 1965; McGugan et al., 1975).

Esterases of LAB can hydrolyze aromatic esters, thioesters, and alkyl esters, as well as hydrolysis of b-naphthyl or p-nitrophenyl esters of FAs, partially hydrolyzed milk fat, and synthetic glycerides. It has been stated that esterases of Lb. casei, Lc. Lactis, and Lb. helveticus are able to hydrolyze ethyl esters of FAs of C2 to C6, acetate esters of hexanol, propanol, and butanol, as well as aromatic esters, such as phenyl acetate and phenyl thioacetate. Researchers could increase ester production by lowering water activity (0.8 5 and 0.9) and increasing the levels of substrates (up to 1000 ppm of FFA and alcohol) in model system of cheese containing esterases from several LAB. According to studies, each esterase results in hydrolysis of a different flavor-active ester (Fenster et al., 2000, 2003a, 2003b). *Candida lipolytica* was used in the manufacture of Blue cheese (Price et al., 2014). Moschopoulou (2011) successfully indicated the flavor of Pecorino cheese by using lipolytic enzymes derived from microorganisms obtained from the abomasum of lambs. Rhizopus lipase has been applied to development of artificial butter flavors and in yogurt manufacture (Singh & Banerjee, 2017).

Generally, it is known that esterification of FFAs with ethanol results in ester formation and these esters impart aromatic molecules in foods, specifically in cheeses. Ethyl esters or methyl esters derived from short-chain fatty acids mostly carry significant fruity flavors, whereas thioesters from thiols have sulfur and cabbage aromas (Collins et al., 2003; Fox & Wallace, 1997).

It is known that LAB have little contribution to lipolysis, but additional cultures, such as molds usually have high activities in fat conversion in the case of surface-ripened cheeses (Molimard & Spinnler, 1996). The derived flavors from the fat conversion are predominantly important in Camembert and Roquefort (soft cheeses).

Esterases of LAB are also known as alcohol acyltransferases due to their ability in transferring fatty acyl groups from a glyceride to either water, which is called hydrolysis, or alcohols, which is called alcoholysis, in aqueous systems. Moreover,

esters synthesizing in aqueous systems can be done through alcoholysis from other alcohols and glycerides. For instance, it is found that *St. thermophilus* is able to synthesize aromatic esters (2-phenyl ethyl hexanoate and 2-phenyl ethyl octanoate) from an aromatic alcohol (2-phenyl ethanol) and two glycerides [monooctanoin (mono-caprylin, C8) and dihexanoin (dicaproin, C6)](Liu et al., 2003a). Generally, di- and monoglyceride substrates are preferred for alcoholysis by LAB esterases which have the same specificity in hydrolysis (Liu et al., 2003a).

In cheese medium ester synthesizing can also done by selecting specific starters that have high esterase or transferase activity, by changing the mono-, di-, and triglyceride composition of the cheese fat glycerides, or by controlling ethanol availability. For instance, in cheese made with mesophilic starters, by increasing the ethanol level, ethyl hexanoate and ethyl butanoate concentrations are significantly increased (Crow et al., 2002). In other study by spiking 1 M ethanol in Cheddar-type cheese curd, during an incubation of 2 months, ethyl butanoate and ethyl hexanoate are synthesized by the recombinant esterase from *Lb. rhamnosus*. Availability of ethanol is possibly an essential factor in controlling ester formation in cheese. Also primed cheeses with prehydrolyzed milk fat contain more esterases and this is because of the presence of more di- and monoglycerides and it is found that without addition of ethanol there is no ester formation in these cheeses. Moreover, higher ester levels in cheese curd result in fruity aroma. In addition, some commercial fungal lipases also found to synthesize esters from glycerides and ethanol in cheese medium; also it is shown that they also have similar mechanism to LAB (Liu et al., 2003b). Moreover, yeasts and molds have effect on ester formation in cheese by producing the alcohols needed for ester synthesizing in cheese.

Salvadori (1961) could double the flavor of Pecorino cheese prepared with ewe's milk by addition of lipases taken from bacteria which were isolated from the abomasum of lambs. In addition, it is found that esterase from *Mucor miehei* also shows lipolytic activity in cheese similar to that achieved by pregastric oral esterases (Richardson & Nelson, 1968).

In a study by comparing flavor characteristics of Romano cheese and Fontina cheese containing *Mucor miehei* esterase to that of cheeses prepared with pregastric esterase, it was found that by using fungal esterase and pregastric calf esterase at the same lipolytic activity in the Fontina cheese, the desired flavor develops in both cheeses. However, based on lipolytic activity, in Romano cheese it was necessary to add a five time greater of fungal esterase, rather than pregastric kid esterase. According to Baillargeon (1990), lipase from *Geotrichum candidum* which is a dairy yeast has selectivity towards unsaturated fatty acids with cis-9 double bonds, such as oleic, linoleic, and linolenic acids. Also, *Yarrowia lipolytica* (a dairy yeast) could majorly release oleic and palmitic, from milk fat (Suzzi et al., 2001). It was also previously found that *G. candidum and Y. lipolytica* can selectively release unsaturated long-chain fatty acids from milk fat, in the cheese environment (Das et al., 2005). However, these FAs have not favorable flavor and they impart a soapy flavor to cheese, but they can be converted to conjugated linoleic acid (CLA) by *Propionibacteria* which are usually used in Swiss cheese production (Jiang et al., 1998). However, in another study researchers used three strains of

Propionibacterium freudenreichii ssp. Shermanii as adjunct strains which are able to convert linoleic acid to CLA in laboratory media, with strains of *Yarrowia lipolytica, Geotrichum candidum,* and *Lactobacillus fermentum* (to produce ethanol from lactose) for preparation of washed-curd, dry-salted cheese, but the results showed the failure of the *Propionibacteria* strains to form CLA from linoleic acid, in the cheese media (Das et al., 2005). CLA introduction in dairy products been also done by lipase immobilization method (Baianu et al., 2003).

Based on different cheese varieties and production conditions, nonstarter LAB and the derived products can be varied. Psychrotrophic bacteria produce heat-stable lipases which can adsorb onto the milk fat globules and resist pasteurization. They may contribute to lipolysis in cheese made from milk prior to pasteurization (Cousins et al., 1977). *Penicillium sp.* (a Psychrotrophic bacteria) can produce extracellular lipases which are predominantly responsible for lipolysis in mold-ripened cheeses (Cousins et al., 1977; Smit et al., 2005). Facultative hetero-fermentative lactobacilli, such as *Lb. casei, Lb. paracasei and Lb. plantarum*, which are dominant in cheese varieties as nonstarter LAB, are weakly lipolytic. However, obligatory homo-fermentative lactobacilli, such as *Lb. delbrueckii subsp. lactis, Lb. helveticus,* and *Lb. delbrueckii subsp. bulgaricus*, which are used as starters, also produce esterases (El Soda et al., 1986). According to Bhowmik and Marth (1990) *Pediococcus* and *Micrococcus* are also weakly lipolytic. The effect of FFA on the flavor of hard Italian cheese varieties is more than Blue mold-ripened cheeses, which is probably due to neutralization by increasing the pH during ripening, and also the principal effect of methyl ketones on the flavor of Blue mold cheeses. Actually alkan-2-ones (2-methyl ketones) is the dominant flavor of Blue mold cheeses which is derived from FFA. β-oxidation, which is the pathway of alkan-2-onesformation, involves the liberation of FAs by lipolysis, and their oxidation to β-keto acids. After decarboxylation of them, alkan-2-ones with one less C atom are formed. This compound can be reduced (which is reversible in aerobic conditions) and leads to the formation of corresponding secondary alcohols (alkan-2-ols).

Intramolecular esterification of hydroxy fatty acids results in the formation of lactones. For instance, γ- and δ-lactones, which are strongly flavored, stable, and the main lactones present in cheese, are formed from δ- or γ-hydroxy fatty acids. According to Urbach (1993) in full-fat cheeses, δ-decalactone increases to a maximum concentration (in 14 weeks) and then its concentration decreases; however, in low-fat cheeses, the δ-decalactone level remains constant during ripening. However, Cheddar cheese flavor is improved by decreasing the δ-decalactone level, indicating that δ-decalactone plays an insignificant role in flavor of Cheddar cheese (Dimos et al., 1996). In a study, lipase from a *Sporidiobolus pararoseus* strain was derived and it was found that the specificity of the enzyme for triglyceride was comparable to pregastric esterase. Using this microbial lipase during the process of mozzarella cheese imparted a strong flavor to the cheese. Hence, the lipase taken from *S. pararoseus* can be used instead of pregastric esterase in dairy industry (Mase et al., 2010). In a study six lipases/esterases, related to buttery flavor production, were compared. It was shown that *Candida cylindracea* lipase has the most

specificity to butanoic acid and production of buttery flavor in cream. Moreover, this enzyme led to the highest concentration of free fatty acids (FFAs) (Saerens et al., 2008).

Based on literatures milk lipases have insignificant importance in cheeses prepared with pasteurized milk, and milk bacterial lipases, which are resistant to thermal condition, can act better in cheese production from pasteurized milk. Moreover, both bacterial and milk lipases have importance in milk fat hydrolysis, whereas bacterial lipases and esterases (from Streptococcus cremoris) have significant role in cheeses made from pasteurized milk (Chambers et al., 2010).

Using pregastric esterase in pasteurized milk for Cheddar cheese production results in more FFA and flavor improvement; however, enzyme's activity is lower than its activity in raw milk. Also it was shown that the flavor of Cheddar cheese is associated to a balance between acetate and FFAs and the ratio of 1.0 to 0.55 of acetate: FFA results in the most favorable flavor. Microbial lipases increase FFAs which affect the flavor of cheese (Collins et al., 2003). Kosikowski (1975) also studied the prospective application of microbial lipases in continuous cheese processing, and suggested good flavor development in Cheddar cheese from different fungal lipases and in Blue cheese from *Aspergillus sp* lipase. However, organoleptic properties of Cheddar cheeses are increased in the presence of gastric lipase in their production (Wolf et al., 2009). Moreover, there are studies about the effect of lipase application in Feta (Karami et al., 2009) and Samsoe cheese (Jensen, 1970) in their flavor development.

Lane and Hammer (1936) were the first people suggesting that homogenization of cows' raw milk or cream which is used in Blue cheese production can activate the milk lipase in it; therefore, they concluded that homogenization has a great impact in cheese flavor development and concentrations of volatile acids are two to fourfold greater in cheese produced by homogenized milk. Studies showed that *P. roqueforti* lipase acts specifically on triglycerides with smaller molecular weight. Secondary alcohols and methyl ketones are fundamental compounds for the Blue cheese flavor characteristics and it is known that they are derived from FFAs which are abundant in Blue cheese (Tunick, 2007). Other researchers found out that methyl-n-amyl ketone (2-heptanone) has a significant role in Blue cheese flavor characteristics, which is suggested to be derived through the P. roqueforti activity on caprylic acid. The methyl ketones' formation and their metabolism by fungi in mold-ripened cheeses act through different enzymatic mechanisms, such as release of FFAs from triglycerides of milk fat by lipases (Walker & Mills, 2014). Dwivedi and KINSELLA (1974) studied the continuous addition of lipolyzed milk fat and gradual release of FFAs, in Blue cheese through the action of milk lipase. As a result, a product was formed which was suitable for application in salad appetizers, dressings. Its amount was 7- to 12-fold more than the ketone amount in a decent Blue cheese, and its flavor efficacy was 4 times higher. Also these researchers reported the production of methyl ketone by *P. roqueforti* from lipolyzed milk fat and suggested that higher concentrations of FA had an impermanent inhibitory effect on the FAs metabolization by enzyme.

Researchers, who studied the effect of pregastric esterases from lamb, kid, and calf on bovine milk fat substrate, showed that these lipases have preference for releasing short-chain FFAs. Each esterase had specificity to release butyric and other short-chain FFAs and they were different in the release ratio of short-chain to long-chain FFAs producing specific flavor characteristic while using cheese. It was found that kid esterase has the highest specificity for butyric and other short-chain FFAs.

Studies on Provolone cheeses showed a direct relationship between FFAs (such as butyric acid) and flavor quality and acceptance in Provolone and Romano cheeses (Christensen, 1964; Collins et al., 2003). According to application of both lamb pregastric esterase with lamb gastric extracts, results in more suitable "Provolone-like" flavors, showing that addition of gastric lipase makes Provolone cheeses organoleptically more acceptable. Overall, the extensive use of lipolytic enzymes and commercial rennet paste in Italian cheese manufacture indicates the importance of controlled lipolysis in this industry (Richardson & Nelson, 1968). It is found that gastric lipase also has application for accelerating the cheese ripening and its flavor development in different cheeses, such as Provolone, Ras, and Cheddar cheeses. Addition of calf lipase increases the release of FAs (C4–C6) rate and accelerates the development of flavor. The drawback of this method is that the lipase remains active after ripening and can lead to development of an intense rancid flavor. By addition of the mixture of fungal protease and lipase, relatively high-soluble proteins and FFAs will develop in various cheeses which show better development of flavor in 3 months of ripening. Also the amount of enzyme addition is of high importance and high level of it results in unfavorable characteristics and decrease in the yield (Jahadi et al., 2016).

Literatures indicated that using liposome technology for cheese ripening decreases the losses in yield and bitterness (Jahadi et al., 2015; Vafabakhsh et al., 2013). In cheese made from unpasteurized milk, the lipase which is inherent in the cheese has significant effect in lipolytic action (Jahadi et al., 2020). *P. roqueforti* and *P. camemberti* in Camembert and Blue-veined cheeses are considered as lipolytic cultures and are responsible for lipases production, that accounts for lipolysis. Moreover, lipases are often added to Italian cheese, such as Romano, Provolone, and Paramesan to increase their flavor (Ghosh et al., 1996). Through ripening, there is a constant rise in the concentration of released FAs and total soluble nitrogen (Jahadi et al., 2012). Lipases are able to liberate the FAs from triglycerides, and in this way they can trigger cheese flavor development (Maia et al., 1999; Mohammadi et al., 2015).

In Gouda and Cheddar cheeses, esters, such as ethyl butyrate, have high contribution to their flavor. Moreover, extra esters in Cheddar can impart fruity defect (Caspia et al., 2006). Furthermore, there are compounds in Cheddar and Camembert cheese, resulting from degradation of phenylalanine (such as phenylacetaldehyde, 2-phenylethanol, and the ester phenylethyl acetate), playing role in its floral rose-like aroma and desirable floral note (Carunchia Whetstine et al., 2005).

There is an increasing interest for lipolytic enzymes in baking industry. Findings suggested (phospho) lipases application as alternatives for producing emulsifying lipids due to the enzymes ability to degrade polar wheat lipids to produce

emulsifying lipids in situ (Collar et al., 2000; Kirk et al., 2002). Lipase was mainly used to increase the flavor in bakery products through esterification (releasing short-chain fatty acids). In addition to flavor development, it also extended the shelf life of most of the bakery products. Also softness and texture can be developed by lipase catalyzation (Laboret & Perraud, 1999). Lipase from *A. oryzae* was also used in baking industry, as processing aid (Greenough et al., 1996). Hydrolytic enzymes, such as lipase, were discovered to be effective in decreasing the initial firmness and enhancing the specific volume of breads (Laboret & Perraud, 1999). According to Sánchez et al. (2002), yeasts with LIP A (bacterial lipase gene) result in enzyme with high productivity which can be used in bread as an additive. Butter flavor increases, by hydrolyzing the butterfat with an appropriate lipase in baked foods.

Recent studies have investigated the possibility of application of various enzyme systems for milk fat modification to incorporate them into bakery products. Generally, lipases of animal origin were found to be most suitable for milk fat modification and including milk fat that is modified by these enzymes in bakery products led to the formation of better flavors in them rather than milk fats modified by other enzymes. *Penicillium roqueforti, Geotrichum candidum,* and *Acromobacter lipolyticurn* lipases were mostly inappropriate due to the liberation of a large concentrations of medium- or long-chain fatty acids and production of musty or soapy flavors. Also lamb and kid esterases were not suitable due to the production of excessive quantities of short-chain FAs imparting rancid flavors in bread formulations (Arnold et al., 1975).

Vanillin (4-hydroxy-3-methoxybenzaldehyde), which is a generally desirable flavor, mostly is present in Vanilla Planifolia beans. This flavor is extensively used in foods (Priefert et al., 2001). Ferulic acid is precursor of vanillin and feruloyl esterase has been recognized as the critical enzyme in the biosynthesis of it. This enzyme can be produced in microbial cultures of several fungi grown on various pretreated cereal brans (Mathew & Abraham, 2005). The metabolism of ferulic acid in some microorganisms has also been investigated (Falconnier et al., 1994; Narbad & Gasson, 1998).

Carboxyl esterases (carboxyl ester hydrolases) are enzymes extensively used in different industries (Ewis et al., 2004). These enzymes prefer to catalyze the hydrolysis of esters of short-chain FAs, but they also are able to catalyze the synthesis of ester and transesterification reactions (Bornscheuer, 2002). Amid these esters, flavor acetates from primary alcohols have a great application due to their characteristic flavor and fragrance (Romero et al., 2005). Isoamyl acetate is also one of the most significant flavor compounds used in the food industries and is produced using lipases. In a research, the ability of type II esterase enzyme to catalyze the esterification of isoamyl alcohol to isoamyl acetate which is less toxic compound was studied. This compound is one of the most substantial flavor compounds used in the food industries due to its distinctive banana flavor (Krishna et al., 2001). This ester is widely used as a flavoring compound in several foods and drinks, such as artificial coffee, honey, butterscotch, and alcoholic beverages. Production of isoamyl acetate is typically doable through Fischer esterification

mechanism (Welsh et al., 1989) and all of the enzymatic synthesis reactions of this compound were taken place using lipases (Krishna et al., 2001). Also there are applications of lipases in ice cream and single cell protein in which C. rugosa lipase has significant role in their flavor characteristics (Sánchez et al., 2002).

Tomato flavor, which is composed of a complex mixture of volatiles containing multiple acetate esters, is resulted from esterases activity. It is believed that red-fruited species of the tomato clade collects a quite low content of acetate esters rather than the green-fruited species and the difference is related to the insertion of a retrotransposon adjacent to the most enzymatically active member of family of esterases, which results in higher expression of the esterase and decrease in the levels of multiple esters that have negative impact on human preferences for tomato (Goulet et al., 2012).

Studies on the flavor and odor characteristics of Suanyu (a traditional Chinese fermented freshwater fish), in the presence of the esterase activity of Saccharomyces cerevisiae 31, Lactobacillus plantarum 120, and Staphylococcus xylosus 135, indicated that all strains displayed esterase activities in both intracellular and extracellular fractions against p-NP-butyrate, p-NP-octanoate, and p-nitrophenyl (p-NP)-acetate. Moreover, it was found out that a low Aw (water activity), a low pH, and also a high temperature would be more suitable for Suanyu processing, which results in more flavor compounds which are favorable for consumers (Gao et al., 2017).

Also in wine industry high levels of 4-vinylguaiacol and 4-vinylphenol were identified from grape juice primarily treated with two enzymes. Firstly, cinnamoyl esterase activity releases cinnamic acids from the related tartaric acid esters. Secondly, decarboxylase activity by the yeasts transforms cinnamic acids into 4-vinyl phenol and 4-vinyl guaiacol (Dugelay et al., 1993).

In oil industry *Cooney and Emerson strains of Mucor miehei* esterase has some functions. This enzyme have impact on natural fats such as lard oil, beef tallow, and vegetable oils and some synthetic substrates, such as sorbitol esters of FAs. Also fatty acid profiles which are resulted from the hydrolysis of beef tallow and soy oil at pH 8.0 with both pancreatic lipase and M. miehei esterase, are comparable (Moskowitz et al., 1977).

Protease

Proteases are enzymes able to break down the peptide bonds of proteins; they are categorized as alkaline, neutral, and acid proteases. Animals, plants, and microorganisms can be potential sources for these enzymes, in different conditions, such as high salt concentrations. Wouters et al. (2016) explained that the flavor and odor of the protein constituents have significant impact in food industry. In fact, peptides and amino acids, besides other molecules, such as salts or sugars, define the taste of foods. The human gustatory system is able to detect 5 basic flavors, such as sweetness, bitterness, saltiness, sourness, and savoriness or umami. Sweetness, bitterness, and savoriness are the major tastes associated with peptides (Iwaniak et al., 2016). Bitterness needs a particular care, due to its cause for product rejection. The peptides' flavor can change based on their amino acid sequence. Studies have

indicated a correlation between the chain length of the peptides and their bitterness. Their bitterness increases when they are made by up to 8 amino acid groups. Furthermore, this is affected by its general hydrophobicity and the particular amino acid positioned at the N- and C-terminus. For instance, the terminus position of tyrosine or phenylalanine can determine the bitter taste (Iwaniak et al., 2016; Maehashi & Huang, 2009). Therefore, selecting the right protease to accomplish the hydrolysis of protein extract can reduce the unpleasant flavor in the final product and also produce peptides with suitable tastes. For instance, during cheese production and ripening, a continuous proteolysis is normally considered to be a required condition for producing right flavor of cheeses (particularly Camembert or Brie) (Singh et al., 2016). Moreover, proteolysis occurs during the main biochemical modifications in cheese making, considering that in most circumstances, the first step is an enzymatic coagulation. In the next steps, during the cheese ripening, a progressive transformation of flavor and texture occurs. In fact, proteolysis is not taking place only by the activity of external added enzymes but also it can be done by enzymes of microorganisms. In Blue-veined cheeses, Blue molds generate a typical odor and flavor through the conversions catalyzed by their enzymes. The same happens at the moldy surfaces of cheeses, such as Camembert and Brie (Seratlić et al., 2011).

Glutaminase

L-Glutamic acid is a verified flavor-enhancing amino acid which is extensively used as a fundamental seasoning in food service, home cooking, and processed food industries around the world. On the other hand, L-glutamic acid is originated from proteins present in raw materials in fermented foods. L-Glutamic acid can also be produced by the hydrolysis of L-glutamine in the protein hydrolysate; however, in the absence of glutaminase, most of the released L-glutamine is transformed to pyroglutamic acid which is tasteless (Fig. 12.5).

Soy sauce is a product known for its unique and strong umami taste, which is created through its hydrolysis and fermentation. Umami taste induced by monosodium L-glutamate has been widely accepted as one of the basic kinds of tastes which is also named as savory, broth-like, or meaty taste. In fact, this taste is linked to the taste characteristics and the chemical structure of L-glutamyl oligopeptides. The presence of glutamic acid in di- or tripeptides is strongly correlated with umami taste, because of the fact that the anionic L-glutamyl oligopeptides can be present as umami taste (Zhang et al., 2017). Glutaminase derived from *Aspergillus oryzae* is often used for the soy sauce fermentation, and

Glutamine + CH3-CH2-NH2 (Ethylamine) → Thaenine + NH3

Fig. 12.5 Formation of pyroglutamic acid in the absence of glutaminase (tasteless)

high salt concentrations in the soy sauce fermentation process can inhibit its activity. Thus, salt-tolerant type of this enzyme have been studied for application in soy sauce fermentation. Glutaminase enzymes from *Micrococcus luteus, Bacillus subtilis,* and Aspergillus oryzae have been widely studied (Masuo et al., 2005). The glutaminase of *B. subtilis* is commercially available. Zhuang and others (Zhuang et al., 2016) indicated the significance of peptides' effect on the umami taste of soy sauce. They showed that the amino acid sequences of the peptides (Glu-Ala-Gly-Ile-Gln, Ala-Leu-Pro-Glu-Glu-Val, Ala-Gln-Ala-Leu-Gln-Ala-Gln-Ala, Glu-Gln-Gln-Gln-Gln, and Leu-Pro-Glu-Glu-Val), with the presence of glutamic acid/glutamine persistent, are responsible for the umami taste in soy sauce.

Theanine (γ-glutamylethylamide) is an amino acid derived from a nonprotein source that initially was isolated from green tea leaves (Sakato, 1949). This amino acid is considered to be a distinctive amino acid in nature due to its limited occurrence to the *Camellia genus*, especially the tea-producing plant, *C. sinensis* (Nathan et al., 2006). Theanine is acknowledged for its unique umami taste. Therefore, teas containing high theanine are normally considered to have higher quality. Theanine can be formed from ethylamine and glutamate using sugar fermentation reaction of baker's yeast as an ATP regenerating system and bacterial glutamine synthetase reaction (Nandakumar et al., 2003). There are some beneficial effects for theanine, including improvement of learning ability, memory, and relaxation, strengthening the immune system vascular diseases, prevention of cancer, and weight gain (Kimura et al., 2007; Owen et al., 2008). In a mixture of γ-glutamyl donor and γ-glutamyl acceptor, glutaminase from *Pseudomonas nitroreducens* has been found to catalyze a γ-glutamyl transfer reaction. This enzymatic method has been industrialized to produce theanine using glutaminase enzyme from *P. nitroreducens*. For the enzymatic synthesis of theanine, GGT (γ-glutamyl transpeptidase) catalyzes the hydrolysis of γ-glutamyl compounds and transmissions of γ-glutamyl moiety from γ-glutamyl compounds, such as glutathione, to amino acids and peptides. Also GGT was responsible for synthesizing other physiologically significant γ-glutamyl compounds, including γ-glutamyl taurine and γ-Glutamyl-L-3,4-dihydroxyphenylalanine (γ-glutamyl DOPA). Additionally, bitter taste of some amino acids can be decreased by the mean of γ-glutamylization by GGT (Suzuki et al., 2002).

Thermolysin

Synthetic sweeteners, involving anticariogenic and noncaloric sweeteners, have been suggested to be a substitute to natural, calorie-containing sweeteners to decrease the risk of cardiovascular diseases, diabetes, and obesity. For instance, saccharin, aspartame, acesulfame potassium, and sucralose are examples of sugar substitutes regularly used alone or with other natural sweeteners as food additives, in several foods and soft drinks. Aspartame (α-aspartyl-phenylalanine methyl ester), which is a peptide-type artificial sweetener, has been broadly used in processed and pet foods, and beverages and drinks, such as tea, coffee, and sports drinks. The sweetness degree of this sweetener is around 200 times higher than that of sucrose. Aspartame thermolysin is a neutral protease from *Bacillus thermoproteolyticus* and

it is revealed that its enzymatic activity enhances by increasing NaCl concentration. The reverse reaction of hydrolysis of peptide bonds in the mixture of N-carbobenzoxy-L-aspartic acid and DL-phenylalanine methyl ester can be catalyzed by this enzyme (Inouye et al., 2007; Ogino et al., 2010). At the end, the N-carbobenzoxy-protecting group is detached chemically, and unreacted D-phenylalanine methyl ester is altered to DL-phenylalanine methyl ester which is its racemic form.

β-Galactosidase

D-glucoside glucohydrolase, also called lactase or β-Galactosidase, is able to catalyze the hydrolysis of the galactosyl moiety from the nonreducing end of several galactosyl-oligosaccharides. This enzyme is usually used in the dairy industry for hydrolyzing lactose, Gal-(β1,4)-Glc, which is the main saccharide naturally present in milk (Dutra Rosolen et al., 2015). Moreover, this reaction (hydrolyzing lactose to galactose and glucose by β-galactosidase) inhibits the lactose crystallization in condensed and frozen milk products besides strengthening the sweetness. Moreover, β-galactosidase is capable of catalyzing transgalactosylation, in which a galactose moiety is transmitted to the saccharides, emerging different galacto-oligosaccharides (GOs). GOs have some advantageous effects, such as decreasing blood serum cholesterol level, suppressing the colon cancer progress, and improving the mineral absorption (Vulevic et al., 2013). The initial application of glycosidases was in wines which was used to liberate flavor compounds from glycosidic precursors. The first reason for this is that significant flavor compounds in wines from *Vitis vinifera* cultivars are present as flavorless glycoconjugates in grapes, and the second reason is that the glucose inhibitory effect of -glucosidases restricts their use to the media containing low levels of glucose (<1 g/L), such as wine. However, due to the presence of flavor glycoconjugates in various fruits, enzyme applications have also been reported for fruit juices.

A. Wine Flavor Enhancement: During grape juice fermentation, there is only a slight decrease in the amount of glycosidic flavor precursors. This can be described by the low levels of glycosidase activities of enological yeast strains and grapes (Hernandez-Orte et al., 2008). Therefore, there is a special interest for the use of enzymes, such as pectinases and hemicellulases, which are commercially available fungal enzymes (mainly derived from *Aspergillus spp*.) that also have glycosidase activities. However, recent regulations do not accept including the enzymes in the wine. The hydrolysis of monoterpenyl glycosides in muscat cultivars releases odorous alcohols that can be identified by sensory analysis and can be analyzed by GC/MS. Generally, the addition of glycosidase during wine making increases in the concentration of monoterpenes, shikimate-derived compound, and C13-norisoprenoids (Hernandez-Orte et al., 2008).

B. Fruit Juices: There are few studies about the use of glucosidases in fruit juices which is due to the inhibition of them by glucose of the "key enzyme" glucosidase in enzyme derived from fungi. According to a study curing passion and mango fruit juices with a glucosidase derived from *Candida cacaoi* resulted in a rise in monoterpene concentrations (Chanprasartsuk & Prakitchaiwattana, 2015). Nevertheless,

this increase was pretty low rather than acid hydrolysis. Moreover, immobilization of the enzyme on sodium alginate beads, released less terpenols rather than the free enzyme. The addition of immobilized *C. molischiana*-derived glucosidase (onto Duolite A-568 resin) to apple juices, mango, strawberry, apricot, and peach, caused a rise in the concentration of monoterpene alcohols, such as 2-phenylethanoal, geraniol, terpineol, benzyl alcohol, and linalool (Pogorzelski & Wilkowska, 2007). However, there were no differences in the flavor liberations using immobilized and free glucosidase from *C. molischiana*. The immobilized enzyme was stated to be much more stable in fruit juice settings. In passion fruit juice, an immobilized (onto cellulose or acrylic beads) endo glucosidase derived from *A. niger* increased the concentration of volatile compounds, such as benzyl alcohol, benzaldehyde, and linalool. Generally, an aroma improvement was determined in the treated juices (Pogorzelski & Wilkowska, 2007).

Although by application of glucosidase, the levels of volatile compounds in fruit juices have increased, there were two significant parameters restricting the effectiveness of the process: firstly, it is known that there are lots of flavor compounds in fruit juice, such as diglycosides, that in general, fungal glucosidases are not able to hydrolyze them; secondly, the examined fungal glucosidases are prevented by glucose, which might then constraint their action in fruit juices. There is a report about the process that the amount of glycosides alters in a sweet wine, with 30 g/L glucose, in the presence of fungal glycosidases. It is shown that the monoterpene diglycosides' level decreases remarkably in the enzyme-treated wine, which can be described by the activity of exoglycosidases. The produced monoterpenyl-D glucosides were not hydrolyzed and consequently they were accumulated in the wine. This clearly shows that the fungal glucosidase activity is inhibited by glucose, which significantly limits the release of volatiles as it is approved by their analysis. In contrary, the hydrolysis of monoglucosides was very efficient though a dry wine was made using the same conditions, such as the same enzyme dosage and grape juice (Pogorzelski & Wilkowska, 2007).

Naringinase(α-L-Rhamnosidase)

Naringinase (EC 3.2.1.40) is mostly used for the naringin' breakdown which is the main bitter flavanone glycoside present in citrus fruits (Puri, 2012). Through the activity of α-rhamnosidase and β-glucosidase, naringin is converted to a naringenin and rhamnose. The sources for naringinase are mostly fungal isolates, such as *Circinella, Eurotium, Aspergillus niger, Rhizopus, Trichoderma, Fusarium,* and *Penicillium,* and bacteria, such as *Pseudomonas paucimobilis, Bacillus sp., Thermomicrobium roseum, Bacteriodes distasonis,* and *Burkholderia cenocepacia* (Puri, 2012). Naringinase derived from fungi has more application rather than the bacterial ones due to increased yield. Naringinase, as a debittering enzyme, has a principle role in food processing when it is added to fruit juices, in both immobilized and free forms. There are several food additives, such as sweeteners and biopolymers, which can be produced by naringinase or rhamnosidase. Moreover, there is another application for naringinase in addition to arabinosidase and β-glucosidase to develop the aroma of wine (Custodio et al., 1996). It is also stated

that naringinase can be used in preparation of tomato pulp and prunin, and treatment of kinnow peel waste (Puri, 2012).

Citrus juice is considered as one of the most commonly used juices in the world. However, mostly the taste of it is too bitter and this bitterness is mainly because of the presence of the naringin in the citrus fruits. Naringinase (α-L-rhamnosidase) is used to diminish the bitterness by breaking naringin into rhamnose and prunin and it is regularly used in the industrial production process of citrus juice (Puri et al., 2005). β-glucosidase, which is responsible for hydrolyzing the prunin to naringenin (a flavorless flavanone) and glucose, is usually used with naringinase, for a successful debittering (Table 12.1).

12.2 Preservation

12.2.1 Introduction

It is undeniable that food is important for mankind and there is not any way of living without eating. Therefore, it is highly important for the health of everyone to eat food. Although the need to feed did not change during the years, the way of eating has seen a lot of changes. Nowadays, food additives are needed to make various foods that are meeting the progressively challenging market and legal demands (Saltmarsh & Saltmarsh, 2013). Foods can be made in different facilities and then can be transported to local markets that are inside the same country or even in distant ones (Atkins & Bowler, 2016). Food transportation in suitable conditions needs special equipment and considerations, such as controlled packaging, refrigeration, or using some additives to prevent food spoilage and diminish food alterations. In today's global market which is highly competitive, it is desirable to use the cheapest method of food preservation and food additives can be a good choice.

12.2.2 Lysozyme

Antimicrobials with animal origin are compounds, such as proteins and enzymes that are isolated from animals. Currently, the only certified natural antimicrobial with animal origin that is used in US and the EU is lysozyme (E-1105). This enzyme is derived from eggs (Carocho et al., 2015), and it has high antimicrobial activity against gram-negative bacteria. The reason for this higher activity is that gram-negative bacteria are mostly (90%) composed of peptidoglycan, and lysozyme's antimicrobial activity is dependent on the hydrolysis of the β-1,4 linkage site of the peptidoglycan in the bacterial walls. This enzyme has only a moderate effect against gram-positive bacteria that have much less peptidoglycan in their wall, and apparently it has no activity against fungi or yeasts (Barbiroli et al., 2012). It is reported that the principal commercial use of this natural antimicrobial is in the cheese industry, that is used for prevention of "late blowing" of cheese; also there are studies about its application in eggs, beef (200 mg/90 mg), and milk (2 mg/mL in

Table 12.1 Enzyme role related to food flavor

Enzyme role	Enzyme	Bound volatiles	Flavor like	Ref
	B-glucosidase	Benzaldehyde, benzyl alcohol, 2-henylethanol, CI3-norisoprenoid, monoterpenes, CI3-nonsoprenoids, Marmelactones, hexanol, 2-henylethanol, linalool, dienediols, nerol	Papaya, apple, apricot, peach, yellow plum	Eriksson (2012)
	Endo-glucosidase	Terpenes (linalool)	**Fruity**	Martinez Ortega (2015)
	ß-glucosidase	Vanillin	Vanilla	Esparan (2015); Lim (2012)
Enhance or produce flavor	Tannase	Terpenes (linalool)	**Fruity**	Beniwal et al. (2013)
	Pectinase	2-phenyl ethanol, benzyl alcohol, benzoic acid	Tomato	de Andrade Júnior and Andrade (2015)
	Anthocyanase	Terpenes (linalool)	**Fruity**	Yang et al. (2020)
	Glucosidase	Norisoprenoids from lutein, violaxanthin, neoxanthin	Grape	Williams et al. (1992)
	Lactoperoxidase, xanthine oxidase, proteinase, lipase	Complex (methyl esters, ketones, aldehydes, and FFA)	Milk and milk products	Campbell and Drake (2013)
	Sulfhydryl oxidases	Oxidase the volatile thiol compounds	Removal of cooked flavor in UHT milk	Faccio et al. (2011)
	Limonoate dehydrogenase	Oxidize limonin (a bitter compound of grapefruit and orange) to nonbitter limonoate A-ring lactone	Debittering	Kola et al. (2010)
	Diacetyl reductase	Reduce diacetyl (responsible of unpleasant butter-like note) to 2,3 butylene glycol	Removal of diacetyl from beer	Krogerus and Gibson (2013)
Elimination of off-flavors	Naringinase	Hydrolyzes the naringin (responsible for the bitter taste) to prunin and rhamnose	Debitterize kumquat, grapefruit juice or	Kiefl et al. (2017); Zeng and Huang (2011)

(continued)

Table 12.1 (continued)

Enzyme role	Enzyme	Bound volatiles	Flavor like	Ref
			grapefruit concentrate	
	Laccase	Significant reduction of the concentrations of guaiacol and 2,6-dibromophenol	Elimination of musty, earthy, or like roots	Schroeder et al. (2008)
	(poly)phenol oxidases	Oxidation of poly phenols	Debittering of coffee, cacao, and olives	Kongor et al. (2016)
	Lipoxygenase	Hexanal, nonanal, nonenal, and 1-octen-3-ol	Beany-grassy flavor in soy bean	Iassonova et al. (2009)
Production of off-flavor	Lipoxygenase	2-methoxy-3-isopropyl-(5 or 6)-methyl pyrazine, hexanal, (E,E)-2,4-nonadienal, and (E,E)-2,4-decadienal	Off-flavor in pea milk	Zhang et al. (2020)
	Lipoxygenase	1-octen-3-one, hexanal, (E,E)-2,4-nonadienal, and (E,E)-2,4-decadienal	Off-flavor in soy milk	Zhang et al. (2020)

25 mL milk) (Sung et al., 2011). Lysozyme has also been assessed for making edible coatings and biofilms (Barbiroli et al., 2012) and its synergistic effects were studied in corporation with other natural antimicrobials (Bayarri et al., 2014).

12.2.3 Oxidoreductase

Redox enzymes have applications for extending the shelf life of food products. These enzymes include those that able to remove oxygen or reactive oxygen species, such as H2O2 and superoxide anion, and those that are capable of generating antimicrobial agents. Therefore, the stability of foods can considerably be improved with regard to their appearance, taste, and microbial spoilage.

12.2.3.1 Lactoperoxidases

Lactoperoxidase which belongs to the superfamily of peroxidase-cyclooxygenase enzymes is the most important enzyme present in the bovine milk in concentrations of approximately 30 mg/L. This enzyme shows its antimicrobial activity through the lactoperoxidase system, including lactoperoxidase, hydrogen peroxide, and thiocyanate, which are referred to as the lactoperoxidase system (LPS). The intermediate compounds formed through the oxidation of thiocyanate have antimicrobial activity (Silva et al., 2014). The main application of lactoperoxidase enzyme is to preserve raw milk, specifically in places that it is not readily available to refrigerate it. By

addition of thiocyanate to the milk, the LP system will start showing its antimicrobial activity. It is necessary to add thiocyanate, because of the low amount of thiocyanate in the milk which is not enough to initiate the antimicrobial activity. The antimicrobial activity of the LP system is activated by production of thiocyanate (SCN) oxidation products, primarily hypothiocyanate ions (OSCN), that are able to attack the sulfhydryl groups of major metabolic enzymes of the microorganisms. Moreover, the LP system does not affect the mammalian cells. Usually barely 10–20 ppm of lactoperoxidase is necessary for an efficient system. Also the required amount of thiocyanate and H2O2 is pretty low which are 10–25 ppm and 10–15 ppm, respectively. H2O2 is also bactericidal, but it shows its antimicrobial activity at higher concentrations (300–900 ppm) (Martin et al., 2014). Hence, lactoperoxidase (LPO) is usually used in combination with enzymes that generate H2O2. Moreover, in the LP system the amounts of cofactors and also the oxidation products are toxicologically harmless. LP system has applications in dental products, veterinarian products such as antidiarrheal, milk replacers, and antimastitis, and food products such as functional foods, cheese, fish, meat, poultry products, and liquid milk. There are claimed applications of this system alone or in combination with other systems for pickled foods, fish products, Lactobacillus fermented milk products, and white mold cheeses (Laane & Bruggeman, 2002). Moreover, there is an interest for its effect on yogurt. By addition of LPO to yogurt, the production of unnecessary acid of LAB in it is stifled (Masud et al., 2010). The mentioned applications are becoming accessible now that isolating the lactoperoxidase of milk is doable with high purity on an industrial scale. This system is commercially available now, at considerably low costs. Latest applications recommended for lactoperoxidase are preservation of fruit juices, and also using as coating of foodstuffs (Cissé et al., 2015).

12.2.3.2 Xanthine Oxidases

Xanthine oxidase (XO, EC 1.2.3.2) is broadly spread in microorganisms, plants, and animals. It is able to catalyze the oxidation of hypoxanthine to xanthine and consequently conversion of xanthine to uric acid. Moreover, XO is capable of oxidizing a wide range of pteridines, purines, and aldehydes, with simultaneous reduction of O2 to H2O2. Under definite circumstances, XO also is able to produce the highly reactive superoxide anion. Bovine milk is a good source of XO containing 35 mg. L of it. (Lin et al., 2015). XO also produces superoxide anion during oxidation of its substrates and in this way it involves the oxidative deterioration dairy products (Zarepour et al., 2010). This enzyme can also make a bactericidal or bacteriostatic agent in milk by producing H2O2 which is used in the lactoperoxidase system of milk (Whitaker et al., 2002).

12.2.3.3 Superoxide Dismutases and Catalases

Superoxide dismutase (SOD; EC 1.15.1.1) and catalase (EC 1.11.1.6) are the enzymes in milk that are capable of removing reactive oxygen species formed by other chemical or biochemical processes (Koh & Kim, 2001). Application of SOD is for catalyzing the superoxide anion (produced by XO) reduction, to O2 and H2O2. Consequently, catalase is able to convert H2O2 to oxygen and water. So application

of exogenous SOD, together with catalase, is a very efficient antioxidant in dairy products (Koh & Kim, 2001). Also it was shown that SOD can be a protection against free radical damage in beer (G Yao et al., 1987). Clearly, the commercial application of SOD as an antioxidant depends on the cost, especially rather than chemical antioxidants. However, it is known that SOD has not commercial application as an antioxidant in food systems.

Catalase have application for cold sterilization of milk in areas without refrigeration and also it is used for the treatment of cheese milk in developed countries (Czyzewska & Trusek, 2018). Sweet potato, *Aspergillus niger*, liver, and beef are good sources of catalase. There is also growing interest in using immobilized catalase reactors for milk pasteurization or glucose oxidase–catalase reactions (McSweeney, 2016). In addition to the application of SOD and catalase for elimination of reactive oxygen species, there are other enzymes, such as ascorbic acid oxidase, alcohol oxidase, glucose oxidase, and D-amino acid oxidase, that can be used to remove them but in lower amounts. The drawback of these enzymes is that they produce H2O2, which is a potent oxidant. Addition of catalase can be a way for removing H2O2, but then oxygen is produced again. Lately, new enzymes (such as laccase from PPOs) have been stated to use for deoxygenation of juices and beer (Nunes & Kunamneni, 2018). These enzymes remove oxygen more efficiently and do not produce H2O2 which is a benefit of using them; therefore, it is not needed to combine them with catalase. Catalase is usually used in combination with glucose oxidases for food preservation. Ough (1975) used a catalase with glucose oxidase for removing the oxygen from wine prior to bottling and assessed the acetaldehydes formation. Results indicated that proper treatment of the wine with the enzyme makes the amount and color of acetaldehyde stable (Röcker et al., 2016). Catalase is also used in milk processing industry to remove peroxide of the milk; it is used in bakery industry for removing glucose from egg white. Also in food wrappers this enzyme is used to inhibit and control oxidation and perishability of food (Röcker et al., 2016). However, this enzyme has limitations for using in cheese industry (Sindhu Raveendran et al., 2018).

12.2.3.4 Glucose Oxidase

Glucose oxidase is usually used in baking industry for making better dough due to its oxidizing effects. Moreover, this enzyme increases the aroma, flavor, and stability of food products by eliminating oxygen and glucose from egg white and diabetic drinks. Glucose oxidase modifies the flavor, texture, color, and shelf life of food products and inhibits decay and spoilage. Glucose oxidase is used to extend storage life of food products during packaging, by eliminating oxygen (Hanft & Koehler, 2006).

12.3 Conclusion and Future Prospect

Recently, there is a developing attention for food research by the international scientific community, and the principal areas of their focus include the increased production of food products, modification of foods with improved nutritive value, flavor development, increasing shelf life, strengthening food protection with procedures to preserve food from food pathogens, packaging technology and materials, and quality control. Food industry researchers are directing serious researches about the improvement of modified foods with additives (Chandrasekaran, 2012). The World Health Organization (WHO) food safety unit has stated the role of microorganisms and derived enzymes from them for getting safe, secure, and nutritive foods. Therefore, this can be applied to food processing industries. It is important to determine and select the proper fermentative processing technologies and then to improve, and apply them in the food industries. These technologies can be used for preparation and storage of food, in addition to transforming the vast amount of waste materials, resulting in the environmental damage.

Using of bioprocesses involving enzymes in food processing can decrease the antinutritive factors and toxins of the by-products, besides solving the environmental issues and increasing their consumer acceptance by enhancing their nutritive value. Thus, food industries are changing their functions toward green processes. However, widespread research and control on the stability of bioprocessed products, food-borne illness, development of fermentation process, and risk of food contamination are needed (Chandrasekaran, 2012). Currently, industry is considering the enzymatic catalyzes as a way to produce high amount of its products in environmentally friendly green processes, also as a tool to make additional value to the final product and increase the economical production. Therefore, the increased need for enzymes and enzymatic processes is making a new movement in enzyme technology, supported by improvements in chemical sciences biotechnology.

References

Alvarez-Macarie, E., & Baratti, J. (2000). Short chain flavour ester synthesis by a new esterase from Bacillus licheniformis. *Journal of Molecular Catalysis B: Enzymatic, 10*, 377–383.

Antrim, R. L., & Taylor, J. (1990). *Deodorization of a water-inoil emulsion containing fish oil using pure alcohol and aldehyde oxidases/dehydrogenase in combinations with a cofactor*. Patent US4961939.

Aravindan, R., Anbumathi, P. and Viruthagiri, T., 2007. Lipase applications in food industry.

Arnold, R., Shahani, K., & Dwivedi, B. (1975). Application of lipolytic enzymes to flavor development in dairy products. *Journal of Dairy Science, 58*, 1127–1143.

Arpigny, J. L., & Jaeger, K.-E. (1999). Bacterial lipolytic enzymes: Classification and properties. *Biochemical Journal, 343*, 177–183.

Asunción Longo, M., & Sanromán, M. (2005). Production of food aroma compounds: Microbial and enzymatic methodologies. *Food Technology and Biotechnology, 44*, 335–353.

Atkins, P., & Bowler, I. (2016). *Food in society: Economy, culture, geography*. Routledge.

Baianu, I., Lozano, P., Prisecaru, V., & Lin, H. (2003). *Novel techniques and their applications to agricultural biotechnology, health foods and medical biotechnology* (p. 13). University of Illinois at Urbana, ACES College.

Baillargeon, M. W. (1990). Purification and specificity of lipases from Geotrichum candidum. *Lipids, 25*, 841–848.

Barbiroli, A., Bonomi, F., Capretti, G., Iametti, S., Manzoni, M., Piergiovanni, L., & Rollini, M. (2012). Antimicrobial activity of lysozyme and lactoferrin incorporated in cellulose-based food packaging. *Food Control, 26*, 387–392.

Barrett, D., Garcia, E., Russell, G., Ramirez, E., & Shirazi, A. (2000). Blanch time and cultivar effects on quality of frozen and stored corn and broccoli. *Journal of Food Science, 65*, 534–540.

Basset, F. (1990). Arômes naturels: Oui aux biotechnologies. *Parfums, Cosmétiques, Arômes*, 17–26.

Bayarri, M., Oulahal, N., Degraeve, P., & Gharsallaoui, A. (2014). Properties of lysozyme/low methoxyl (LM) pectin complexes for antimicrobial edible food packaging. *Journal of Food Engineering, 131*, 18–25.

Beniwal, V., Kumar, A., Sharma, J., & Chhokar, V. (2013). Recent advances in industrial application of tannases: A review. *Recent Patents on Biotechnology, 7*, 228–233.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal, 11*, 259–274.

Bhowmik, T., & Marth, E. H. (1990). Rote of Micrococcus and Pediococcus species in cheese ripening: A review. *Journal of Dairy Science, 73*, 859–866.

Bigelis, R. (1992). *Flavor metabolites and enzymes from filamentous fungi*. Food technology (USA).

Bills, D., Morgan, M., Libbey, L., & Day, E. (1965). Identification of compounds responsible for fruity flavor defect of experimental Cheddar cheeses. *Journal of Dairy Science, 48*, 1168–1173.

Bornscheuer, U. T. (2002). Microbial carboxyl esterases: Classification, properties and application in biocatalysis. *FEMS Microbiology Reviews, 26*, 73–81.

Campbell, R., & Drake, M. (2013). Invited review: The effect of native and nonnative enzymes on the flavor of dried dairy ingredients. *Journal of Dairy Science, 96*, 4773–4783.

Carocho, M., Morales, P., & Ferreira, I. C. (2015). Natural food additives: Quo vadis? *Trends in Food Science & Technology, 45*, 284–295.

Carunchia Whetstine, M. E., Cadwallader, K. R., & Drake, M. (2005). Characterization of aroma compounds responsible for the rosy/floral flavor in Cheddar cheese. *Journal of Agricultural and Food Chemistry, 53*, 3126–3132.

Caspia, E., Coggins, P., Schilling, M., Yoon, Y., & White, C. (2006). The relationship between consumer acceptability and descriptive sensory attributes in cheddar cheese. *Journal of Sensory Studies, 21*, 112–127.

Chambers, D. H., Esteve, E., & Retiveau, A. (2010). Effect of milk pasteurization on flavor properties of seven commercially available French cheese types. *Journal of Sensory Studies, 25*, 494–511.

Chandrasekaran, M. (2012). *Future prospects and the need for research, valorization of food processing by-products* (pp. 786–801). CRC Press.

Chang, R.-C., Chou, S.-J., & Shaw, J.-F. (2001). Synthesis of fatty acid esters by recombinant Staphylococcus epidermidis lipases in aqueous environment. *Journal of Agricultural and Food Chemistry, 49*, 2619–2622.

Chanprasartsuk, O.-O., & Prakitchaiwattana, C. (2015). Impacts of allochthonous and autochthonous yeast starters: Case studies in fruit wine fermentations. *Food Microbiology: Fundamentals, Challenges and Health Implications*, 117–160.

Charoenprasert, S., & Mitchell, A. (2012). Factors influencing phenolic compounds in table olives (Olea europaea). *Journal of Agricultural and Food Chemistry, 60*, 7081–7095.

Chin, H. W., & Lindsay, R. (1993). Volatile sulfur compounds formed in disrupted tissues of different cabbage cultivars. *Journal of Food Science, 58*, 835–839.

Chin, H.-W., & Lindsay, R. C. (1994a). Mechanisms of formation of volatile sulfur compounds following the action of cysteine sulfoxide lyases. *Journal of Agricultural and Food Chemistry, 42*, 1529–1536.

Chin, H.-W., & Lindsay, R. C. (1994b). *Modulation of volatile sulfur compounds in cruciferous vegetables*. ACS Publications.

Christensen, V. W. (1964). Flavor development and control in Provolone and Romano cheese. *Presented at Marschall Invitational Italian Cheese Seminar*.

Cissé, M., Polidori, J., Montet, D., Loiseau, G., & Ducamp-Collin, M. N. (2015). Preservation of mango quality by using functional chitosan-lactoperoxidase systems coatings. *Postharvest Biology and Technology, 101*, 10–14.

Collar, C., Martinez, J., Andreu, P., & Armero, E. (2000). Effects of enzyme associations on bread dough performance. A response surface analysis/Efectos de las asociaciones enzimáticas sobre la calidad funcional de masas panarias. Análisis de superficies de respuesta. *Food Science and Technology International, 6*, 217–226.

Collins, Y. F., McSweeney, P. L., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *International Dairy Journal, 13*, 841–866.

Consultants, I. (2018). *An overview of the global flavors and fragrances market*, 11th edition. Retrieved from www.ialconsultants.com.

Cousins, C., Sharpe, M., & Law, B. (1977). *The bacteriological quality of milk for Cheddar cheesemaking*. Dairy Industries International.

Crow, V., Curry, B., Christison, M., & Hellier, K. (2002). Raw milk flora and NSLAB as adjuncts. *Australian Journal of Dairy Technology, 57*, 99.

Crow, V., Curry, B., & Hayes, M. (2001). The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal, 11*, 275–283.

Custodio, M. V. G., Otamendi, F. P., Vidal, D. R., & Alventosa, S. V. (1996). Production and characterization of an Aspergillus terteus α-l-rhamnosidase of oenological interest. *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 203*, 522–527.

Custry, F., Fernander, N., & Shahani, K. (1987). Role of lipases and other enzymes in flavour development. In *Proceedings of 24th annual Miles-Marschall Italian cheese seminar*. pp. 16–17.

Czyzewska, K., & Trusek, A. (2018). Encapsulated catalase from Serratia genus for H2O2 decomposition in food applications. *Polish Journal of Chemical Technology, 20*, 39–43.

Das, S., Holland, R., Crow, V., Bennett, R., & Manderson, G. (2005). Effect of yeast and bacterial adjuncts on the CLA content and flavour of a washed-curd, dry-salted cheese. *International Dairy Journal, 15*, 807–815.

de Andrade Júnior, M. C., & Andrade, J. S. (2015). Changes in pectinases, dietary fibers, and physicochemical indices related to the flavor of cubiu fruits during ripening. *Acta Scientiarum Agronomy, 37*, 171–179.

Desjardins, J. J., Duby, P., Dupart, P., Wood R. D., & Zurcher, U. (1997). *Producing of starchy material with a-amylase and lipoxygenase to produce a toasted or cookielike flavor*. Patent EP772980.

Dimos, A., Urbacha, G., & Miller, A. (1996). Changes in flavour and volatiles of full-fat and reducedfat cheddar cheeses during maturation. *International Dairy Journal, 6*, 981–995.

Dominguez, A., Sanroman, A., Fucinos, P., Rua, M., Pastrana, L., & Longo, M. (2004). Quantification of intra-and extra-cellular thermophilic lipase/esterase production by Thermus sp. *Biotechnology Letters, 26*, 705–708.

Dugelay, I., Gunata, Z., Sapis, J. C., Baumes, R., & Bayonove, C. (1993). Role of cinnamoyl esterase activities from enzyme preparations on the formation of volatile phenols during winemaking. *Journal of Agricultural and Food Chemistry, 41*, 2092–2096.

Dutra Rosolen, M., Gennari, A., Volpato, G., & Volken de Souza, C. F. (2015). Lactose hydrolysis in milk and dairy whey using microbial β-galactosidases. *Enzyme Research, 2015*.

Dwivedi, B. K., & Kinsella, J. E. (1974). Carbonyl production from lipolyzed milk fat by the continuous mycelial culture of Penicillium roqueforti. *Journal of Food Science, 39*, 83–87.

El Soda, M., Abd El Wahab, H., Ezzat, N., Desmazeaud, M., & Ismail, A. (1986). The esterolytic and lipolytic activities of the Lactobacilli. II. Detection of the esterase system of Lactobacillus helveticus, Lactobacillus bulgaricus. Lactobacillus lactis and Lactobacillus acidophilus. *Le Lait, 66*, 431–443.

Eriksson, C. (2012). Flavor modification. *Flavor of Foods and Beverages: Chemistry and Technology, 1*.

Esparan, V. (2015). *Fungal enzymes for the production of vanillin, divanillin & lignans*. Gottfried Wilhelm Leibniz Universität Hannover.

Ewing, T. A., Van Noord, A., Paul, C. E., & Van Berkel, W. J. (2018). A xylenol orange-based screening assay for the substrate specificity of flavin-dependent para-phenol oxidases. *Molecules, 23*, 164.

Ewis, H. E., Abdelal, A. T., & Lu, C.-D. (2004). Molecular cloning and characterization of two thermostable carboxyl esterases from Geobacillus stearothermophilus. *Gene, 329*, 187–195.

Faccio, G., Nivala, O., Kruus, K., Buchert, J., & Saloheimo, M. (2011). Sulfhydryl oxidases: Sources, properties, production and applications. *Applied Microbiology and Biotechnology, 91*, 957–966.

Falconnier, B., Lapierre, C., Lesage-Meessen, L., Yonnet, G., Brunerie, P., Colonna-Ceccaldi, B., Corrieu, G., & Asther, M. (1994). Vanillin as a product of ferulic acid biotransformation by the white-rot fungus Pycnoporus cinnabarinus I-937: Identification of metabolic pathways. *Journal of Biotechnology, 37*, 123–132.

Fenster, K., Parkin, K., & Steele, J. (2000). Characterization of an arylesterase from Lactobacillus helveticus CNRZ32. *Journal of Applied Microbiology, 88*, 572–583.

Fenster, K., Parkin, K., & Steele, J. (2003a). Intracellular esterase from Lactobacillus casei LILA: Nucleotide sequencing, purification, and characterization. *Journal of Dairy Science, 86*, 1118–1129.

Fenster, K., Parkin, K., & Steele, J. (2003b). Nucleotide sequencing, purification, and biochemical properties of an arylesterase from Lactobacillus casei LILA. *Journal of Dairy Science, 86*, 2547–2557.

Fox, P., & Wallace, J. (1997). Formation of flavor compounds in cheese. *Advances in Applied Microbiology, 45*, 17–86.

Fuciños, P., Domínguez, A., Angeles Sanromán, M., Longo, M. A., Luisa Rúa, M., & Pastrana, L. (2005). Production of thermostable lipolytic activity by Thermus species. *Biotechnology Progress, 21*, 1198–1205.

Gao, P., Jiang, Q., Xu, Y., & Xia, W. (2017). Esterase activities of autochthonous starter cultures to increase volatile flavour compounds in Chinese traditional fermented fish (Suan yu). *International Journal of Food Properties, 20*, S663–S672.

Gao, W., Wu, K., Chen, L., Fan, H., Zhao, Z., Gao, B., Wang, H., & Wei, D. (2016). A novel esterase from a marine mud metagenomic library for biocatalytic synthesis of short-chain flavor esters. *Microbial Cell Factories, 15*, 41.

Ghosh, P., Saxena, R., Gupta, R., Yadav, R., & Davidson, S. (1996). Microbial lipases: Production and applications. *Science Progress (1933)*, 119–157.

Goulet, C., Mageroy, M. H., Lam, N. B., Floystad, A., Tieman, D. M., & Klee, H. J. (2012). Role of an esterase in flavor volatile variation within the tomato clade. *Proceedings of the National Academy of Sciences, 109*, 19009–19014.

Greenough, R., Perry, C., & Stavnsbjerg, M. (1996). Safety evaluation of a lipase expressed in Aspergillus oryzae. *Food and Chemical Toxicology, 34*, 161–166.

Hanft, F., & Koehler, P. (2006). Studies on the effect of glucose oxidase in bread making. *Journal of the Science of Food and Agriculture, 86*, 1699–1704.

Hansen, A. P., Welty, R. E., & Shen, R.-S. (1973). Free fatty acid content of cacao beans infested with storage fungi. *Journal of Agricultural and Food Chemistry, 21*, 665–670.

Hernandez-Orte, P., Cersosimo, M., Loscos, N., Cacho, J., Garcia-Moruno, E., & Ferreira, V. (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chemistry, 107*, 1064–1077.

Huang, S., Chang, H., & Goto, M. (1998). Preparation of surfactant-coated lipase for the esterification of geraniol and acetic acid in organic solvents. *Enzyme and Microbial Technology, 22*, 552–557.

Iassonova, D. R., Johnson, L. A., Hammond, E. G., & Beattie, S. E. (2009). Evidence of an enzymatic source of off flavors in "lipoxygenase-null" soybeans. *Journal of the American Oil Chemists' Society, 86*, 59–64.

Inouye, K., Kusano, M., Hashida, Y., Minoda, M., & Yasukawa, K. (2007). Engineering, expression, purification, and production of recombinant thermolysin. *Biotechnology Annual Review, 13*, 43–64.

Iwaniak, A., Minkiewicz, P., Darewicz, M., & Hrynkiewicz, M. (2016). Food protein-originating peptides as tastants-Physiological, technological, sensory, and bioinformatic approaches. *Food Research International, 89*, 27–38.

Jahadi, M., Khosravi-Darani, K., Ehsani, M., Mozafari, M., Saboury, A., Seydahmadian, F., & Vafabakhsh, Z. (2012). Evaluating the effects of process variables on protease-loaded nanoliposome production by Plackett-Burman design for utilizing in cheese ripening acceleration. *Asian Journal of Chemistry, 24*, 3891.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., Pimentel, T., Gomes, A., & Mozafari, M. R. (2020). Accelerating ripening of Iranian white brined cheesesusing liposome encapsulated and free proteinases. *Journal of Open Access (Biointerface Research in Applied Chemistry), 10*, 4966–4971.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., Mozafari, M. R., Saboury, A. A., & Pourhosseini, P. S. (2015). The encapsulation of flavourzyme in nanoliposome by heating method. *Journal of Food Science and Technology, 52*, 2063–2072.

Jahadi, M., Khosravi-Darani, K., Ehsani, M. R., Mozafari, M. R., Saboury, A. A., Zoghi, A., & Mohammadi, M. (2016). Modelling of proteolysis in Iranian brined cheese using proteinase-loaded nanoliposome. *International Journal of Dairy Technology, 69*, 57–62.

Jayathilaka, L., Gupta, S., Huang, J.-S., Lee, J., & Lee, B.-S. (2014). Preparation of (+)-trans-isoalliin and its isomers by chemical synthesis and RP-HPLC resolution. *Journal of biomolecular techniques: JBT, 25*, 67.

Jensen, F. (1970). Free fatty acids in lipase activated Samsoe cheese. In *XVIII International Dairy Congress, Sydney*.

Jiang, J., Björck, L., & Fonden, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *Journal of Applied Microbiology, 85*, 95–102.

Kamenetsky, R. (2007). Garlic: Botany and horticulture. *Horticultural Reviews-Westport Then New York, 33*, 123.

Karami, M., Ehsani, M., Mousavi, S., Rezaei, K., & Safari, M. (2009). Microstructural properties of fat during the accelerated ripening of ultrafiltered-Feta cheese. *Food Chemistry, 113*, 424–434.

Kiefl, J., Kohlenberg, B., Hartmann, A., Obst, K., Paetz, S., Krammer, G., & Trautzsch, S. (2017). Investigation on key molecules of Huanglongbing (HLB)-induced orange juice off-flavor. *Journal of Agricultural and Food Chemistry, 66*, 2370–2377.

Kimura, K., Ozeki, M., Juneja, L. R., & Ohira, H. (2007). L-Theanine reduces psychological and physiological stress responses. *Biological Psychology, 74*, 39–45.

Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current Opinion in Biotechnology, 13*, 345–351.

Koh, M., & Kim, H.-J. (2001). The effects of metallothionein on the activity of enzymes involved in removal of reactive oxygen species. *Bulletin-Korean Chemical Society, 22*, 362–366.

Kola, O., Kaya, C., Duran, H., & Altan, A. (2010). Removal of limonin bitterness by treatment of ion exchange and adsorbent resins. *Food Science and Biotechnology, 19*, 411–416.

Kongor, J. E., Hinneh, M., Van de Walle, D., Afoakwa, E. O., Boeckx, P., & Dewettinck, K. (2016). Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile—A review. *Food Research International, 82*, 44–52.

Kosikowski, F. V. (1975). Potential of enzymes in continous cheesemaking. *Journal of Dairy Science, 58*, 994–1000.

Kragl, U., Kruse, W., Hummel, W., & Wandrey, C. (1996). Enzyme engineering aspects of biocatalysis: Cofactor regeneration as example. *Biotechnology and Bioengineering, 52*, 309–319.

Krishna, S. H., Divakar, S., Prapulla, S., & Karanth, N. (2001). Enzymatic synthesis of isoamyl acetate using immobilized lipase from Rhizomucor miehei. *Journal of Biotechnology, 87*, 193–201.

Krogerus, K., & Gibson, B. R. (2013). 125th Anniversary review: Diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing, 119*, 86–97.

Kwon, D. Y., Hong, Y.-J., & Yoon, S. H. (2000). Enantiomeric synthesis of (S)-2-methylbutanoic acid methyl ester, apple flavor, using lipases in organic solvent. *Journal of Agricultural and Food Chemistry, 48*, 524–530.

Laane, C., & Bruggeman, Y. (2002). Applications of oxidoreductases in foods. *Handbook of Food Enzymology, 122*, 161.

Laane, C., Rietjens, I., Haaker, H., & Van Berkel, W. (1997). Generation of Taste through (Redox) Biocatalysis. *Special Publication-Royal Society of Chemistry, 214*, 137–152.

Laboret, F., & Perraud, R. (1999). Lipase-catalyzed production of short-chain acids terpenyl esters of interest to the food industry. *Applied Biochemistry and Biotechnology, 82*, 185–198.

Lane, C., & Hammer, B. (1936). The manufacture of Blue cheese (Roquefort type) from homogenized cows' milk. *Iowa State College Journal of Science, 10*, 391.

Langrand, G., Rondot, N., Triantaphylides, C., & Baratti, J. (1990). Short chain flavour esters synthesis by microbial lipases. *Biotechnology Letters, 12*, 581–586.

Larios, A., García, H. S., Oliart, R. M., & Valerio-Alfaro, G. (2004). Synthesis of flavor and fragrance esters using Candida antarctica lipase. *Applied Microbiology and Biotechnology, 65*, 373–376.

Lim, T. (2012). *Vanilla planifolia, edible medicinal and non-medicinal plants* (pp. 106–114). Springer.

Lin, S., Zhang, G., Liao, Y., Pan, J., & Gong, D. (2015). Dietary flavonoids as xanthine oxidase inhibitors: Structure–affinity and structure–activity relationships. *Journal of Agricultural and Food Chemistry, 63*, 7784–7794.

Liu, S.-Q., Holland, R., & Crow, V. (2003a). Ester synthesis in an aqueous environment by Streptococcus thermophilus and other dairy lactic acid bacteria. *Applied Microbiology and Biotechnology, 63*, 81–88.

Liu, S.-Q., Holland, R., & Crow, V. (2003b). Synthesis of ethyl butanoate by a commercial lipase in aqueous media under conditions relevant to cheese ripening. *The Journal of Dairy Research, 70*, 359.

Lopez-Gallego, F., Betancor, L., Hidalgo, A., Dellamora-Ortiz, G., Mateo, C., Fernández-Lafuente, R., & Guisán, J. M. (2007). Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. *Enzyme and Microbial Technology, 40*, 278–284.

Maehashi, K., & Huang, L. (2009). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences, 66*, 1661–1671.

Maia, M. M. D., de Morais, M. M. C., de Morais, M. A., Jr., Melo, E. H. M., & de Lima Filho, J. L. (1999). Production of extracellular lipase by the phytopathogenic fungus Fusarium solani FS1. *Revista de Microbiologia, 30*, 304–309.

Mandal, S. (2012). *Functional characterization of lipoxygenase isozymes in relation to off flavor development in soybean (glycine max)*. IARI, Division of Biochemistry.

Markus, P. A., Peters, A. L. J., & Roos, R. (1991). *Enzymatic process for preparation of phenylacetaldehyde compounds especially vanillin by conversion of starting compounds in the presence of lipoxidase*. Patent EP542348.

Martin, N. H., Friedlander, A., Mok, A., Kent, D., Wiedmann, M., & Boor, K. J. (2014). Peroxide test strips detect added hydrogen peroxide in raw milk at levels affecting bacterial load. *Journal of Food Protection, 77*, 1809–1813.

Martinez Ortega, A. (2015). Most significant biotechnological improvements on the production process of wine, beer and bread.

Mase, T., Sonoda, M., Morita, M., & Hirose, E. (2010). Characterization of a lipase from Sporidiobolus pararoseus 25-A which produces cheese flavor. *Food Science and Technology Research, 17*, 17–20.

Masud, T., Khalid, S., Maqsood, S., & Bilal, A. (2010). Preservation of raw buffalo's milk by the activation of lactoperoxidase system and its effect on yogurt preparation. *Journal of Food Processing and Preservation, 34*, 241–254.

Masuda, R., Kaneko, K., & Saito, M. (2012). Accumulation of alkyl-cysteine derivatives in Crucifers. *Biology and Biotechnology of the Plant Hormone Ethylene, II*, 439.

Masuo, N., Yoshimune, K., Ito, K., Matsushima, K., Koyama, Y., & Moriguchi, M. (2005). Micrococcus luteus K-3-type glutaminase from Aspergillus oryzae RIB40 is salt-tolerant. *Journal of Bioscience and Bioengineering, 100*, 576–578.

Mathew, S., & Abraham, T. E. (2005). Studies on the production of feruloyl esterase from cereal brans and sugar cane bagasse by microbial fermentation. *Enzyme and Microbial Technology, 36*, 565–570.

McGugan, W., Blais, J., Boulet, M., Giroux, R., Elliott, J., & Emmons, D. (1975). Ethanol, ethyl esters, and volatile fatty acids in fruity Cheddar cheese. *Canadian Institute of Food Science and Technology Journal, 8*, 196–198.

McSweeney, P. (2016). Enzymes exogenous to milk in dairy technology: Catalase, glucose oxidase, glucose isomerase and hexose oxidase.

Menzel, M., & Schreier, P. (2007). *Enzymes and flavour biotechnology* (pp. 489–505). Flavours and Fragrances, Springer.

Mohammadi, R., Mahmoudzadeh, M., Atefi, M., Khosravi-Darani, K., & Mozafari, M. (2015). Applications of nanoliposomes in cheese technology. *International Journal of Dairy Technology, 68*, 11–23.

Molimard, P., & Spinnler, H.-E. (1996). Compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. *Journal of Dairy Science, 79*, 169–184.

Moschopoulou, E. (2011). Characteristics of rennet and other enzymes from small ruminants used in cheese production. *Small Ruminant Research, 101*, 188–195.

Moskowitz, G. J., Cassaigne, R., West, I. R., Shen, T., & Feldman, L. I. (1977). Hydrolysis of animal fat and vegetable oil with Mucor miehei esterase. Properties of the enzyme. *Journal of Agricultural and Food Chemistry, 25*, 1146–1150.

Nagodawithana, T., & Reed, G. (1993). *Preface to the third edition, enzymes in food processing* (p. xix). Elsevier.

Nandakumar, R., Yoshimune, K., Wakayama, M., & Moriguchi, M. (2003). Microbial glutaminase: Biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic, 23*, 87–100.

Narbad, A., & Gasson, M. J. (1998). Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly-isolated strain of Pseudomonas fluorescens. *Microbiology, 144*, 1397–1405.

Nathan, P. J., Lu, K., Gray, M., & Oliver, C. (2006). The neuropharmacology of L-theanine (N-ethyl-L-glutamine) a possible neuroprotective and cognitive enhancing agent. *Journal of Herbal Pharmacotherapy, 6*, 21–30.

Nunes, C. S., & Kunamneni, A. (2018). *Laccases—Properties and applications, Enzymes in human and animal nutrition* (pp. 133–161). Elsevier.

Obenland, D., & Aung, L. (1996). Cystine lyase activity and anaerobically-induced sulfur gas emission from broccoli florets. *Phyton*.

Ogino, H., Tsuchiyama, S., Yasuda, M., & Doukyu, N. (2010). Enhancement of the aspartame precursor synthetic activity of an organic solvent-stable protease. *Protein Engineering, Design & Selection, 23*, 147–152.

Okazawa, S., Hashimoto, M., & Hatsutori, Y. (1993). *Treatment of adzuki bean with enzymes*. Patent JP0524488.

Ough, C. (1975). Further investigations with glucose oxidase-catalase enzyme systems for use with wine. *American Journal of Enology and Viticulture, 26*, 30–36.

Owen, G. N., Parnell, H., De Bruin, E. A., & Rycroft, J. A. (2008). The combined effects of L-theanine and caffeine on cognitive performance and mood. *Nutritional Neuroscience, 11*, 193–198.

Parker, K., Salas, M., & Nwosu, V. C. (2010). High fructose corn syrup: Production, uses and public health concerns. *Biotechnology and Molecular Biology Reviews, 5*, 71–78.

Petropoulos, S., Ntatsi, G., & Ferreira, I. (2017). Long-term storage of onion and the factors that affect its quality: A critical review. *Food Reviews International, 33*, 62–83.

Pico, J., Bernal, J., & Gómez, M. (2015). Wheat bread aroma compounds in crumb and crust: A review. *Food Research International, 75*, 200–215.

Pogorzelski, E., & Wilkowska, A. (2007). Flavour enhancement through the enzymatic hydrolysis of glycosidic aroma precursors in juices and wine beverages: A review. *Flavour and Fragrance Journal, 22*, 251–254.

Price, E. J., Linforth, R. S., Dodd, C. E., Phillips, C. A., Hewson, L., Hort, J., & Gkatzionis, K. (2014). Study of the influence of yeast inoculum concentration (Yarrowia lipolytica and Kluyveromyces lactis) on blue cheese aroma development using microbiological models. *Food Chemistry, 145*, 464–472.

Priefert, H., Rabenhorst, J., & Steinbüchel, A. (2001). Biotechnological production of vanillin. *Applied Microbiology and Biotechnology, 56*, 296–314.

Puri, M. (2012). Updates on naringinase: Structural and biotechnological aspects. *Applied Microbiology and Biotechnology, 93*, 49–60.

Puri, M., Banerjee, A., & Banerjee, U. (2005). Optimization of process parameters for the production of naringinase by Aspergillus niger MTCC 1344. *Process Biochemistry, 40*, 195–201.

Raghuvanshi, R. S., & Bisht, K. (2010). 18 uses of soybean: Products and preparation. *The Soybean, 404*.

Ramírez, E. C., & Whitaker, J. R. (1999). Biochemical characterization of cystine lyase from broccoli (Brassica oleracea Var. Italica). *Journal of Agricultural and Food Chemistry, 47*, 2218–2225.

Raveendran, S., Parameswaran, B., Beevi, S., Amith, U., Anil, A., Mathew, K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of microbial enzymes in food industry. *Food Technology and Biotechnology, 56*(1), 16–30.

Ricard, P. B. P. (1991). *Synthesis of cis-3-hexenol from unsaturated aliphatic acid—Using combined action of natural enzyme system and yeast*. Patent EP481147.

Richardson, G., & Nelson, J. (1968). Rapid evaluation of milk coagulating and flavor producing enzymes for cheese manufacture. *Journal of Dairy Science, 51*, 1502–1503.

Röcker, J., Schmitt, M., Pasch, L., Ebert, K., & Grossmann, M. (2016). The use of glucose oxidase and catalase for the enzymatic reduction of the potential ethanol content in wine. *Food Chemistry, 210*, 660–670.

Romero, M., Calvo, L., Alba, C., Daneshfar, A., & Ghaziaskar, H. (2005). Enzymatic synthesis of isoamyl acetate with immobilized Candida antarctica lipase in n-hexane. *Enzyme and Microbial Technology, 37*, 42–48.

Ruiz-Matute, A. I., Weiss, M., Sammataro, D., Finely, J., & Sanz, M. L. (2010). Carbohydrate composition of high-fructose corn syrups (HFCS) used for bee feeding: Effect on honey composition. *Journal of Agricultural and Food Chemistry, 58*, 7317–7322.

Saerens, K., Descamps, D., & Dewettinck, K. (2008). Release of short chain fatty acids from cream lipids by commercial lipases and esterases. *Biotechnology Letters, 30*, 311–315.

Sakato, Y. (1949). The chemical constituents of tea; III. A new amide theanine. *Nippon Nogeikagaku Kaishi, 23*, 262–267.
Saltmarsh, M., & Saltmarsh, M. (2013). *Essential guide to food additives*. Royal Society of Chemistry.
Salvadori, P. (1961). The use of lipolytic enzyme of microbiological origin in the manufacture of Pecorino cheese. *Latte, 35*, 177.
Sánchez, M., Prim, N., Rández-Gil, F., Pastor, F. J., & Diaz, P. (2002). Engineering of baker's yeasts, E. coli and Bacillus hosts for the production of Bacillus subtilis lipase A. *Biotechnology and Bioengineering, 78*, 339–345.
Schroeder, M., Pöllinger-Zierler, B., Aichernig, N., Siegmund, B., & Guebitz, G. (2008). Enzymatic removal of off-flavors from apple juice. *Journal of Agricultural and Food Chemistry, 56*, 2485–2489.
Schwab, W., Davidovich-Rikanati, R., & Lewinsohn, E. (2008). Biosynthesis of plant-derived flavor compounds. *The Plant Journal, 54*, 712–732.
Scrimgeour, C., Gao, Y., Oh, W. Y., & Shahidi, F. (2005). Chemistry of fatty acids. *Bailey's Industrial Oil and Fat Products*, 1–40.
Seitz, E. W. (1974). Industrial application of microbial lipases: A review. *Journal of the American oil chemists' society, 51*, 12–16.
Seratlić, S. V., Miloradović, Z. N., Radulović, Z. T., & Maćej, O. D. (2011). The effect of two types of mould inoculants on the microbiological composition, physicochemical properties and protein hydrolysis in two Gorgonzola-type cheese varieties during ripening. *International Journal of Dairy Technology, 64*, 408–416.
Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances, 19*, 627–662.
Shieh, C.-J., & Chang, S.-W. (2001). Optimized synthesis of lipase-catalyzed hexyl acetate in n-hexane by response surface methodology. *Journal of Agricultural and Food Chemistry, 49*, 1203–1207.
Shimada, Y., Hirota, Y., Baba, T., Sugihara, A., Moriyama, S., Tominaga, Y., & Terai, T. (1999). Enzymatic synthesis of steryl esters of polyunsaturated fatty acids. *Journal of the American Oil Chemists' Society, 76*, 713–716.
Silva, F., Gibbs, P., Nunez, H., Almonacid, S., Simpson, R., Batt, C., & Tortorello, M. (2014). *Encyclopedia of food microbiology, Thermal processes: Pasteurization* (pp. 577–595). Elsevier Netherlands.
Singh, A., & Banerjee, R. (2017). *Challenges in lipase mediated synthesis of food flavor ester compounds, developing technologies in food science* (pp. 63–74). Apple Academic Press.
Singh, R., Mittal, A., Kumar, M., & Mehta, P. K. (2016). Microbial proteases in commercial applications. *Journal of Pharmaceutical, Chemical and Biological Sciences, 4*, 365–374.
Small, L. E., & Asquith, T. (1989). *Process for treating coffee beans with enzyme-containing solution under pressure to reduce bitterness*. Patent EP337541.
Smit, G., Smit, B. A., & Engels, W. J. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*, 591–610.
Sung, K., Khan, S. A., Nawaz, M. S., Cerniglia, C. E., Tamplin, M. L., Phillips, R. W., & Kelley, L. C. (2011). Lysozyme as a barrier to growth of Bacillus anthracis strain Sterne in liquid egg white, milk and beef. *Food Microbiology, 28*, 1231–1234.
Suzuki, H., Izuka, S., Miyakawa, N., & Kumagai, H. (2002). Enzymatic production of theanine, an "umami" component of tea, from glutamine and ethylamine with bacterial γ-glutamyltranspeptidase. *Enzyme and Microbial Technology, 31*, 884–889.
Suzzi, G., Lanorte, M., Galgano, F., Andrighetto, C., Lombardi, A., Lanciotti, R., & Guerzoni, M. (2001). Proteolytic, lipolytic and molecular characterisation of Yarrowia lipolytica isolated from cheese. *International Journal of Food Microbiology, 69*, 69–77.
Takemori, T., Ito, Y., Ito, M., & Yoshama, M. (1992). *Flavor and taste improvement of cacao nib by enzymatic treatment*. Patent JP04126037.

Thorpe, C., Hoober, K. L., Raje, S., Glynn, N. M., Burnside, J., Turi, G. K., & Coppock, D. L. (2002). Sulfhydryl oxidases: Emerging catalysts of protein disulfide bond formation in eukaryotes. *Archives of Biochemistry and Biophysics, 405*, 1–12.

Tishel, M., & Mazelis, M. (1966). Enzymatic degradation of L-cystine by cytoplasmic particles from cabbage leaves. *Nature, 211*, 745–746.

Tunick, M. H. (2007). *Origins of cheese flavor*. ACS Publications.

Urbach, G. (1993). Relations between cheese flavour and chemical composition. *International Dairy Journal, 3*, 389–422.

Vafabakhsh, Z., Khosravi-Darani, K., Khajeh, K., Jahadi, M., Komeili, R., & Mortazavian, A. M. (2013). Stability and catalytic kinetics of protease loaded liposomes. *Biochemical Engineering Journal, 72*, 11–17.

Van Haandel, M. J., Saraber, F. C., Boersma, M. G., Laane, C., Fleming, Y., Weenen, H., & Rietjens, I. M. (2000). Characterization of different commercial soybean peroxidase preparations and use of the enzyme for N-demethylation of methyl N-methylanthranilate to produce the food flavor methylanthranilate. *Journal of Agricultural and Food Chemistry, 48*, 1949–1954.

Vandamme, E. J., & Soetaert, W. (2002). Bioflavours and fragrances via fermentation and biocatalysis. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology, 77*, 1323–1332.

Vulevic, J., Juric, A., Tzortzis, G., & Gibson, G. R. (2013). A mixture of trans-galactooligosaccharides reduces markers of metabolic syndrome and modulates the fecal microbiota and immune function of overweight adults. *The Journal of Nutrition, 143*, 324–331.

Waldmann, E., & Parhofer, K. G. (2019). Apheresis for severe hypercholesterolaemia and elevated lipoprotein (a). *Pathology, 51*, 227–232.

Walker, V., & Mills, G. A. (2014). 2-Pentanone production from hexanoic acid by Penicillium roqueforti from blue cheese: Is this the pathway used in humans? *The Scientific World Journal 2014*.

Welsh, F. W., Murray, W. D., Williams, R. E., & Katz, I. (1989). Microbiological and enzymatic production of flavor and fragrance chemicals. *Critical Reviews in Biotechnology, 9*, 105–169.

Whitaker, J. R., Voragen, A. G., & Wong, D. W. (2002). *Handbook of food enzymology, 122*. CRC Press.

Wilding, P., & Woolner, E. (1997). *Reatment of tomato with eroxidase to achieve a fresh flavour profile*. Patent WO9738591.

Williams, P. J., Sefton, M. A., & Francis, I. L. (1992). *Glycosidic precursors of varietal grape and wine flavor*. ACS Publications.

Wolf, I. V., Meinardi, C. A., & Zalazar, C. A. (2009). Production of flavour compounds from fat during cheese ripening by action of lipases and esterases. *Protein and Peptide Letters, 16*, 1235–1243.

Wouters, A. G., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*, 786–800.

Yang, H., Cai, G., Lu, J., & Gómez Plaza, E. (2020). The production and application of enzymes related to the quality of fruit wine. *Critical Reviews in Food Science and Nutrition*, 1–11.

Yao, G., Chen, G., & Fu, J. (1987). *Technique for brewing beer containing SOD, hedgehog polysaccharide and theaflavin*. Patent CH1145404.

Zarepour, M., Kaspari, K., Stagge, S., Rethmeier, R., Mendel, R. R., & Bittner, F. (2010). Xanthine dehydrogenase AtXDH1 from Arabidopsis thaliana is a potent producer of superoxide anions via its NADH oxidase activity. *Plant Molecular Biology, 72*, 301–310.

Zeng, L.-L., & Huang, H.-H. (2011). Optimization of debitterizing of kumquat juice by naringinase with response surface method [J]. *Science and Technology of Food Industry 5*.

Zhang, C., Hua, Y., Li, X., Kong, X., & Chen, Y. (2020). Key volatile off-flavor compounds in peas (Pisum sativum L.) and their relations with the endogenous precursors and enzymes using soybean (Glycine max) as a reference. *Food Chemistry, 127469*.

Zhang, Y., Venkitasamy, C., Pan, Z., Liu, W., & Zhao, L. (2017). Novel umami ingredients: Umami peptides and their taste. *Journal of Food Science, 82*, 16–23.

Zhuang, M., Lin, L., Zhao, M., Dong, Y., Sun-Waterhouse, D., Chen, H., Qiu, C., & Su, G. (2016). Sequence, taste and umami-enhancing effect of the peptides separated from soy sauce. *Food Chemistry, 206*, 174–181.

Enzymes from Genetically Modified Organisms and Their Current Applications in Food Development and Food Chain

13

Senthilkumar Muthusamy, Shilpa Ajit, Asha V. Nath, J. Anupama Sekar, and T. S. Ramyaa Lakshmi

Abstract

The fulfillment of nutrition needs is in high demand due to the increase in population, especially in developing countries with a reduction in arable land. Though the use of natural enzymes from plants, animals, and microbes is high, the demand and modern food processing conditions require improved enzymes both qualitative and quantitatively. Current recombinant DNA technologies such as altering genetic code and introduction of a gene allow genetic modification in an organism, which gives desired products. Some of the genetically modified foods are on the market with rigorous safety assessment and many of them are under development. Genetically modified enzymes that ameliorate the processing of carbohydrates, protein, and lipid are in pipeline to use in various food industries. This chapter emphasizes the enzymes derived from genetically modified organisms; the methods used to generate genetically modified organisms and the use of these enzymes in food industries for the application of various food development and food chain. The chapter will also highlight people's thoughts and safety concerns about the use of enzymes from genetically modified organisms.

Keywords

Enzymes · Recombinant DNA technology · GM foods · Applications

S. Muthusamy · S. Ajit · A. V. Nath · J. Anupama Sekar
Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India

T. S. Ramyaa Lakshmi (✉)
Department of Zoology, Thiagarajar College, Madurai, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_13

13.1 Introduction

Enzymes are proteins (also referred to as biocatalysts) that can be extracted from cells, which catalyze the biochemical reactions in living organisms. They can be used to catalyze a good range of commercially important processes in paper and pulp, textile, chemical and pharmaceutical, agriculture, and food industries. Among food, enzymes represent a major share in the enzyme industry. For many years, enzymes are utilized in food processes, like for the clarification and filtering of wines and beers, for baking, the assembly of cheeses, and far more (Robinson, 2015). Global population is accelerating and is estimated to reach ten billion by 2057. The use of industrial enzymes is also estimated to increase in correlation with the population from 5.8 billion USD in 2020 to 20 billion USD in 2057 (Fig. 13.1). The preferred source of enzymes are plant and animal, which constitutes around 15% and the remaining 85% of industrial enzymes are from microorganisms such as fungi and bacteria. The economic and technical ease put microorganisms forefront of enzyme synthesis.

The development of recombinant technology brought considerable improvements in the enzyme industries by generating Genetically Modified Organisms (GMOs). In today's market, around 50% of the enzymes are derived from these GMOs, which are intended to increase their yield, purity, specificity, and stability (Petersen, 2005; Poulsen & Bucholz, 2003). As per FAO (Food and Agriculture Organization of the United Nations). GMOs are "the organisms those not occur by natural reproduction or natural recombination." GMOs are the major source of enzyme production in food industries that requires a comprehensive understanding of the integration between enzymology, molecular biology, and bioinformatics. The enzymes from GMOs especially from the microorganisms have several safety concerns such as contamination with toxins, allergens, and other impurities (de Santis et al., 2018; Srivastava, 2019). Thus, before getting into the marketing, enzymes from GMOs need approval from regulatory bodies such as the Food and Drug Administration (FDA; USA) and European Food Safety Authority (EFSA; Europe) with rigorous assessment in addition to regular ethical concerns. This chapter deals with the enzymes from GMOs; applications in food industries and regulatory concerns for their use.

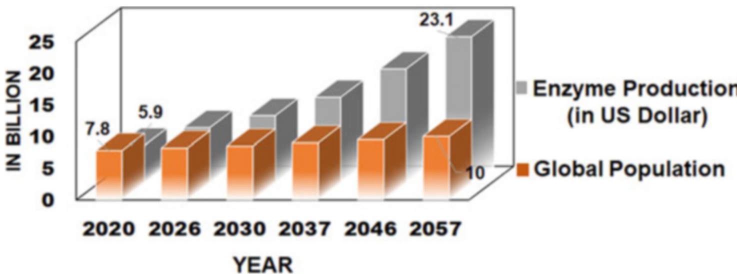


Fig. 13.1 Projected global population and enzyme production

13.2 Use of Genetically Modified Organisms for Enzyme Synthesis

The biological variants form very often due to natural recombination and mutation by changes in the surrounding microenvironment. More than a century, we also exploiting the genetic materials that are naturally present in animals, plants, and microbes. Initially, the geographic distribution of genetic variants occurred unknowingly from one part of the world to another with distinct climate conditions. For example, Asian soybean to America and American potato throughout the world. The history of genetic engineering begins with Charles Darwin's notions of species variation and selection. The evolution of biotechnology leads to the development of biological techniques that help to overcome unbeatable physiological barriers and to exchange genetic materials among all living organisms. In late 1950s and 1960s, the scientists discovered the double helix structure, central dogma, and genetic code which led to the development of DNA recombination technology in 1973 by Cohen et al. The development in the DNA recombination technology showed that the genetically engineered DNA molecules can be transferred among same species and in different species.

13.2.1 Genetically Modified Organisms

According to World Health Organization (WHO), GMOs are the organisms (i.e., plants, animals, or microorganisms) in which the genetic material (Deoxyribonucleic acid) has been altered in a way that does not occur naturally by mating and/or natural recombination. The genetic modification occurs in two ways; either mutation or recombination, which may be spontaneous/natural or induced. The evolution of biotechnology leads to the development of biological techniques that help to overcome unbeatable physiological barriers and to exchange genetic materials among all living organisms. The technology is often called modern biotechnology, recombinant DNA technology, or genetic engineering.

Initially, the first-generation GMOs were developed for improving the quality and yield by increasing their resistance to certain diseases and unfavorable environments. In 1983, the first genetically modified plants antibiotic-resistant tobacco and petunias were produced by three independent research teams (Bevan & Chilton, 1982; Fraley, 1983; Herrera-Estrella et al., 1983). In 1990, China first commercialized genetically modified tobacco and in 1994 USA marketed the genetically modified tomato which is approved by the Food and Drug Administration (FDA). Many transgenic crops, such as Canola with modified oil composition, cotton and soybeans resistant to herbicides, etc. have received FDA approvals. Genetically modified potatoes, eggplants, strawberries, carrots, and many more are available in the market (Bawa & Anilakumar, 2013). The second-generation GMOs were aimed to transfer genetic information from one organism to the other to improve specific product quality and quantity.

13.2.1.1 GM Plants

The Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA) listed more than 3000 plants that have been developed using genetic modification with physical and chemical mutagenic agents. These GM foods are either used for the human consumption or used as feed for livestock and poultry.

The first commercialized food crop is papaya, which was genetically modified for its resistance against papaya ringspot virus. Later, some fruit ripening characteristics have been improved using genetic modification. Polyphenol oxidase is responsible for the production of melanin in apples and potatoes, which cause a brown tint when exposed to air. The restriction of polyphenol oxidase synthesis below 10% by genetic modification allowed the apple and potato remain same after slicing. The herbicide-tolerant varieties of alfalfa, soybean, sugar beet, sweet corn (maize), and cotton have been developed using genetic engineering. GM Golden rice, which contains beta-carotene (precursor of vitamin A) along with enhanced iron content has been approved in various countries. At present, more than 170 million hectares are under cultivation of GM crops worldwide (James, 2006).

13.2.1.2 GM Animals

Though GM animals are in the use of biomedical and pharmaceutical applications to produce certain recombinant proteins and to study selective gene functions, the generation of GM animals for food from a variety of target species traits is still on bench side due to the inefficacy of the production and ethical issues. The major causes to attain genetically modified organisms are; to increase animal health, (Tong et al., 2011; Wheeler et al., 2001), to make them disease resistant (Lyall et al., 2011; Richt et al., 2007), to improve growth rate (Devlin et al., 2009), to increase meat quantity (Lai et al., 2006), to increase milk production and alter composition (Wu et al., 2012), and to increase wool production (Damak et al., 1996). The fast-growing Atlantic salmon is the only GM animal that has been approved for food by health Canada and USA-FDA, in which the growth hormone gene from Chinook salmon was transferred into the Atlantic salmon genome. The use of this fast-growing GM fish for the food in future is still uncertain.

13.2.1.3 GM Microorganisms

Microorganisms, produce endogenous enzymes responsible for several food products like yoghurt, cheese, beer, wine, vinegar, and many more. Humans used these microorganisms even without knowing they are responsible for the fermentation process in various food products (Jay et al., 2005; Zhang et al., 2017). During the eighteenth century, the discovery of the microorganisms responsible for a certain process in food industries began to research efficient ways to use microorganisms (Barnett, 2003). The discovery of DNA followed by recombinant technology allowed genetic alteration to optimize the use of microorganisms in effective way for food production. These genetic modifications either improve the production of existing proteins or increase their potential or produce a new protein with different characteristic features. The advancement in molecular biology led to widespread use of GM microorganisms in food industries and is recognized as an environmentally

friendly and cost-effective method for food production. The yeast *Saccharomyces cerevisiae* was the first GM microorganism used for enzymes synthesis in the dairy and brewing industries. The trypsin and chymosin from the GM microorganisms served as an alternate for animal sources such as pigs and cattle. Replacement of certain plant-based products by GM microorganisms could meet the demand with reduced land usage and waste generation. The GM yeast-based production of vanillin, an alternate for vanilla extract; GM bacteria that produce riboflavin are certain examples of the use of GM microbes in food industries (Brochado et al., 2010; Schwechheimer et al., 2016). Other food ingredients known to be produced by microbes are includes vitamins, amino acids, nutritional proteins, oligosaccharides, flavoring agents, and sweeteners (Adrio & Demain, 2010).

13.2.2 Enzyme Synthesis from GM Microbes

According to legal authorities such as FDA, the European Food Safety Authority (EFSA), the enzymes are considered nutritional additives. The major industrial enzyme market relies on the food and beverage industry, which is expected to reach 2.3 billion USD in 2021 (Raveendran et al., 2018). Although the majority of the organisms including plants and animals produce enzymes, microbial sources become more valuable. Table 13.1 shows various industrial enzymes used in the food and animal feed industries.

13.2.2.1 Advantages of Using Microbes for Enzyme Synthesis

(a) Many MO produce enzymes and release into the medium which simplifies the extraction method during the purification. It is easy to extract intracellular enzymes as well with the disruption of microbial cells compared to the extraction process from animal and plant sources (Robinson, 2015).
(b) Specific microbial strains can be used for the yield of well-characterized enzymes with specific properties (Chang et al., 2016).
(c) Cost-effective production due to shorter time and simple facility requirements. The whole production can be achieved in one location where animals and plants need to be transported to extraction facilities (Raveendran et al., 2018).
(d) High yield can be obtained for microbial enzymes compared to animal and plant sources where the availability depends on a specific period, which could limit the continuous production (Singh et al., 2016).
(e) Microbial enzymes usually have a higher activity and stability (Vieille & Zeikus, 2001).
(f) Genetically modified microbial strains can be easily produced in order to obtain higher yields, optimized enzyme products, and better characteristics. Modifications of animals and plants are difficult and ethically more sensitive (Lievens et al., 2015).

The wild-type microbes are unstable and may not produce desired quality enzyme, especially in fermenter conditions. Sometimes the feedback repression occurs that

Table 13.1 Industrial enzymes used in food and animal feed industries

Industry	Enzyme	Function
Dairy	Lactase (beta galactosidase) Aminopeptidase catalase Proteinase (acid and neutral) Lipase Transglutaminase	Lactose reduction in milk, coagulation, faster cheese ripening, debittering, flavored cheese, protein crosslinking
Baking	Amylase Transglutaminase Lipase Glucose oxidase Xylanase	Bread softening, enhance lifespan, dough conditioning, dough stability, and strengthening
Beverage	Pectinase Cellulase Amylase (Alpha and Beta) Beta Glucanase Protease Glucose oxidase Pullulanase Naringinase Limoninase Aminopeptidases	Pectin digestion, starch hydrolysis fruit liquefaction, protein breakdown, oxygen removal, debittering, restrict mist formation
Animal feed	Beta glucanase Phytase Xylanase	Enhance digestion

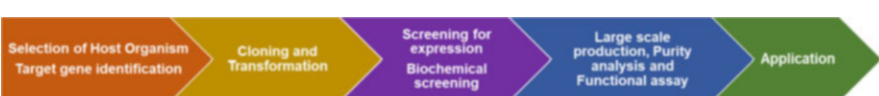


Fig. 13.2 Major steps involved in the enzyme production from genetically modified microbes

ultimately affect the enzyme production. Considering the advantages of using microbial strain for the food enzyme production, the genetic modifications can be easily done in the microbial strains which improve the industrial enzyme production. GM microbes could overcome these drawbacks and enhance the production of quality enzymes. The GM microbes mainly certain bacterial and fungal strains have been optimized for increased enzyme production with a limited synthesis of unwanted secondary metabolites (Deckers et al., 2020). The GM enzymes are mostly expressed in well-established standard microbial model systems like *Escherichia coli, Bacillus,* lactic acid bacteria, filamentous fungi, and yeast. These microbes have advantageous characteristics over other microbes, which resulted in the use of a number of biotechnological applications. The major steps involved in industrial enzyme production are given in Fig. 13.2. Few strategies that are required to produce genetically modified enzymes are;

1. Codon optimization for the heterologous expression and to limit mRNA folding for optimal translation (Rosano & Ceccarelli, 2014).
2. In-depth understanding of enzyme sequence, corresponding amino acids, structure, and related activity. This will allow one to modify the sequence by mutation-like site-directed mutagenesis to alter or increase the enzyme activity, specificity, and structural stability (Hua et al., 2018).
3. Developing an efficient screening protocol for identifying effective variants (Packer & Liu, 2015).
4. Knowledge about fusion and truncation of target sequence to produce an enzyme with multiple functions and to eliminate the unwanted sequences those are not important for the function (Dediu, 2015).

13.2.2.2 Selection of Host Organism

In order to obtain a successful enzyme synthesis, it is important to select a host organism having suitable characteristics for the production and expression of the specific enzyme and that is relatively cheap to culture. The type of expression vectors, the transformation methods, and the selection markers used to select the transformed strains need to be determined are also important for obtaining genetically modified organisms for use in food industries (Olempska-Beer et al., 2006).

Many expression systems are available for the recombination of bacteria resulting in the easy integration of modifications. Bacteria are not able to perform posttranslational modifications resulting in only simple proteins being produced. In the case of fungal strains, correct folding and posttranslational modifications were done by the subcellular organization and they secrete a limited amount of proteins into the medium (Demain & Vaishnav, 2009). The commonly used bacterial host strains are *Escherichia coli, Bacillus subtilis, Bacillus licheniformis, and Bacillus amyloliquefaciens*. Availability of molecular techniques for the construction of an expression system in *E. coli* results in the high-yield enzyme production in the cytoplasm and additional purification steps are required. However, the Bacillus species secrete higher concentrations of the enzymes into the medium (Puetz & Wurm, 2019). *Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei* are commonly used filamentous fungi. A. oryzae used for the production of fermented food, which is also known to produce mycotoxin aflatoxin. The mutant strain was constructed to overcome this issue, in which the production of these products was reduced (Meyer, 2008).

13.2.2.3 Gene Transfer Methods

Expression Vector System

Once a suitable host has been selected, the selected host strain is modified by using the expression vectors. A vector is a DNA molecule that is a vehicle to transfer foreign genetic material into another cell. Plasmids, viral vectors, cosmids, and artificial chromosomes are the different types of vectors. Plasmids are called expression vectors those express transgene in the target cell, and they generally have a promoter sequence that drives the expression of the transgene. A typical expression

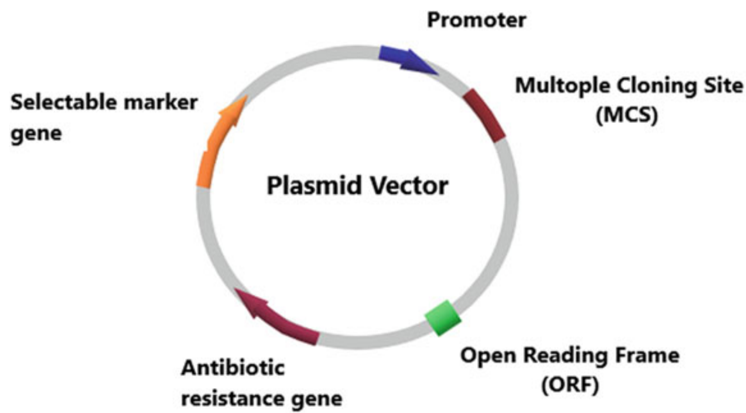


Fig. 13.3 Basic structure of expression vector used in recombinant enzyme synthesis

vector is shown in Fig. 13.3. An expression vector contains, at the least, an origin of replication (ORI), a Multiple Cloning Site (MCS), in which the gene of interest will be integrated, and a selection marker for the selection of the recombinant strain (Fig. 13.1). Promoter and terminator regions are also present to control the expression of the gene of interest and the selection marker. In order to avoid an increase in the metabolic load on the host organism and to avoid reduced plasmid stability, the size of the vector is kept as small as possible (Rieder et al., 2019).

It is important to select the recombinant strain and the selection is based on the selectable marker. A selectable marker is usually a gene that shows resistance to an antibiotic that would otherwise kill the normal cells which lack these genes under culture conditions with a specific antibiotic. Kanamycin (kanR), ampicillin (ampR), and tetracycline (tetR) resistance genes are commonly used in the bacterial expression vector. Antibiotic resistance markers are associated with a public health concern, which is replaced by auxotrophic strains, where it requires specific nutrients for growth. Some auxotrophic markers are amdS, URA3, which are usually used as the selectable marker in fungal strains (Hjort, 2007; Olempska-Beer et al., 2006).

Preparation of GM Microbes

The common method employed in the preparation of GM microbes is to cut open the plasmid DNA; inserting the target DNA molecule (named molecular cloning) and transform it into host microbes, which can efficiently express the target DNA. Dissecting each part, (1) the plasmid expression vector has been chosen based on the host organism; (2) the target DNA has been amplified from the target genome which has restriction enzyme site overhanging on both the ends that matching to the sites present in the multiple cloning site of the plasmid; (3) restriction digestion of the plasmid vector and amplified target DNA molecule using restriction enzymes (which can form either blunt or sticky ends); (4) Purify the restriction digested DNA and plasmid; (5) Ligate the plasmid and target DNA using ligase; and (6) transform the recombined plasmid to the host organism. The recombinant DNA molecule can

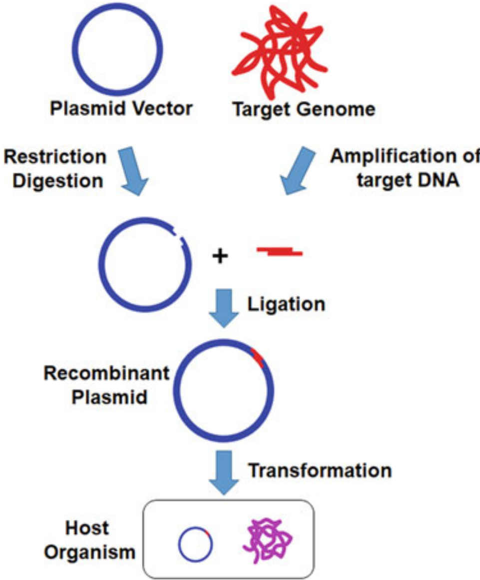


Fig. 13.4 General recombinant technology involved in the recombinant enzyme synthesis

be transformed into cells of higher organisms (Fig. 13.4). Transformation of the target DNA is done by transfection in which the introduction of nucleic acids into cells by nonviral methods. Calcium phosphate, DEAE-dextran, or other substances are nonviral methods of transformation. Physical methods used are electroporation which cellular membranes of an organism are disrupted by using an electrical pulse, direct microinjection into a cell, and biolistic particle disruption into cells using gene guns. The plasmids are the most commonly used DNA carrier molecules and the viral DNA molecule also can be used to stably express the target enzyme. Plasmids are isolated from bacterial cells and specific sequences of DNA molecule are altered in vitro by insertion or deletion. Antibiotic resistance, ß-galactosidase, luciferase, etc. act as gene reporters for the screening of transformed cells.

Other Gene Editing Options

Several molecular tools are used to insert desired DNA sequence directly into the genome or delete unwanted DNA bases from the genome. For example, the insertion of functional genes such as active promoter sequence activates the production of the desired enzyme and the insertion of transporter sequence can improve the transport of enzymes. The source of DNA can be either endogenous or exogenous from closely related/distinct organisms. Currently, the use of synthetic DNA allows fast development of GM microbes rather than isolating and amplifying from the other organisms. Recent techniques allow to insert DNA sequences to specific loci, which can be confirmed by genome sequencing. Today, the discovery of CRISPR (Clustered Regularly Interspaced Palindromic Repeats) facilitates insert or delete DNA sequence at precise position using specifically designed guide RNA sequences that can recognize specific DNA code.

13.2.3 Enzyme Extraction Process

The extraction and purification of enzymes from GM microbes is much easier compared to plant and animal sources. Depending on the enzyme of interest and the host-microbe, the growth environment, such as pH, temperature, gas exchange rate, and substrate can be optimized during the extraction process while using GM microbes in comparison with wild-type organisms. Current genetic engineering techniques enabled the large-scale production of enzymes for use in food and other industrial applications. The level of downstream processing mainly depends on the application of the finished product (Headon & Walsh, 1994; Wheelwright, 1989). The overall enzyme extraction and purification process is illustrated in Fig. 13.5. The enzyme extraction process at the industrial level involved two basics bioreactor-based fermentation methods, which are (1) submerged and (2) solid-state fermentation.

1. **Submerged Fermentation**
   Submerged fermentation (SF) is the most preferred method for industrial-level enzyme production, where the selected GM microbes (bacteria or Fungi) are cultured in the liquid nutrient medium at a higher concentration of oxygen. In this process, the microbes release the enzymes to break down the complex carbohydrates, proteins, and lipids in the medium. A large fermenter with a high capacity of around 1000 cubic meters is used to produce a large quantity of enzymes. Simple batch reactors are used, where the reactor is filled with selected microbe and nutrients and allowed for fermentation. After fermentation, the contents are emptied for further enzyme purification. On the other way, the batch-fed process is a continuous process, where the sterile nutrient mix is added

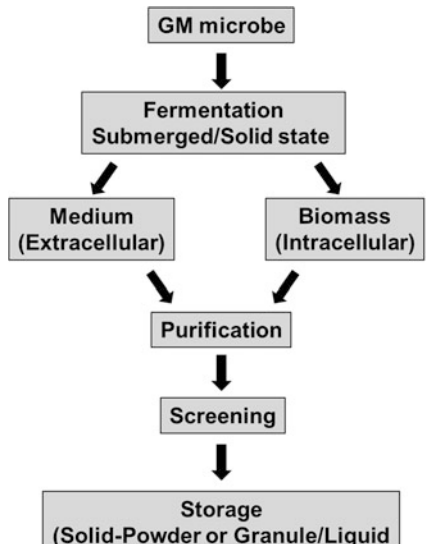


Fig. 13.5 Enzyme extraction and purification process from GM microbe

to the fermenter at the same level of fermented broth leaving for further enzyme purification.

2. **Solid-State Fermentation**
   Solid-state fermentation (SSF) is another method that involves the culture of the GM microbe on a solid substrate, such as pulp, corn, wheat or rice bran, and grains. The advantages of SSF over SF are; a high concentration of enzyme production with less affluent in a simple fermenter. In SSF, the factors such as particle size and moisture play a major role in enzyme production. For example, smaller substrate particles make larger surface area microbial growth, whereas larger particles provide efficient aeration. In SSF the water level should be optimized for enzyme production, as moisture level greatly influences the microbial activity.

The enzyme produced during fermentation should be further processed to purify the enzyme from the biomass and other byproducts. First, the solid waste and the biomass are removed from the medium and the solution is further concentrated using filtration, evaporation, and crystallization. Chromatography method such as ion-exchange chromatography is employed to remove impurities. If the enzyme is localized intracellular, the biomass has to be collected and processed further for enzyme purification. Depending on the nature and use of enzymes, the enzymes can be further stored in lyophilized powder form, liquid form if crude, or granule form. Some enzymes are immobilized on the surface of inner granules.

13.3 GMO-Derived Enzymes in Food Industries

From the ancient period onwards a wide variety of enzymes from a natural source has been used for the production of numerous food products. Natural sources for obtaining enzymes are animals, plants, and microorganism. There are about 3000 known enzymes produced by all animals, green plants, fungi, and bacteria that catalyze about 4000 biochemical reactions (Patel et al., 2017). Among these sources, microorganisms have been involved in the food industry for various applications like the production of alcoholic beverages, ethyl alcohol, organic products, dairy products, and drugs including antibiotics through fermentation (Verma et al., 2018). For example, the usage of chymosin for cheese production and pectinases for fruit and beverage processing (Chandrasekaran, 2015). In modern food processing methods extreme conditions, such as high temperature, high pressure, extreme pH, salinity, etc., are required to improve the commercialization of enzymes and their products (Li et al., 2012). This condition may affect natural enzymes. So, most of the enzymes produced by microbes are no longer native enzymes, but instead engineered versions. Genetic modifications through genetic engineering and recombinant DNA technology creates Genetically Modified Organisms (GMOs) that have specific modifications introduced into genetic material which enable the production of new protein or food ingredient, enhance the production, and modify the characteristics of an existing enzyme for a new application (Maghari &

Table 13.2 Common GM Microorganisms involved in food enzymes production

Microorganism	Species
Bacteria	Escherichia Coli Bacillus megaterium Bacillus brevis Lactobacillus lactis Pichia pastoris
Yeast	Saccharomyces cerevisiae Hansenulapolymorpha, Schizosaccharomycespombe Yarrowialipolytica
Filamentous fungi	Aspergillus oryzae Aspergillus Niger Trichoderma reesei Penicilliumpurpurogenum Rhizopusoryzae

Ardekani, 2011). Thus, the quality and productivity of enzymes can be improved. Enzymes obtained from genetically modified organisms are intended to improve or alter the enzymological properties or to increase the purity and yield of enzymes. Although there are challenges and safety concerns for using recombinant proteins in food processing, genetically modified enzymes are promising because of their potential benefits for the food industry, consumers, and the environment (Zhang et al., 2017).

Food enzymes are commonly used to perform a wide variety of applications in food production, which include; lactase that reduce lactose in food and dairy products, amylases that strengthen dough in bakery, mannase that helps in coffee production, proteases that hydrolyze protiens, chymosin that used to make cheese, glucoamylase, and transglucosidase those reduces starch into simple sugar and phospholipase that refine vegetable oil. Considering the safety and synthesis of other metabolites, selected bacterial, yeast, and fungal species are commonly involved in the recombinant enzyme synthesis (Table 13.2). The list of major enzymes derived from GM microbes and their function are given in Table 13.3. The main industrial enzymes can be classified into three groups: carbohydrases, proteases, and lipases (Castro et al., 2018). Microorganisms secrete a variety of enzymes that plays an important role in the food manufacturing industries (Hanlon & Sewalt, 2020). With the growing research on genetically modified organisms, production, and development of these enzymes are also getting better.

Carbohydrases: Carbohydrases belonging to glycosidase family are a set of enzymes that catalyzes hydrolysis of complex carbohydrates (polysaccharides) into simple sugars (monosaccharides). These enzymes are used in the food industry for baking, brewing, sweetener production, etc. Carbohydrases include amylases, cellulases, glucanases, pectinases, xylanases, invertase, galactosidase, and others (Castro et al., 2018).

Amylase: Amylase catalyzes the hydrolysis of glucosidic linkages of large polysaccharides (starches) into oligosaccharides. These enzymes are used in the

Table 13.3 List of major GM food enzymes and their functions

Food enzymes	Functions
Amylase	Breaks down starches and sugars found in grains, fruits, and vegetables
Galactosidase	Breaks down the polysaccharides and oligosaccharides in foods, such as legumes and cruciferous vegetables
Beta-glucanase	Breaks down plant cell walls (cellulose) beta-linked glucose polymers associated with fibers, grains, and cereals
Cellulase	Breaks down food fiber (cellulose) found in fruits and vegetables
Glucoamylase	Breaks down off long chain carbohydrates or starches (corn, potatoes, wheat and rice) into sugar
Invertase	Breaks down simple sugar (fructose and fruit sugar)
Lactase	Breaks down lactose found in milk
Lipase	Breaks down fats and lipids
Pectinase	Breaks down pectin, a polysaccharide found in plant cells in many fruits and vegetables
Peptidase	Breaks down proteins into amino acids
Phytase	Breaks down carbohydrates; helpful in the breakdown of phytic acids in the leaves of plants, grains, seeds
Protease	Breaks down protein found in meats, fish, poultry, grains, nuts, gluten, milk proteins (caseins)
Xylanase	Breaks down plant nutrients from vegetables with high fibercontent (fibrous veggies, grains, and legumes)

production of maltodextrin, modified starches, glucose and fructose syrups, production of bread, cakes, and pastries (De Souza & Magalhaes, 2010). These enzymes degrade starch in the flour used as dough of bread into smaller dextrins which enhance the rate of subsequent fermentation by the yeast and reduce the viscosity of dough which in turn improves the volume and texture of the product. Through the action of enzyme additional sugar is produced in the dough, which improves the quality of the bread, such as taste, crust color, and toasting qualities (van der Maarel et al., 2002).

The conditions during the development of fermentation processes are very important such as thermostability, pH profile, pH stability, and Ca-independency. Industrial source of α-amylase is the bacteria *Bacillus licheniformis*. Usually, the α-amylase produced by wild-type *Bacillus licheniformis* is thermostable but acid-sensitive. Genetically modifying *Bacillus licheniformis* through directed evolution by replacing active site domain His residues with Arg and Asp residues produces α-amylases having higher activity at pH 4.5 than the wild type enzyme which enhances the properties of an enzyme such as starch liquefaction (production of nutritive sweeteners from starch), saccharification and fermentation processes where the pH of the starch slurry is usually <6.0 (Liu et al., 2014). Genetically modifying *Rhizopus oryzae* using site-saturation mutagenesis of His 286 produces α-amylases having higher optimum temperature (60 °C) and lower optimum pH (4.0–4.5) than

the wild-type enzyme which makes the enzyme more suitable for maltose syrup production (Li et al., 2018).

Glucosidase: These enzymes are involved in the breaking down of starch and glycogen into their monomers. It is used as a flavor enzyme to enhance the flavor of wine, tea, and fruit juice. β-glucosidases play a critical role in plant biomass deconstruction. β-Glucosidases cause flavor liberation by catalyzing the breakdown of β-1, 4-glycosidic linkages and producing free fermentable glucose used for the production of glucose syrups from fruits and other plant tissues. In modern industrial conditions, wild-type fungal β-glucosidases show weak activity at high glucose concentrations limiting enzymatic hydrolysis of plant biomass. Genetic modification of *Trichoderma harzianum* by site-directed mutagenesis efficiently improved the functional properties of a β-glucosidase where the enzyme displayed high glucose tolerance levels by the substitution of two amino acids that act as gatekeepers, changing active-site accessibility and preventing product inhibition. The efficiency of the engineered enzyme was enhanced by the yield of glucose and ethanol (Santos et al., 2019). Genetically modifying *Aspergillus aculeatus* using site-saturation mutagenesis produces β-glucosidase having enhanced hydrolytic efficiency, especially to cellobiose, and improves saccharification of alkaline-pretreated bagasse (Baba et al., 2016).

Xylanses: These enzymes are involved in breaking down the hemicellulose in the plant cell wall, specifically hydrolyzes *xylan* and *arabinoxylan*. It is used in the application of the food industry for bread making, the production of corn starch, clarification of fruit juice and wine, and alcoholic fermentation. In bread-making process, xylanases are added to the dough for stabilizing the dough by hydrolyzing polysaccharides in the wheat and making it more flexible, and enhancing the gluten strength. Treating with xylanase increases moisture retention and shelf life of the dough (Ahmad et al., 2014). It is also used in fruit juice processing where it breaks down the polysaccharide plant cell walls to intensify the juice extraction efficiency and thus extracting more nutrients and aroma compounds. Also, it helps in juice clarification by decreasing cloudiness (Bajaj & Singh, 2010).

Even though xylanases have potential applications there is a need for xylanases that can endure relevant processing conditions and industrial settings. A variant of this enzyme generated using site-directed mutagenesis exhibited higher specific activity and was utilized in the production of xylooligosaccharides from wheat straw under thermal and alkaline conditions (50–65 °C, pH 7–10) (Faryar et al., 2015). Endo-b-1,4-xylanases with modification in secondary binding sites also showed increased specificity toward water-insoluble, but not water-soluble, wheat arabinoxylan, and its application in bread making resulted in increased loaf volumes (Leys et al., 2016).

Glucanase: The glucanase enzymes help in the breakdown of polysaccharide glucan by hydrolyzing the β-glycosidic bonds between glucose molecules in glucans. This enzyme is mainly employed in the brewing industry where it hydrolyses barley gums. It helps in beer brewing by lowering the viscosity of warts, improves clarification by breaking the turbidity system by hydrolyzing the beer haze, and aid in production of clear wart. β-glucanase is usually found

endogenously in barley itself and is called endo-b1, 3-1, 4-glucanases having an optimum pH and temperature values of 6.0 and 45–50 °C; and denaturizes at 60 °C. So, industrial source β-glucanase from microbial organisms is used for the standard production of light beers. Industrial sources for glucanases are *Bacillus amyloliquefaciens* and fungi of the group In the brewing industry β-glucanase hydrolyzes barley gums. Glucanases are also used during wine-making processes where it hydrolyses *Botrytis* and yeast glucans and thus enhance the clarification and wine filterability. β-1, 3-1, 4-glucanases used in malt extract production in the brewing industry are obtained from *Bacillus* species. Genetically modified forms of β-1, 3-1, and 4-glucanases were produced by replacing Lys with Ser and these modified glucanases showed higher efficiency under industrial operating conditions with improved optimal temperature and thermostability and halostability following replacement of hydrophobic Lys 48 with Ala and Leu (Lee et al., 2017).

Invertases: Carbohydrases that hydrolyze sucrose and polysaccharides into fructose and glucose as final products. They are used as a catalytic agent in obtaining an artificial sweetener and so it is used in food industry for confectionery, syrups, condensed milk, infant milk, and beverages (Kotwal & Shankar, 2009). When sucrose is present in higher concentrations invertases are able to synthesize fructo-oligosaccharides through fructotransferase. Though the natural sources of invertases are plants, bees, and microorganisms, the most prominent organisms used for invertase production are the filamentous fungi belonging to the *Aspergillus* genus and yeast such as *Saccharomyces cerevisiae* and *Candida utilis* (Veana et al., 2018). From *Aspergillus* genus *A.niger, A.terreus, A.japonicus, A.versicolor A.tubingensis, A.fumigatus, A.oryzae, A.aculeatinus,* and *A.homomorphus* are the main source.

The most important characteristic required for the invertase application in the industry are pH and thermostabilities (50–75 °C). Fungal invertases have these features, i.e., they have these optimal values and stabilities but low-specific activity. This limitation had been overcome using gene modification in the host organism. Invertase produced by genetically modified *Saccharomyces cerevisiae* where the enzyme was modified by the substitution of hydrophilic residues in the active site region or peripheral loops with hydrophobic amino acids have optimal pH and stability ranges for sucrose breakdown into glucose and fructose (Sainz-Polo et al., 2013) Genetically modified invertase with high transfructosylating activity is used for simple and efficient production of prebiotic fructo-oligosaccharides.

β-galactosidase: commonly known as lactase, is an enzyme that hydrolyzes lactose. This enzyme has wide applications in food processing industries. It helps in the digestion of lactose present in dairy products and converts it to glucose and galactose. This enzyme holds importance in food industries as it manufactures lactose-hydrolyzed products which act as a "predigester" for people with milk intolerance problems, milk destined for cheese and yogurt making, for the production of sweeteners and hydrolyzed whey syrups (Dekker et al., 2019). Lactases are used in the production of ice cream and sweetened flavoured and condensed milks. The sources of β-galactosidases are microbial, plants, and animal origin. Among these sources, microbial lactase shows higher productivity, and it is cost-–effective. Bacteria, fungi, and yeasts are the microbial sources. On a commercial and industrial

scale, the most commonly used sources of β-galactosidase are *Aspergillus* and *Kluyveromyces* (Saqib et al., 2017). The enzyme source is selected depending on the required reaction conditions. Bacterial β-galactosidases work with optimal pH between 2.5–5.4 and are mainly used for acidic whey hydrolysis whereas yeast β-galactosidase shows maximum activity at pH 6.0–7.0 which is more suitable for the hydrolysis of milk and sweet whey. The temperature at which lactose-free milk product is processed is relatively low. The lactase derived from the dairy yeast *Saccharomyces lactis* has optimal process conditions (35–40 °C, pH 6.6–6.8) that are close to the natural temperature and pH of the milk (O'connell & Walsh, 2006). It shows considerable activity at lower temperatures, down to about 4 °C, and slows the growth of spoilage bacteria thus it is the most widely used commercial lactase source. Another source for lactase is fungal lactase derived from *Aspergillus niger*. Its optimal process conditions are around 50 °C and pH 3.5–4.5 so its application is limited to acid (or acidified) whey or lactose.

To satisfy the growing demand for lactase the development of genetic engineering technology makes the large-scale industrial production of lactase possible through the introduction or modification of genes to promote characteristics such as higher enzyme activity or higher production. Modification of lactase produced by microbes through directed evolution had high activity in industrial type conditions for milk processing, that is, substrate lactose, buffer pH 6.75, and 8 °C. The production of recombinant strains using the *lac*A gene from *Aspergillus niger* and *Saccharomyces cerevisiae* can produce a high quantity of lactase (Domingues et al., 2010).

Proteases/peptidases: The major use of proteases in the food industry is the hydrolysis of protein matrices to enhance flavor, texture, or functional properties in dairy, meat, and fish products. Commonly used food proteases are derived from animals which include trypsin, chymotrypsin, chymosin/rennet, pepsin, etc., and papain, bromelain, and ficin from plants and from microorganisms microbial acid protease, proteinase A, alkaline proteases, flavourzyme, etc. (Mazorra-Manzano et al., 2018). Microbial proteases had vital roles in the production of traditional fermented foods.

Trypsin: Trypsin belongs to the serine protease superfamily that cleaves on the carboxyl end (C-terminal) arginine (Arg) and lysine (Lys) on peptide chains. Trypsin is used as a baking enzyme to improve the workability of dough, in the extraction of flavorings from vegetable or animal proteins, to control aroma formation in cheese and milk products, to improve the texture of fish products, to tenderize meat, in the production of hypoallergic food, i.e., proteases can break down specific allergic components present in cow's milk into nonallergenic peptides and diminishes the risk of babies developing milk allergies (Bu et al., 2013).

Usually, trypsin for food uses has been obtained from purified extracts of bovine or porcine pancreatic tissue but the trypsin from mammal sources has several limitations, including high costs, security, and ethical issues. So microbial trypsin is employed for easy preparation and low costs. But there have been a few strains, including those from the genera of *Fusarium*, *Streptomyces*, and *Trichoderma* reported. The reported microbial trypsins show poor specific activity and operational

stability. Thus, trypsin is derived from genetically modified microorganisms for example *F.venenatum* containing the gene for trypsin from *F.oxysporum*, which is used as a food processing aid. The genetic modifications improve serine protease enzyme yields. Studies also showed that trypsin produced by recombinant *Streptomyces griseus* showed higher enzyme activity.

Chymosin (rennin): is an aspartyl proteinase. It is found in the fourth stomach of the unweaned calf. It leads to limited or partial proteolysis of K-casein in milk resulting in clotting. The enzyme is used in the food industry as a processing aid for the manufacture of cheese and curd and other milk coagulants. As an industrial enzyme it is widely used in cheesemaking and it was mainly extracted from dried calf stomachs. But the cheesemaking industry has expanded beyond the supply of available calf stomachs and in order to meet the increasing global demand expression of chymosin has been achieved in many species such as *Escherichia coli*, *Bacillus* species, *Aspergillus* species and yeast (Wei et al., 2016). Thus 80% of fermentation chymosin is produced as recombinant proteins in microorganisms with protein produced in *Aspergillus* and yeast the most widely used.

Expression of yak chymosin in gene-recombinant *Pichiapastoris* is the method used for large-scale production of chymosin (Ersoz & Inan, 2019). Some *Lactococcus* spp. also help in the production of chymosin at an industrial scale. Some other common microorganisms that are involved directly or indirectly in the production of chymosin are *E. coli*, *Kluyveromyces lactis, P. pastoris, Aspergillus awamori.*

Aminopeptidases: Enzymes that hydrolyzes the peptide bond of N-terminal amino acids in peptides and proteins. Cleavages by the peptidases will change the flavors of the proteins in a food matrix. Therefore, aminopeptidases are used in the production of cheese, beverages, flavorings, meats, and milk products (Nandan & Nampoothiri, 2020). They are specifically meant to enhance and optimize aroma and flavor. The addition of aminopeptidases can accelerate the maturing of cheese. Various recombinant aminopeptidases have been produced from *Lactobacillus rhamnosus*, *Lactococcus lactis,* and *Aspergillus sojae*.

The activity and stability of proteases depend on the amino acid composition and their respective position. Protein engineering modifies the protease structure in such a way that enhances the activity, specificity, and stability of protease. Bacillus spp. are the main producers of alkaline proteases with high thermal and pH stabilities. The cold activity at 10 °C and alkali-resistant properties of a *Bacillus alcalophilus* alkaline protease were enhanced through directed evolution using error-prone PCR for use in cold-temperature food processing (Liu et al., 2014). Similarly, an alkaline serine protease from mesophilic *Bacillus pumilus* was engineered to have increased hydrolytic efficiency at 15 °C without compromising thermostability (Zhao & Feng, 2018). Acid protease from a mutant *A. oryzae* strain was produced using solid-state fermentation with potato pulp powder, with enhanced glycine releasing activity (Murthy & Kusumoto, 2015). A truncated neutral protease from *A. oryzae* had optimum pH of 8.0 and an optimum temperature of 55 °C, and its enzymological characterization suggested that it was efficient in antihypertensive peptide production, debittering, and food oil processing (Ke et al., 2012).

Lipases: Lipases are the enzymes that catalyses the hydrolysis of long-chain triglycerides (fats and oils). These are used for the following applications: dairy, baking, cocoa butter substitutes, human milk fat substitutes, egg processing, and edible oil production. In baking and dairy applications, the addition of lipases enhances and accelerates the development of aromatic notes (Goncalves et al., 2019). In vegetal oil processing, lipases allow a significant increase in oil yield and enhance the appearance of end product, i.e., improves their texture and softness. Also, lipases are used to enhance the emulsification properties of egg yolk lipids. These enzymes help in developing new functional ingredients and functional foods, such as cocoa butter equivalents or human milk fat equivalents. Lipases help in the breakdown of milk fats and provide characteristic flavors to cheeses. The flavor is due to the release of free fatty acids produced during the hydrolysis of milk fats. Microbial lipases are produced by fungal, yeast, and bacterial species. *Candida sp., Aspergillus sp., Rhizomucor sp., Rhizopus sp., Humicola sp., Yarrowia lipolytica,* and *Pseudomonas sp.,* etc. are microbial sources for lipase (Tan et al., 2003).

Lipases are used widely for different applications in the food industry and thus it is necessary to meet the increasing demand and enzymes capable of tolerating industrial conditions. Lipase secretion by *Rhizopus oryzae* was improved by rational design of the N-glycosylation sites especially for the use in the edible oil and fat industries (Yu et al., 2017). Genetically modified *Candida rugosa* by site-directed mutagenesis produces lipases having high catalytic efficiency for producing fatty acid methyl esters and diglycerides as food emulsifiers (Chang et al., 2014).

13.4 Regulatory Management of GMO-Derived Enzymes

The Generally Recognized as Safe (GRAS) is well-known process that gives general supporting materials such as methodology involved and safety of the enzymes used in food industries from genetically modified organisms. Section 201(s) of the Act [21 U.S.C. 321(s)] exempts the use(s) of a substance that is GRAS from the definition "food additive." In general, the enzyme preparations should confirm the purity and specifications according to "Enzyme Preparations" of the 6th (2008) or the current edition of the Food Chemicals Codex (FCC). According to the USA, if an enzyme is produced to use in meat and poultry products, FDA has to approve it in consultation with the Food Safety and Inspection Service (FSIS) of the U.-S. Department of Agriculture. The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) is used to prevent food allergens contamination from enzyme. Genetically modified food enzymes, which are produced from genetically modified organisms fall under the regulation, (Regulation (EC) No 1829/2003) according to these regulation food enzymes produced from a part or whole of a genetically modified organism (GMO) but it does not contain GMO. Processing aid of the enzymes is not included in this regulation because food produced with GMO are not included in this regulation. Genetically modified microorganisms (GMM) which are producing food enzymes and are available in commercialized preparations need authorization according to the regulations (EC) No. 1332/2008 and (EC) No.

1829/2003. The European regulation (EC) No. 1829/2003 is only regulation for the material which is produced from genetically modified sources is used in enzyme preparation or food production. Food enzymes fall under two categories either an ingredient or processing aid. The risk assessment performed by the European Food Safety Agency (EFSA) for food additives requires European regulation (EC) No 1129/2011.

There are many other European regulations for the safety evaluation for use of food enzymes in terms of food additives, enzymes, flavorings they are (1) (EC) No. 1332/2008: Food Enzymes (2) (EC) No. 1334/2008: Food Flavourings (3) (EC) No. 1331/2008: Common Authorization Procedure (4) (EC) No. 1333/2008: Food Additives (The European Parliament and the Council of the European Union, 2008). Some specific food enzymes had already been given authorization for use according to regulation (EU) No. 231/2012 (The European Parliament and the Council of the European Union 2008), which allows the specifications according to regulations (EC) No. 1333/2008 and (EC) No. 1493/1999 lysozyme and invertase are authorized for use in food additives. Lysozyme, Urease, and β-glucanase are authorized for use in wine production according to regulation (Deckers et al., 2020).

13.4.1 Safety Concern for GMO-Derived Enzymes

Deriving food enzymes from Genetically Modified organisms have raised concerns regarding the chance of contaminating the food with bacterial toxins or mycotoxins, or some uncharacterized extraneous substances that may act as allergens (de Santis et al., 2018). Since the organisms itself is genetically modified there is also a chance for modifying their allergenic properties which increases the safety concerns (Olempska-Beer et al., 2006) There are situations in which industrial workers exposed to environments using genetically modified enzymes have developed type I sensitization (Budnik et al., 2017). The regulation of enzymes including those produced by genetic engineering is a major concern in the food industry. So before bringing genetically modified food enzymes into the market, it is necessary to obtain approval from various regulatory bodies such as the US Food and Drug Administration, the Association of Manufacturers and Formulators of Enzyme Products, the European Food Safety Authority, and these processes vary in different countries. Also, ethical and religious concerns are raised for genetically modified enzymes, such as the raw materials used in the fermentation of microorganisms for the production of enzymes should be halal (Ermis, 2017).

Genetically modified food enzymes are typically better suited for specific industrial applications than their native counterparts, and research on their enzymological properties in comparison to wild-type enzymes enables us to better understand how to optimize structure-function relationships. Currently, novel genetically modified food enzymes are mostly used for applications in food processing involving carbohydrates, followed by lipids. Although there are challenges for safe use, genetic modification strategies for the development of novel food enzymes are highly promising for the future, and we expect that more genetically modified

enzymes will be introduced in various aspects of food processing. The production of enzymes by the process of genetic engineering emphasizes on economic production, enhanced enzyme purity, and naturally responsive production processes. Genetically modified microbes are used to produce a wide variety of products, ranging from synthetic insulin to vitamin C and enzymes like chymosin involved in food application. Appropriate purification, concentration, and formulation of enzymes with appropriate substances are crucial for further use in food industries (Hanlon & Sewalt, 2020).

13.4.2 Pros and Cons of GM Food Enzymes

13.4.2.1 Improvement in Food Processing

Increased scarcity of arable lands with increased population forced to use recombinant DNA technology in food industries. Several platforms are working together to make and deliver GM food enzymes as risk-free safe product (Fig. 13.6). The achievements in molecular biology in the past several decades have led to a widespread use of GM microbes in the production of food substances including enzymes. Currently, these genetically modified enzymes are employed in several food industries to improve in food processing. Manipulation of regulatory genes, restrict feedback inhibition, increase the shelf life, overcome the rate-limiting steps, reduce the process cost and production time, environment friendly are some of the

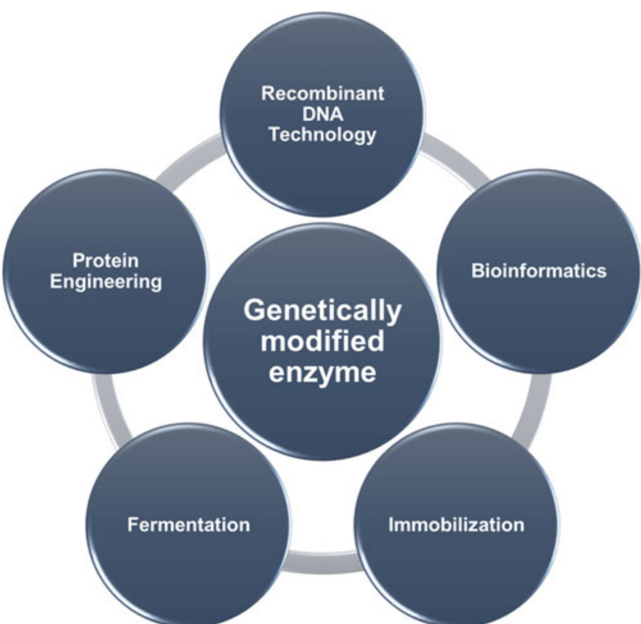


Fig. 13.6 Improved technologies currently involved in genetically modified enzyme production

major improvements that GM microbes could provide. For example, the use of enzyme Amino peptidases to modify by debituminize proteins which helps in the large scale production of food products such as milk, cheese meat, beverages and flavoring (Molinaro et al., 2005; Raksakulthai & Haard, 2003; Izawa et al., 1997). Pectinolytic enzymes are also widely used in the food industry for large-scale production of wine and juice production (Semenova et al., 2006).

13.4.2.2 Health and Ecological Risks Associated with GM Food Enzymes

Smaller recombinant enzymes are easily over-expressed in *E.coli,* but the larger proteins and the proteins that require posttranslation modifications cannot be expressed. Major health-related risks associated with GM foods enzymes are allergenicity, genetic hazards, and toxicity. These problems are associated with the change in pleiotropic effects when a new gene is inserted into the organism which may also cause the deletion of natural genes in the modified organism (Bawa & Anilakumar, 2013). Ecological risks associated with the food enzymes are resistant to antibiotics and in this genetic modification, antibiotics are frequently used as selection markers to distinguish whether the genes got transformed into bacteria. Thus genetically modify an organism have the risk of transferring the genes of antibiotics resistance.

13.5 Conclusion and Future Aspects

It has been more than 15 years, since the use of genetically modified enzymes in the food industry. With the development of genetic engineering and recombinant DNA technology, the production of enzymes enhances the natural responsive production and purity of enzymes. It also offers safe handling and cost-effective production of enzymes in the commercial market. The prime factor to be considered before commercialization of a food enzyme is the host organism that expresses an enzyme. A microbe used in enzyme production should be well characterized, which should not make any adverse effects by synthesizing byproducts like toxins. The safety and hazardous risks of the enzymes from genetically modified organisms have to be evaluated similarly to other enzymes those in use.

References

Adrio, J. L., & Demain, A. L. (2010). Recombinant organisms for production of industrial products. *Bioeng Bugs, 1*, 116–131.

Ahmad, Z., Butt, M. S., Ahmed, A., Riaz, M., Sabir, S. M., Farooq, U., & Rehman, F. U. (2014). Effect of aspergillus Niger xylanase on dough characteristics and bread quality attributes. *Journal of Food Science and Technology-Mysore, 51*, 2445–2453.

Baba, Y., Sumitani, J., Tanaka, K., Tani, S., & Kawaguchi, T. (2016). Site-saturation mutagenesis for beta-glucosidase 1 from Aspergillus aculeatus to accelerate the saccharification of alkaline-pretreated bagasse. *Applied Microbiology and Biotechnology, 100*, 10495–10507.

Bajaj, B. K., & Singh, N. P. (2010). Production of xylanase from an alkali tolerant Streptomyces sp. 7b under solid-state fermentation, its purification, and characterization. *Applied Biochemistry and Biotechnology, 162*, 1804–1818.

Barnett, J. A. (2003). Beginnings of microbiology and biochemistry: The contribution of yeast research. *Microbiology-Sgm, 149*, 557–567.

Bawa, A. S., & Anilakumar, K. R. (2013). Genetically modified foods: Safety, risks and public concerns-a review. *Journal of Food Science and Technology-Mysore, 50*, 1035–1046.

Bevan, M. W., & Chilton, M. D. (1982). Multiple transcripts of T-DNA detected in nopaline crown gall tumors. *Journal of Molecular and Applied Genetics, 1*, 539–546.

Brochado, A. R., Matos, C., Moller, B. L., Hansen, J., Mortensen, U. H., & Patil, K. R. (2010). Improved vanillin production in baker's yeast through in silico design. *Microbial Cell Factories, 9*, 84.

Bu, G. H., Luo, Y. K., Chen, F. S., Liu, K. L., & Zhu, T. W. (2013). Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Science & Technology, 93*, 211–223.

Budnik, L. T., Scheer, E., Burge, P. S., & Baur, X. (2017). Sensitising effects of genetically modified enzymes used in flavour, fragrance, detergence and pharmaceutical production: Cross-sectional study. *Occupational and Environmental Medicine, 74*, 39–45.

Castro, A. M. D., Santos, A. F. D., Kachrimanidou, V., Koutinas, A. A., & Freire, D. M. G. (2018). Solid-state fermentation for the production of proteases and amylases and their application in nutrient medium production. In A. Pandey, C. Larroche, & C. R. Soccol (Eds.), *Current developments in biotechnology and bioengineering*. Elsevier.

Chandrasekaran, M. (2015). *Enzymes in food and beverage processing*.

Chang, M., Chu, X., Lv, J., Li, Q., Tian, J., & Wu, N. (2016). Improving the thermostability of acidic pullulanase from Bacillus naganoensis by rational design. *PLoS One, 11*, e0165006.

Chang, S. W., Huang, M., Hsieh, Y. H., Luo, Y. T., Wu, T. T., Tsai, C. W., Chen, C. S., & Shaw, J. F. (2014). Simultaneous production of fatty acid methyl esters and diglycerides by four recombinant Candida rugosa lipase's isozymes. *Food Chemistry, 155*, 140–145.

Damak, S., Su, H. Y., Jay, N. P., & Bullock, D. W. (1996). Improved wool production in transgenic sheep expressing insulin-like growth factor 1. *Bio-Technology, 14*, 185–188.

De Santis, B., Stockhofe, N., Wal, J. M., Weesendorp, E., Lalles, J. P., Van Dijk, D. J., Kok, E., De Giacomo, M., Einspanier, R., Onori, R., Brera, C., Bikker, P., Van Der Meulen, J., & Kleter, G. (2018). Case studies on genetically modified organisms (GMOs): Potential risk scenarios and associated health indicators. *Food and Chemical Toxicology, 117*, 36–65.

De Souza, P. M., & Magalhaes, P. D. E. (2010). Application of microbial alpha-amylase in industry—A review. *Brazilian Journal of Microbiology, 41*, 850–861.

Deckers, M., Deforce, D., Fraiture, M. A., & Roosens, N. H. C. (2020). Genetically modified micro-organisms for industrial food enzyme production: An overview. *Food, 9*, 326.

Dediu, D. (2015). *An introduction to genetics for language scientists: Current concepts, methods and findings*.

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-free dairy products: Market developments, production, nutrition and health benefits. *Nutrients, 11*, 551.

Demain, A. L., & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances, 27*, 297–306.

Devlin, R. H., Sakhrani, D., Tymchuk, W. E., Rise, M. L., & Goh, B. (2009). Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (Oncorhynchus kisutch). *Proceedings of the National Academy of Sciences of the United States of America, 106*, 3047–3052.

Domingues, L., Guimaraes, P. M., & Oliveira, C. (2010). Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. *Bioeng Bugs, 1*, 164–171.

Ermis, E. (2017). Halal status of enzymes used in food industry. *Trends in Food Science & Technology, 64*, 69–73.

Ersoz, F., & Inan, M. (2019). Large-scale production of yak (Bos grunniens) chymosin A in Pichia pastoris. *Protein Expression and Purification, 154*, 126–133.

Faryar, R., Linares-Pasten, J. A., Immerzeel, P., Mamo, G., Andersson, M., Stalbrand, H., Mattiasson, B., & Karlssona, E. N. (2015). Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant xylanase. *Food and Bioproducts Processing, 93*, 1–10.

Fraley, R. T. (1983). Liposome-mediated delivery of tobacco mosaic virus RNA into petunia protoplast: Improved conditions for liposome-protoplast incubations. *Plant Molecular Biology, 2*, 5–14.

Goncalves, D., Silva, A. G., & Guidini, C. Z. (2019). Lipases: Sources, immobilization methods, and industrial applications. *Applied Microbiology and Biotechnology, 103*, 7399–7423.

Hanlon, P., & Sewalt, V. (2020). GEMs: Genetically engineered microorganisms and the regulatory oversight of their uses in modern food production. *Critical Reviews in Food Science and Nutrition.*

Headon, D. R., & Walsh, G. (1994). The industrial-production of enzymes. *Biotechnology Advances, 12*, 635–646.

Herrera-Estrella, L., Block, M. D., Messens, E., Hernalsteens, J. P., Montagu, M. V., & Schell, J. (1983). Chimeric genes as dominant selectable markers in plant cells. *The EMBO Journal, 2*, 987–995.

Hjort, C. (2007). *Industrial enzyme production for food applications. Novel enzyme technology for food applications*. Woodhead Publishing.

Hua, W., Sheikha, A. F. E., & Xu, J. (2018). Molecular techniques for making recombinant enzymes used in food processing. In A. F. E. Sheikha, R. Levin, & J. Xu (Eds.), *Molecular techniques in food biology: Safety, biotechnology, authenticity and traceability*. John Wiley & Sons.

Izawa, N., Tokuyasu, K., & Hayashi, K. (1997). Debittering of protein hydrolysates using Aeromonas caviae aminopeptidase. *Journal of Agricultural and Food Chemistry, 45*, 543–545.

James, C. (2006). *Global status of commercialized biotech/GM crops, 2006*. Ithaca N.Y. New Delhi, International Service for the Acquisition of Agri-Biotech Applications; Publication orders, ISAAA South Asia Office.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern food microbiology*. Springer.

Ke, Y., Huang, W. Q., Li, J. Z., Xie, M. Q., & Luo, X. C. (2012). Enzymatic characteristics of a recombinant neutral protease I (rNpI) from Aspergillus oryzae expressed in Pichia pastoris. *Journal of Agricultural and Food Chemistry, 60*, 12164–12169.

Kotwal, S. M., & Shankar, V. (2009). Immobilized invertase. *Biotechnology Advances, 27*, 311–322.

Lai, L. X., Kang, J. X., Li, R. F., Wang, J. D., Witt, W. T., Yong, H. Y., Hao, Y. H., Wax, D. M., Murphy, C. N., Rieke, A., Samuel, M., Linville, M. L., Korte, S. W., Evans, R. W., Starzl, T. E., Prather, R. S., & Dai, Y. F. (2006). Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nature Biotechnology, 24*, 435–436.

Lee, J. M., Moon, S. Y., Kim, Y. R., Kim, K. W., Lee, S. J., & Kong, I. S. (2017). Improvement of thermostability and halostability of beta-1,3-1,4-glucanase by substituting hydrophobic residue for Lys(48). *International Journal of Biological Macromolecules, 94*, 594–602.

Leys, S., Pauly, A., Delcour, J. A., & Courtin, C. M. (2016). Modification of the secondary binding site of xylanases illustrates the impact of substrate selectivity on bread making. *Journal of Agricultural and Food Chemistry, 64*, 5400–5409.

Li, S., Yang, Q., Tang, B., & Chen, A. (2018). Improvement of enzymatic properties of Rhizopus oryzae alpha-amylase by site-saturation mutagenesis of histidine 286. *Enzyme and Microbial Technology, 117*, 96–102.

Li, S., Yang, X., Yang, S., Zhu, M., & Wang, X. (2012). Technology prospecting on enzymes: Application, marketing and engineering. *Computational and Structural Biotechnology Journal, 2*, e201209017.

Lievens, A., Petrillo, M., Querci, M., & Patak, A. (2015). Genetically modified animals: Options and issues for traceability and enforcement. *Trends in Food Science & Technology, 44*, 159–176.

Liu, Y. H., Zhang, T., Zhang, Z. M., Sun, T. Y., Wang, J. L., & Lu, F. P. (2014). Improvement of cold adaptation of Bacillus alcalophilus alkaline protease by directed evolution. *Journal of Molecular Catalysis B-Enzymatic, 106*, 117–123.

Lyall, J., Irvine, R. M., Sherman, A., Mckinley, T. J., Nunez, A., Purdie, A., Outtrim, L., Brown, I. H., Rolleston-Smith, G., Sang, H., & Tiley, L. (2011). Suppression of avian influenza transmission in genetically modified chickens. *Science, 331*, 223–226.

Maghari, B. M., & Ardekani, A. M. (2011). Genetically modified foods and social concerns. *Avicenna Journal of Medical Biotechnology, 3*, 109–117.

Mazorra-Manzano, M. A., Ramirez-Suarez, J. C., & Yada, R. Y. (2018). Plant proteases for bioactive peptides release: A review. *Critical Reviews in Food Science and Nutrition, 58*, 2147–2163.

Meyer, V. (2008). Genetic engineering of filamentous fungi—Progress, obstacles and future trends. *Biotechnology Advances, 26*, 177–185.

Molinaro, G., Carmona, A. K., Juliano, M. A., Juliano, L., Malitskaya, E., Yessine, M. A., Chagnon, M., Lepage, Y., Simmons, W. H., Boileau, G., & Adam, A. (2005). Human recombinant membrane-bound aminopeptidase P: Production of a soluble form and characterization using novel, internally quenched fluorescent substrates. *Biochemical Journal, 385*, 389–397.

Murthy, P. S., & Kusumoto, K.-I. (2015). Acid protease production by Aspergillus oryzae on potato pulp powder with emphasis on glycine releasing activity: A benefit to the food industry. *Food and Bioproducts Processing, 96*, 180–188.

Nandan, A., & Nampoothiri, K. M. (2020). Therapeutic and biotechnological applications of substrate specific microbial aminopeptidases. *Applied Microbiology and Biotechnology, 104*, 5243–5257.

O'connell, S., & Walsh, G. (2006). Physicochemical characteristics of commercial lactases relevant to their application in the alleviation of lactose intolerance. *Applied Biochemistry and Biotechnology, 134*, 179–191.

Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., & Dinovi, M. J. (2006). Food-processing enzymes from recombinant microorganisms—A review. *Regulatory Toxicology and Pharmacology, 45*, 144–158.

Packer, M. S., & Liu, D. R. (2015). Methods for the directed evolution of proteins. *Nature Reviews. Genetics, 16*, 379–394.

Patel, A. K., Ania, R. R. S., & Pandey, A. (2017). Production, purification, and application of microbial enzymes. In G. Brahmachari (Ed.), *Biotechnology of microbial enzymes*. Academic Press.

Petersen, S. B. (2005). Protein engineering: Design and engineering on the nano scale. In A. J. J. Straathof & P. Adlercreutz (Eds.), *Applied biocatalysis*. Harwood Academic Publications.

Poulsen, P. B., & Bucholz, K. (2003). History of enzymology with emphasis on food production. In J. R. Whitaker, A. Voragen, & D. W. S. Wong (Eds.), *Handbook of food enzymology*. Marcel Dekker.

Puetz, J., & Wurm, F. M. (2019). Recombinant proteins for industrial versus pharmaceutical purposes: A review of process and pricing. *PRO, 7*, 476.

Raksakulthai, R., & Haard, N. F. (2003). Exopeptidases and their application to reduce bitterness in food: A review. *Critical Reviews in Food Science and Nutrition, 43*, 401–445.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of microbial enzymes in food industry. *Food Technology and Biotechnology, 56*, 16–30.

Richt, J. A., Kasinathan, P., Hamir, A. N., Castilla, J., Sathiyaseelan, T., Vargas, F., Sathiyaseelan, J., Wu, H., Matsushita, H., Koster, J., Kato, S., Ishida, I., Soto, C., Robl, J. M., & Kuroiwa, Y. (2007). Production of cattle lacking prion protein. *Nature Biotechnology, 25*, 132–138.

Rieder, L., Teuschler, N., Ebner, K., & Glieder, A. (2019). Eukaryotic expression systems for industrial enzymes. In A. Vogel & O. May (Eds.), *Industrial enzyme applications*. Wiley.

Robinson, P. K. (2015). Enzymes: Principles and biotechnological applications. *Essays in Biochemistry, 59*, 1–41.
Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in Escherichia coli: Advances and challenges. *Frontiers in Microbiology, 5*, 172.
Sainz-Polo, M. A., Ramirez-Escudero, M., Lafraya, A., Gonzalez, B., Marin-Navarro, J., Polaina, J., & Sanz-Aparicio, J. (2013). Three-dimensional structure of saccharomyces invertase role of a non-catalytic domain in oligomerization and substrate specificity. *Journal of Biological Chemistry, 288*, 9755–9766.
Santos, C. A., Morais, M. A. B., Terrett, O. M., Lyczakowski, J. J., Zanphorlin, L. M., Ferreira, J. A., Tonoli, C. C. C., Murakami, M. T., Dupree, P., & Souza, A. P. (2019). An engineered GH1 ss-glucosidase displays enhanced glucose tolerance and increased sugar release from lignocellulosic materials. *Scientific Reports, 9*, 4903.
Saqib, S., Akram, A., Halim, S. A., & Tassaduq, R. (2017). Sources of beta-galactosidase and its applications in food industry. *3 Biotech, 7*, 79.
Schwechheimer, S. K., Park, E. Y., Revuelta, J. L., Becker, J., & Wittmann, C. (2016). Biotechnology of riboflavin. *Applied Microbiology and Biotechnology, 100*, 2107–2119.
Semenova, M. V., Sinitsyna, O. A., Morozova, V. V., Fedorova, E. A., Gusakov, A. V., Okunev, O. N., Sokolova, L. M., Koshelev, A. V., Bubnova, T. V., Vinetskii, Y. P., & Sinitsyn, A. P. (2006). Use of a preparation from fungal pectin lyase in the food industry. *Applied Biochemistry and Microbiology, 42*, 598–602.
Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: Industrial progress in 21st century. *3 Biotech, 6*, 174.
Srivastava, N. (2019). Production of food-processing enzymes from recombinant microorganisms. In M. Kuddus (Ed.), *Enzymes in food biotechnology*. Academic Press.
Tan, T. W., Zhang, M., Wang, B. W., Ying, C. H., & Deng, L. (2003). Screening of high lipase producing Candida sp and production of lipase by fermentation. *Process Biochemistry, 39*, 459–465.
The European Parliament and the Council of the European Union. (2008) Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. *Official Journal of the European Union, 354*, 7–50.
Tong, J., Wei, H. X., Liu, X. F., Hu, W. P., Bi, M. J., Wang, Y. Y., Li, Q. Y., & Li, N. (2011). Production of recombinant human lysozyme in the milk of transgenic pigs. *Transgenic Research, 20*, 417–419.
Van Der Maarel, M. J. E. C., Van Der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. *Journal of Biotechnology, 94*, 137–155.
Veana, F., Flores-Gallegos, A. C., Gonzalez-Montemayor, A. M., Michel-Michel, M., Lopez-Lopez, L., Aguilar-Zarate, P., Ascacio-Valdés, J. A., & Rodríguez-Herrera, R. (2018). Invertase: An enzyme with importance in confectionery food industry. In M. Kuddus (Ed.), *Enzymes in food technology*. Springer.
Verma, D. K., Kimmy, G., Singhal, P., Vij, R., Mahato, D. K., Patel, A., & Srivastav, P. P. (2018). Genetically modified organisms (Gmos) produced enzymes: Multifarious applications in food manufacturing industries. In D. K. Verma, A. R. Patel, & P. P. Srivastav (Eds.), *Bioprocessing technology in food and health: Potential applications and emerging scope* (1st ed.). Apple Academic Press.
Vieille, C., & Zeikus, G. J. (2001). Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews, 65*, 1-+.
Wei, Z. Y., Zhang, Y. Y., Wang, Y. P., Fan, M. X., Zhong, X. F., Xu, N., Lin, F., & Xing, S. C. (2016). Production of bioactive recombinant bovine chymosin in tobacco plants. *International Journal of Molecular Sciences, 17*, 624.

Wheeler, M. B., Bleck, G. T., & Donovan, S. M. (2001). Transgenic alteration of sow milk to improve piglet growth and health. *Reproduction (Cambridge, England) Supplement, 58*, 313–324.

Wheelwright, S. M. (1989). The design of downstream processes for large-scale protein-purification. *Journal of Biotechnology, 11*, 89–102.

Wu, X., Ouyang, H. S., Duan, B. A., Pang, D. X., Zhang, L., Yuan, T., Xue, L., Ni, D. B., Cheng, L., Dong, S. H., Wei, Z. Y., Li, L., Yu, M., Sun, Q. Y., Chen, D. Y., Lai, L. X., Dai, Y. F., & Li, G. P. (2012). Production of cloned transgenic cow expressing omega-3 fatty acids. *Transgenic Research, 21*, 537–543.

Yu, X. W., Yang, M., Jiang, C. H., Zhang, X. F., & Xu, Y. (2017). N-glycosylation engineering to improve the constitutive expression of rhizopus oryzae lipase in Komagataella phaffii. *Journal of Agricultural and Food Chemistry, 65*, 6009–6015.

Zhang, Y. H. P., Sun, J. B., & Ma, Y. H. (2017). Biomanufacturing: History and perspective. *Journal of Industrial Microbiology & Biotechnology, 44*, 773–784.

Zhao, H. Y., & Feng, H. (2018). Engineering Bacillus pumilus alkaline serine protease to increase its low-temperature proteolytic activity by directed evolution. *BMC Biotechnology, 18*, 34.

14 Current Applications of Enzymes in GM (Genetically Modified) Food Development and Food Chain

Nafiseh Sadat Naghavi, Fatemeh Mahmoodsaleh, and Masoumeh Moslemi

Abstract

New applications are introduced for food enzymes with regard to their impact on the improvement of organoleptic attributes and quality of foods other than their cost-effectiveness compared to chemical alternatives. Along with technological advances, novel and specified enzymes have been widely used in food industries. The enzymes may be extracted from plants and animals or be produced through biotechnological processes. In this regard, microbial fermentation is preferred compared to the other sources to some extent because it is a convenient process, needs lower cost, and is conducted consistently in the laboratory. Moreover, the fermentation process can be optimized by the use of genetically modified (GM) microorganisms or the addition of recombinant enzymes through which the production yield will be increased significantly. This chapter overviews the use of GM microorganisms in order to produce food enzymes. Advantages of the biological systems including recombinant DNA and metabolic engineering for the production of food enzymes are also discussed. Due to the importance of changes that occurred in the structure of GM enzymes, their halal status is further studied. In addition, the main regulations set by countries and the safety evaluations by risk assessment agencies on GM enzymes are addressed.

N. S. Naghavi
Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

F. Mahmoodsaleh
Department of Biology, Faculty of Sciences, Shahid Chamran University, Ahvaz, Iran

M. Moslemi (✉)
Halal Research Center of IRI., Ministry of Health and Medical Education, Tehran, Iran

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_14

Keywords

GM enzymes · Halal status · Recent developments · Regulation · Safety concern

14.1 Introduction

It was predicted that the market volume of industrial enzymes will have a significant growth in the early future years (Robinson, 2015). The utilization of enzymes especially for the production of food and beverages represents a wide industrial enzyme market. For industrial use, it is possible to extract enzymes from plant and animal sources or obtained by fermentation using Wild-Type (WT) or Genetically Modified (GM) strains of bacteria or fungi for which both have been manipulated to enhance protein production and limit the production of unfavorable secondary metabolites. In addition to genetic modifications of the desired strain, it is also possible to modify the enzyme itself for the purpose of obtaining an improved yield and improve the characteristics of enzymes (Singh et al., 2016). On the other hand, by genetic modification of producing microorganisms, the enzyme can be modified to have better performance and improved properties.

14.2 Food Enzymes Production by Genetically Modified Microorganisms

Foods that contain, or manufactured from genetically modified organisms (GMOs) are defined as GM foods (Robinson, 2015). The utilization of GM for the industrial production of food enzymes (FE) is beneficial because it allows suitable production sources to be combined with the production of the desired enzymes. For example, in the case of the native enzyme producer strains that are not comfortable for industrial applications, such as the enzymes producing pathogenic strains, the coding genes can be extracted and introduced into other organisms that are more appropriate according to food safety for the production of enzymes. Genetic modifications are also used to inhibition the production of unwanted secondary metabolites such as toxins, by deletion of their native genes to prevent their expression. To build an effective recombinant strain, various points must be taken into account. First, it is crucial to select a host organism which has the proper properties for the expression and production of the desired enzyme and has a relatively inexpensive culture. Then, vital parameters are used to catch the optimal GM for industrial use including the expression vector, the methods employed for transformation, and the selection markers to choose the transformed strains must be specified (Liu & Kokare, 2017; Deckers et al., 2020).

14.2.1 Selection of Host Organism

The reasonable host strain is selected on a case-by-case analysis. The most used host are discussed below.

14.2.1.1 Bacteria

A simple and inexpensive host organisms are bacteria, with many expression systems suitable for recombination, leading to an easy combination of modifications. However, because of protein folding issues, only simple proteins are made in bacteria. In addition, bacteria are unable to make posttranslational modifications (Deckers et al., 2020).

Commonly used bacterial host strains are including *Escherichia coli*, *Bacillus subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*. For *E. coli*, many molecular toolboxes are identified to ease the construction of expression systems available for high-yield enzyme production. However, proteins are produced in the cytosol, leading to the requirement for extra purification steps. Nevertheless, various species of Bacillus can secrete higher concentrations of the enzymes such as amylase and proteases into the medium (Zhang et al., 2019; Trono, 2019).

14.2.1.2 Fungi

Fungal host strains contain a subcellular organization, which lets proteins be folded properly and perform posttranslational modifications. Moreover, fungi secrete few amounts of their own secondary metabolites into the medium, easing the subsequent purificationsteps. Any other way, filamentous fungi generally produce low numbers of non-fungal recombinant proteins (Deckers et al., 2020).

Filamentous fungi that commonly used for the production of recombinant proteins are *Aspergillus niger*, *A. oryzae*, and *Trichoderma reesei*. *A. oryzae*, for instance, has been utilized for decades to produce fermented foods, but the production of the mycotoxin, aflatoxin is also known. To solve this problem, the mutant strain BECh2 was built, in which the cluster of the aflatoxin gene and the cyclopiazonic acid coding gene was removed and the production of Kojic acid was declined (Fernandes, 2010; Deckers et al., 2020).

14.2.2 Construction of Expression Vectors and Transformation Processes

Expression vectors are used for introducing modifications indifferent host strains. The expression vector usually includes at least One Origin of Replication (ORI), a Multiple Cloning Site (MCS), where the gene is integrated, and a marker for selecting the modified strain (Fig. 14.1). Besides, the promoter and terminator regions are available to control the expression of the desired gene and selector marker. To prevent an increase in metabolic expressions in the host organism and a decrease in plasmid stability, small size is considered for the vector (Patel et al., 2017).

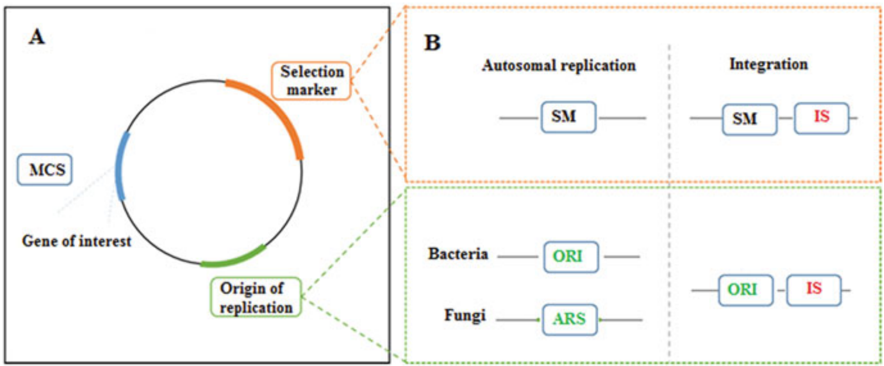


Fig. 14.1 Schematic representation of the expression vector. (**a**) Expression vectors include the origin of replication (ORI), the multiple cloning site (MCS) in which the desired gene is integrated, and a Selection Marker (SM). (**b**) An episomal plasmid and an integrated plasmid, containing a flanked integration site can be formed. ARS is an independent replication sequence and IS represents the integration site (Patel et al., 2017)

Bacterial expression vectors can either be used in the form of integration into the host genome (integrative plasmid) or they can be extra-chromosomal, utilizing independent replicating plasmids (episomal vector). Yeast and fungal expression vectors are usually integrative. In addition, shuttle vectors are commonly used for eukaryotes. These vectors can inter both prokaryotes and eukaryotes, and contain selective markers that work in both MB groups. To insert the host genome, the integrative plasmid consists of two Integration Sites (IS) that flanks via the Multiple Cloning Site (MCS), allowing the cleavage of the vector and then consolidation by recombination in the host genome containing homologous ordering to the IS. The integration usually takes place at a unique locus. In such a case, an extension homologous to a specific desired site needed to be added to the expression cassette (Patel et al., 2017).

Episomal plasmids contain an origin of replication (ORI) that is homologous via the ORI of the host type. Autonomous replication in eukaryotes utilizes an Autonomous Replication Sequence (ARS), which is the same as the ORI found in prokaryotes. Different methods of transformation are employed to introduce the vector DNA into the host organism. For bacterial host strains, this method contains conjugation that needs cell-to-cell contact, electroporation that is done by using competent cells, or vector incubation by constructing protoplasts. Protoplast fusion is mainly utilized for gene transfer into eukaryotes (Deckers et al., 2020).

14.2.3 Induction of Gene Expression

In order to the purpose of catch a high enzyme yield, an effective expression of the interested gene is required. One of the most common strategies used to upgrade the expression level is done by mix integrations (Patel et al., 2017). For this purpose,

various procedures are used, such as the use of high quantities of DNA in the transformation process; high antibiotic concentration pressure that putting to use; or simultaneous or sequential integration of multiple expression vectors that involve different markers for the selection of transforms or via applying copies of two preferred genes in a bidirectional vector (Deckers et al., 2020).

Other important factor affecting gene expression is the promoter strength. The expression is usually conducted by a group of inducers and suppressors that distinguish constitutive promoters from tunable promoters. Constitutive promoters are independent of the transcription factors that brought about environmental agents and regulate the transcription from basal genes. Tunable promoters, as opposed to constitutive promoters, are induced or suppressed by the biotic or abiotic factors in the environment (Fitz et al., 2018). For example, amyL and amyMare, respectively used as promoters for *B. stearothermophilus* maltogenic amylase and *B. licheniformis* α-amylase genes (Deckers et al., 2020), and TAKA amylase promoter is usually utilized in *A. oryzae* (Vieille & Zeikus, 2001). The last tunable promoter is activated via the transcription induceramyR (amylolytic gene expression) and is suppressed via the regulator creA (carbon catabolite suppressor) (Nedovic et al., 2011). Another promoter that is commonly used by *T. reesei* or some other fungi, is the promoter of cellobiohydrolase I gene (cbh1), and activated by cellulose (Olempska-Beer et al., 2006; Trono, 2019).

A simple solve for tandem repetition of one-directional expression cassettes is to apply bidirectional promoters (BDPs). These primers lead to the bidirectional gene transcription from one or more than one gene (Hui et al., 2006). Combination expression of the desired gene and the selective marker is also available (Rieder et al., 2019) while in order to use industrially, larger groups of accessed promoters are required. These promoters control variable expression rates as well as combining various regulatory profiles that are required in each expression direction (Yan & Wu, 2017).

14.2.4 New Technologies to Introduce Genetic Modifications

Recently, new technologies become involved in genetic engineering. The example of these technologies is "Clustered Regularly Interspaced Short Palindromic Repeat" and "CRISPR-associated protein 9" (CRISPR/Cas9), that derived from archaeal and bacterial cells (Meyer, 2008). The Cas9 endonuclease makes Double-Stranded Breaks (DSBs) in the desired DNA. By the application of endogenous repair routes, the genomic DNA can either be conveyed via offering transformation or by the insertion of a particular donor sequence. This is done through the Non-Homologous End-Joining (NHEJ) DNA repair processes or by the Homologous Repair (HR) technique (Song et al., 2019; Deckers et al., 2020). Opposed to eukaryotes, the NHEJ DNA repair system is not present in bacterial cells. A, these bacteria are not able to remedy the CRISPR/Cas9-mediated leakages, leading to the demise of wild-type strains. Therefore, the CRISPR/Cas9 system can request an election of recombinant types as a substitute for antimicrobial coding marker genes (Deckers

et al., 2020). Using this method, the production of extracellular pullulanase has been induced in a strain of *B. subtilis*. Because proteases can debase other enzymes, like pullulanase, the genes involved in protease generation have been removed (Dijck et al., 2003). The genes responsible for the production of unfavorable products during the fermentation such as foam, or involved in spore formation may also be disrupted to construct a more suitable strain for food enzyme production (Clyne & Kelly, 1997). CRISPR/Cas can also be requested for the transformation of eukaryotes. For instance, a strain of *Penicillium subrubescens* has been constructed in which the ku70 gene was deleted. This gene has participated in the non-homologous end-joining (NHEJ) DNA rebuild system. With this deletion, the homologous repair (HR) system is preferred, and for this reason, the subjunction of a wanted DNA sequence into the leakage site is performed (Zou et al., 2012).

14.3 Recombinant Food Enzymes

The origin of enzymes usage in industries of food processing dates back to 1874, when Christian Hansen obtained rennin (chymosin) from calves' stomach and used it for cheese production (Petersen et al., 2006). Chymosin is now made up of microorganisms that involve the bovine prochymosin gene, which is imported by recombinant deoxyribonucleic acid (rDNA) technology. The bovine chymosin, expressed in *E. coli* K-12, is the first recombinant enzyme approved by the Food and Drug Administration (FDA) for use in the food industry (Flamm, 1991).

Different enzymes that are utilized in food staffs are developed in recombinant microorganisms. Enzyme producers use new genetic technologies to obtain enzymes through desired properties. These kinds of enzymes most of the time are produced by microorganisms that are fastidious in growing on laboratory or industrial scales. By selecting suspicious host microorganisms, it is possible to produce recombinant strains and produce enzymes that are significantly free of unwanted enzymes or other microbial metabolites. Advanced enhancement of food processing resulted in a request for a wide range of food processing enzymes via properties reasonable for food processing conditions (Beilen & Li, 2002). For instance, usually applied sweeteners like glucose or fructose syrups are usually made from corn starch utilizing hydrolyzing enzymes. In the first stage of starch hydrolysis, the starch is recognized by using α-amylase for 2–5 min via heating at 105 °C and next put at 90–100 °C for around 1–2 h. Advances in rDNA technology have made it catchable to engineer α-amylases by improving the stability of heat and high compatibility with other parameters of the liquefaction stage. These improvements have been made by understanding the α-amylase amino acid sequences via the DNA sequence of the α-amylase genes. Another enzyme recently used in food processing has also been cached using rDNA technology. These enzymes are well-known commercially as enzyme ministration. An enzyme preparation usually contains the interested enzyme and varied additional substances, such as preservatives, diluents, and stabilizers. These extra substances are usually well-known ingredients that are accepted for utilization in food staffs (Olempska-Beer et al., 2006).

There are simultaneous techniques that are possible to applied to reproduce recombinant enzymes, such as rational design, directed evolution, and semi-rational design, based on the amount of information about the conformational structure and task of the enzyme, the enzyme sequences, and the alterations to be made to catch the expected result (Rajamanickam et al., 2017; Yang et al., 2013; Vogl et al., 2018).

The basis of rational design is the replacement, insertion, or removing of a unique DNA coding the fragment for an amino acid in relation to a specific activity that needs to be developed. The knowledge about the structural conformation of the protein and its chemical interactions is vital. Most of the time, site-directed mutagenesis is beneficial for changes of a specific amino acid by another to gain the requested activity (Rajamanickam et al., 2017; Vogl et al., 2018; Song et al., 2019). For instance, a thermostable pullulanase from *B. naganoensis* was made from a rational design. This enzyme is applied to hydrolyze amylopectin and make amylose in starch (Donohoue et al., 2018).

Directed evolution is consisting of two steps. First, is genetic diversity which is produced by random mutagenesis, and is an imitation of evolution. This can be done by error-prone PCR, mutagenesis via chemicals, or UV irradiation. In the second step, a thorough screening and detection process, which is usually performed by agar-plate method, to select the modified enzymes that have the desired characteristics. For this case, it is not necessary to have knowledge about the enzyme's structure and activities (Rajamanickam et al., 2017; Vogl et al., 2018).

The semi-rational design combines both the above techniques. In order to this reason, information is catchable for the enzyme's structure and tasks. Plus, there is no understanding about the frequency to be made to gain the desired results. Therefore, in the first step, amino acids undergo mutations by a specific fragment (in relation to the desired properties). Then a screening and evaluation step is completed, like the evaluation of directing (Song et al., 2019; Rajamanickam et al., 2017).

14.4 Application of Metabolic Engineering and Synthetic Biology in Food Enzyme Production

Metabolic engineering is used to optimize cellular procedures, native to a particular organism, to make a combination of interest from a desirably inexpensive and simple substrate. Utilizes different collections of data, a set of compositions and conditions are applied to maximize the generalization of the desired compound and to prevent chemicals and conditions that inhibit the update rate and other specific critical activities available to reach these goals; metabolic fluxes modifications show the main option. In summary, metabolic engineering is known as the directorial advancement of cellular properties by modifying specific biochemical reactions or the introduction new reactions using recombinant DNA technology. Some common strategies used in metabolic engineering are overexpression of enzyme-encoding genes that limit the rate of the biosynthetic pathway, block rival metabolic pathways,

heterologous gene expression, and enzyme engineering (Stephanopoulos, 2012; Kim et al., 2020).

Synthetic biology point to design genetic composition libraries including promoters, codons, terminal sequences, transcriptional factors, assembling devices, genetic circuits, and even a perforated organism; in addition to obtain quantitative information to create models that can demonstrate the behavior of biological systems (Cameron et al., 2014).

While synthetic biology provides champers and information on various biological processes, metabolic engineering tries to utilize all of this information to become optimal for the biological production of the desired compound. It is possible to note that some instances of synthetic biology can be arranged as examples of genetic engineering. However, these two areas are depending on the development of real methods, and tools for DNA transformation. These fields of research seek to achieve basic needs such as arbitrary changes in DNA sequences, production of specific mutations, the recombination between a variety of genetic circuits or biosynthetic pathways, the deletions of genes, and placement of DNA fragments into the organism genome or in a plasmid. Although PCR and its related methods are one of the superior equipment for making some necessary changes (specifically applied for extracting parts of a particular area) they are ineffective for other purposes (Boyle & Silver, 2012). Some examples of employing metabolic engineering processes to evolve in the production of food enzymes are discussed here.

By replacement of the bacterial genes with yeast-preferred genes, Wang et al. (2011) performed mutation and synthesis of tyrosine ammonia lysine (TAL), which elevated translation and vigorously increased p-coumaric acid and resveratrol biosynthesis. They showed that a low-affinity, high-powerful bacterial araE transporter could increase resveratrol yield, without direct resveratrol transfer. Yeast cells containing the araE gene generated up to 2.44-fold more resveratrol than control cells. They offered the transformed yeast cells for industrial applications in fresh grape juice production.

Microbial Cell Factories (MCFs) have appeared as evolving technology for the combination of previous single techniques and complicated multistep procedures into a unit self-replicating microorganism (Dhamankar & Prather, 2011). The usual way to create an MCF is to create a different or artificial de novo biosynthetic pathway in a chassis organism to achieve a new composition. The goal product could be native to a microorganism that is fastidiously cultured/engineered. The product also can obtain from a complex organism such as a plant that industrial production by inexpensive, or from a manner non-native to all microorganisms as a compound from an artificial metabolism. The MCF has also developed a convenient route to generate new derivatives of the desired compound. For example, phenylpropanoids, such as resveratrol, are natural secondary metabolites produced by plants that have been shown to have therapeutic effects and are commercially valuable. Most bioavailable derivatives of resveratrol have also been obtained from an MCF. A new biosynthetic route for resveratrol constitution in *E. coli* was designed using different enzymes obtained from bacteria and plants (Choi et al., 2010), and afterward evolved by adding a glycosyltransferase (from Bacillus), which led to the generation of

resveratrol glucoside derivatives like resveratrol 3-O-glucoside and res-veratrol 4′-O-glucoside) in an *E. coli* MCF (Choi et al., 2014). It is with reckless enzymes that a new combination of enzymes can give rise to new de novo biosynthetic routes that lead to the acquisition of new chemicals by using MCFs. So long as there are different published examples containing the yield of isobutanol (Atsumi et al., 2008a, 2008b), styrene modification (Pugh et al., 2011), production of 3-hydroxybutyric acid (Tseng et al., 2009), or native silk protein (Xia et al., 2010). Most enzymes in nature are able to react on a spectrum of substrates in order to maximize evolutionary compatibility and this development may be engineered (Khersonsky & Tawfik, 2010).

Rational design is the purification of a target DNA sequence that codes a protein to desired mutations because of improve their catalytic task, consistency, or some other representative like linking domain specificity. Devises such as Gibson assembly, the BioBricks or Golden Gate can make different genetic systems to shape genetic circuits, expression cassettes, or equipment (Casini et al., 2015). Finally, the utilization of Lambda-red, CRISPR-Cas9, and other new combination systems lead to imbed varied structures into expression vectors or in vital loci of the genome of the desired organism. It is possible to use some of these techniques to introduce genes of a partial or complete metabolic route for the synthesis of a special compound, and they can also be applied to delete genes from an organism that are antagonistic to the desired synthesis. Moreover, these methods let point mutations be performed that reduce the function or expression of native proteins to alter the metabolic flux. However, in order to the generation of all of these changes, it is necessary to have specific information about the enzymes captive in the pathway that contains these actions and information about the organism in which the modifications happen. (Esvelt & Wang, 2013; Liu et al., 2015). Thus, the genome sequence of organisms, the characteristics of proteins, and the metabolic studies make very beneficial equipment and information (Fig. 14.2).

Additional techniques and procedures have been crucial to reach other goals in the progression of both areas. Some instances include BioBricks, Gibson Assembly, Gap-repair, Lambda-red, MAGE, Recombinase techniques (integrases), and CRISPR-Cas9 (Stephanopoulos, 2012). Simultaneous sequencing of defined and non-defined organisms (metagenomics), allows for gaining more completed information. For example, the list of enzymes and processes has been developed, and some biological phenomena (e.g., infections by viruses or phages) have been reached (Goodwin et al., 2017).

14.5 Halal Status of Recombinant Enzymes

Halal term is a familiar concept in Muslim countries which have been interested in non-Muslim populations in recent decades. Today, it is globally accepted that a halal certificate refers to both religiously permitted and safe/qualified commodities. Therefore, the halal logo facilitates international trade (Ab Talib, 2017). However, it makes some difficulties for non-Muslim countries' exporters such as European

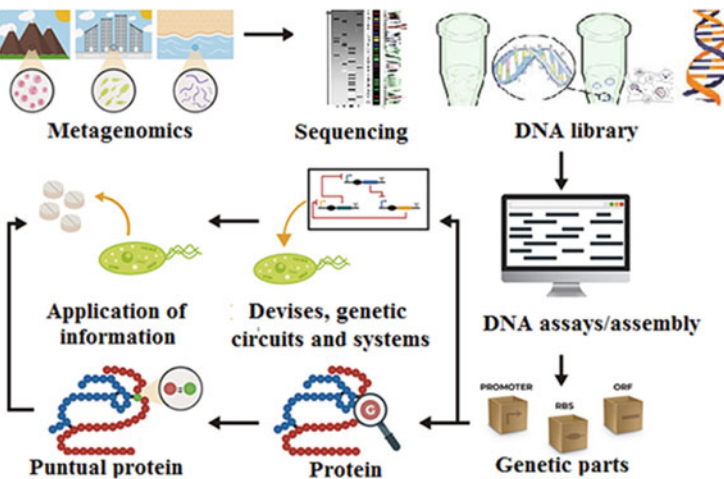


Fig. 14.2 Various steps for the progression of synthetic biology and metabolic engineering procedures

because of their lower knowledge about halal (Tieman, 2017) As a global rule, foods and their ingredients should be free of compounds that originated/are made from haram sources or exclude alcohol. On the other hand, the animals should be slaughtered in a way that is accepted by Islam (named as Dhabihah or Zabiha) (Alzeer & Abou Hadeed, 2020).

Halal commitment is a critical issue in the whole food chain at the international level. In 2001 a Japanese company was condemned due to the production of non-halal monosodium glutamate as a flavor enhancer to achieve better economical productivity. The process was included using BactoSoytoneas a culture media prepared by the enzyme derived from porcine that is not acceptable in the halal concept. This violation was further conceded by the manufacturers that were given the halal certificate by Indonesian authorities for their products. The event obliged other enzyme manufacturers to be approved by the halal certification bodies in the world (Fischer, 2011). There are two types of certificating for halal foods including 1) site registration to evaluate the ability of suppliers in fulfilling the requirements and 2) certifying the imported products (Hanzaee & Ramezani, 2011). Unfortunately, the halal certifiers grow slowly compared to other scientific associations in the world due to the disagreements on the halal concept (Halim & Salleh, 2012). On the one hand, the term Istahala has different understanding and interpretations among communities. There is not a consensus on the final products produced from the non-halal origin when they underwent chemical transformation up to microstructures or molecular level. On the other hand, there is a doubt about the synthetic genes designed in the laboratory which are modeled from haram sources such as pork (Riaz & Chaudry, 2019).

Enzymes are food components that are traded worldwide and their halal assurance is a concern among Muslims. Food enzymes are produced from different

sources of plants, animals, and microbes. Several points are considered in their eligibilities by Islamic traders. The enzymes should be extracted from halal sources and produced in media containing halal raw materials. Furthermore, fermentation media should contain the approved organisms other than halal ingredients if a microbial source is used for enzyme production. It will be important when genetically modified (GM) microorganisms activate for enzyme synthesis. Based on the World Health Organization's definition, "genetically modified organisms can be defined as organisms (i.e., plants, animals, or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination" (WHO, 2014). The source of genes and method of preparation determines the fate of GM products. For example, accordance of GM foods with religious remarks is the most important factor for Malaysians in preparation of their food basket (Oz et al., 2017). It is also a deterministic factor in Islamic countries such as Iran through which the GM foods and components such as enzymes first checked whether they are halal followed by safety evaluation. In this way, the genome of host cells that is manipulated to encode the enzyme should be originated from a halal source (Ermis, 2017). Some steps are generally monitored in halal control points of enzymes extraction and production including the source (animal or microbial), cleanliness of the production utilities, the agents released into the environment, acceptable processing-aid, substrate or raw materials, and packing process. However, up to 0.5% of alcohol may be acceptable when an optimum activity is required (Riaz & Chaudry, 2019).

Use of animal-based enzymes is limited in some cases such as restrictions on the dietary intake of vegans other than halal uncertainties (Nugraha et al., 2015). In comparison, the enzymes extracted from conventional plants are not doubtful (Khattak et al., 2011) and there is no concern about plant-derived genes inserted in transgenic microbes (Riaz & Chaudry, 2019). However, microbial sources are used extensively for enzyme production because of their cost-effectiveness and ease of process control for optimization. Furthermore, it eliminates the concerns of animal origin and slaughtering methods to meet halal criteria in enzyme production (Riaz & Chaudry, 2019). Microbial enzymes are commonly produced by submerged fermentation (Khattak et al., 2011). Several recombinant food enzymes have been engineered to promote resistance of the wild type against harsh conditions of temperature/pH and better reactivity of the GM enzyme with substrates in the environment. *Pichia pastoris* (which was recognized as safe by US Food and Drug Administration) (Espinoza-Molina et al., 2016) and *E. coli* are two species commonly used as a host cells in this regard (Espinoza-Molina et al., 2016; Zhang et al., 2019). In addition, GM *Streptococcus thermophiles*, as a common starter culture of yogurt, capable of antioxidant enzymes' expression was evaluated by Carmen et al. in vitro and positive results were observed in the suppression of oxidative stress in the colon cancer model (del Carmen et al., 2017). The bacteria was manipulated by insertion of the gene responsible for antioxidant enzyme' production isolated from *Lactobacillus casei*BL23 as an anticancer and anti-inflammatory probiotic bacterium (del Carmen et al., 2014; Jacouton et al., 2017) that is considered as a halal source. Moreover, transfer of the encoding gene of

chymosin from camel (Aboulnaga, 2019) and bovine (Ulusu et al., 2016) to *E. coli* and from calve to *E. coli*, *Kluyveromyces marxianus* and *A. niger* (Fernandez-Salguero et al., 2003) in coagulation of milk protein are other examples. At the end of fermentation, the enzyme will be in accordance with halal merits if the aforesaid factor is met in the processing. AbdLatip and Nordin in 2018 listed endemic psychrophilic bacteria of South Pole as a halal source able to produce enzymes resistant against the cold condition. In their opinion, bioengineering of these microorganisms toward specific characteristics would be of interest in the future because their low-temperature resistance limits the side reactions and lowers the required activation energy (Abd Latip & Hadry Nordin, 2018).

Compared to foods, therapeutic agents may be exempted from halal regulations, occasionally. For example, Alzeer and Hadeed in 2020 listed the orally administered capsule of “Nutrizym 22” containing the pancreatic enzymes for pancreatitis treatment as a highly critical pharmaceutical in view of halal because of its porcine origin (Alzeer & Abou Hadeed, 2020). It is expected that there would be less restrictions on therapeutic compounds when there are no halal alternatives.

A big deal in halal assurance is that labeling regulations in some countries suffer from adequate liabilities. In this case, there is no obligation on listing the ingredients of GM foods that might be originated from non-halal sources (Alhariri, 2020). With regard to the enzymes, it is due to the fact that they may decompose during the process, and no residue is left in the final products. Moreover, some regulatory bodies believe that the labeling (if the product does not include significant changes in the composition or make an adverse impact on the consumers) may threaten the products’ sales because of consumers’ misconception about health risks arising from GM ingredients (Uzogara, 2000).

14.6 Regulation on GM Enzymes

GM foods were firstly released to the market in 1994 in the United States by introducing a GM tomato containing the gene expressing an enzyme responsible for modulation of the ripening process (Ujj, 2016). Since then, the countries have had different strategies in accepting or withdrawal of such products. In general, European countries have more restrictions on GM foods than America, and no GM products are approved and marketed without thorough risk assessment or clear labeling. Although, the products processed by GM enzymes or the animals fed by GM feeds may be considered lenient (Yuen-Ting Wong & Wai-Kit Chan, 2016). For example, the bakery products prepared by GM amylase do not require labeling in the EU (European Commission, 2003a). In 2019, a member of the European Commission explained the EU regulation on GM foods. Other than the risk assessment, market surveillance, and labeling, the regulation includes risk communication of the results of safety evaluation performed by the European Food Safety Authority (EFSA) which is responsible for risk assessment in the EU. The regulation was implemented by the authorities for the engineered targeted gene not conventional breeding with a long history of safety in humans, animals, and the environment.

However, like many other populations, people are not interested in GM foods in Europe and the most quantities of authorized GM products are used for animal feeding (Bruetschy, 2019). The list of authorized GM cops by the EU is available on the European Commission website (European Commission, 2020a) and EU regulation on genetically modified food enzymes is addressed in the directives of EC No. 1332/2008 and EC No. 1829/2003 (European Commission, 2003b, 2008). As mentioned, safety evaluations of GM food enzymes in Europe are done by EFSA and the results are freely published to be available within the EU and for other countries. The approved enzyme by EFSA can be commercialized under the rule of the two above directives. The EU directive on GM modified food and feed, which is in accordance with the Cartagena Protocol on Biosafety, clearly mentioned that the processing aids are out of the scope of the directive and considered differently (European Commission, 2003b). Therefore, if the food enzyme is used as a processing aid so that irreversibly denatures, degrades, or removed before consumption, is not covered by the directive. On the contrary, if the food enzyme is an ingredient or additive in the food, it will fall under the scope of the directive of GM food and feed that should be mandatorily listed on the labeling. Importantly, there is an exception about the enzymes are or possibly contain allergenic or intolerant compounds that should be mentioned on the labeling every time (European Commission, 2014).

The United States has the biggest share in the production of GM foods in the world (Oz et al., 2017). It was estimated that the arable land in the United States is 1.4 times more than that Europe but the GM crops are cultured approximately 600 times in the United States (Ujj, 2016).

Three governmental bodies in the US are involved in the safety evaluation of GM foods from farm to fork that in turn included the Department of Agriculture (USDA), Environmental Protection Agency (US EPA), and Food and Drug Administration (US FDA). The first one engages in the protection of GM plants in the farm from pests and diseases and also protects the environment against possible risks arisen by GM crops. The second body is responsible for the safety of both environment and humans faced with the GM traits. The last administration is responsible for the safety of GM foods and feeds taken by humans and animals (Schiemann et al., 2019). The US regulatory bodies focus on the GM characteristics rather than the method of product's synthesis. The USDA classified the GM seeds to regulated and deregulated articles which are those that require direct- and exempted from overseeing, respectively. It is based on the known safety level of the seeds. A joint collaboration of USDA and US EPA is required for the safety evaluation of GM edible crops containing pesticides. In this way, the producer should examine and present the amount of pesticides existed in the final food to US EPA. The producer is allowed by US EPA to conduct a field trial of the edible pest-resistant GM crops on a farm of 0.5 hectares or larger under US EPA consultation. The USDA studies source of the gene producing the toxin, its characteristics, the environmental impact, and its impact on nontarget organisms while the US EPA is responsible for toxicological, allergenicity, and gastrointestinal fate studies on the toxin in the GM corps (Craig et al., 2012). Other than plant-sourced biotechnological products, the safety

investigation of GM microorganisms and their byproducts such as enzymes and other chemicals is led by US EPA (Wozniak et al., 2012). After approval, the product can be commercialized preferably under the consultation of the US FDA which oversights the safety of GM foods through which the producer is the holder of safety responsibility (Craig et al., 2012). List of FDA-approved GM crops is freely available on its website for the readers (US FDA, 2019).

Less restrictions exist in the United States on foods containing GM ingredients that are overseen by the US FDA. The administration has stated that GM foods are as safe as or better than their non-bioengineered counterparts to some extent (US FDA, 2020a). The agency supposed that GM food does not impose new/different risks to the consumer compared to its counterpart. Therefore, no need for pre-market approval is required unless there would be evidence of safety concerns of the bioengineered product other than those expected in the wild type. The US FDA believes that an initial assessment of the food additives was undertaken and further evaluation on the GM counterparts will not be necessary if the new event and trait introduce no side-effect in humans (Yuen-Ting Wong & Wai-Kit Chan, 2016) It is against the public perception about this kind of foods. Scott et al. in 2016 reported that nearly 64% of Americans did not agree with GM foods and most of them would not be affected by further informing strategies conducted on risks and benefits (Scott et al., 2016).

Commercialization of GM foods in the US can be accompanied by FDA consultation but is not compulsory to the industries. The US FDA persists in pre-market examinations conducted by the producers to find out whether the GM foods containing the gene extracted from the allergenic source makes an immune response in humans. It would be well feasible for the known allergenic foods such as Brazil-nut but will be a big deal for unknown sources (Jacobson, 2000).

Nonetheless, no obligation is assigned for labeling GM foods in the United States because the US FDA believes that the bioengineered food is not majorly different from the wild type and labeling may make the additional cost to the consumer (Marchant & Cardineau, 2013). However, some states in the New England region enacted local rules on GM labeling. For example, Vermont State in 2016 and Maine in 2013 (northeast of the US) were obliged listing the GM ingredients when they are added more than 0.9% to foods or Connecticut State (northeast of the US) in 2013 mandated GM infant formula labeling (Oz et al., 2017).

China is a competitor of genetic modification in the world with a strict rule for approval by using widespread research. Initiation of GM foods' investigation in China was later than the European Union and the United States. However, the current president of China in 2013 emphasized the both issues of safety and innovation in bioengineering so that the marketed GM products should be approved by strict regulations and the country does maintain its superior position in GM commercialization (Feng & Yang, 2019). China is one of the most consumers of GM crops especially grains in the world. The Chinese government tried to speed up the commercialization of GM foods in the country but it did not succeed as expected because of consumers' concern (Yuen-Ting Wong & Wai-Kit Chan, 2016). Direct responsible for agricultural genetic modification in China is the Ministry of

Agriculture and the duties are delegated by the ministry to other bodies. They are included in Inter-Ministerial Joint Meeting for integration and coordination of GM issues, National Biosafety Committee for safety evaluation of GM products, the Standardization Committee to amend the safety standards, testing institutions responsible for characterization and safety examination of GM products, and other peer governmental departments responsible for a comprehensive inspection of final products (Jian-ping et al., 2015). There are several steps of evaluation of GM foods before their commercialization in China. The first one is biosafety assessment which covers laboratory to field investigations. The committee in charge of biosafety evaluation categorized the products into 1 to 4 risk groups based on their threat to the life cycle (Yuen-Ting Wong & Wai-Kit Chan, 2016). In this regard, no or negligible risk, low risk, moderate risk, and high risk refer to classes 1 to 4 respectively (European Commission, 2020b). Obviously, a restricted regulation is set for classes 3 and 4. China makes an obligation on GM foods' labeling through which the GM ingredients or those additives degraded under processing (such as an enzyme) should be named on the label (Yuen-Ting Wong & Wai-Kit Chan, 2016). To the contrary, Feng and Yang stated that adequate specification has not been made in Chinese law about labeling GM food (Feng & Yang, 2019). Safety regulation on GM foods in China undertakes three issues of risk assessment (back to the early twenty-first century for GM agricultural foods), labeling of GM foods to alarm the consumers about the content, and risk communication (Sun, 2019). However, the results of a survey showed that the Chinese population tended to lower its intake of GM foods in 2013 compared to 2002. It may be due to their increased knowledge about these types of foods. The authors believed that the oriented advertisement of Chinese media (that are managed by the government) against GM foods might be an important reason. However, the increased significant rate of neutral citizens on GM foods in 10 years along with strict regulations and fast-growing research on GM sources in China are effective factors on GM food development in the country (Zheng et al., 2017).

14.7 Safety Concerns of Recombinant Genes and Enzymes and the Allergenicity Assessments for Development of GM Foods

Genetic engineering is a polar issue in the world so there are several advocates and critics for and against the technology, respectively. The advocates are interested in the promising events evolved in new products by the activity of recombinant genes compared to native ones. Today, other technologies are served to increase the functionality of recombinant proteins. For example, recombinant dextransucrase and dextranase were protected by encapsulation techniques to be more efficient under intended use. The enzymes were coated with edible polymers of alginate and pectin (Sharma et al., 2019). Such polymers which are nontoxic, biodegradable, well-defined film-former, and resistant to higher temperatures and gastrointestinal conditions (Moslemi et al., 2018; Cheraghali et al., 2018; Sharma et al., 2019),

protected the core in favor of non-digestible oligosaccharides' higher production from carbohydrates under harsh condition (Sharma et al., 2019). In comparison, the critics of genetic engineering mainly worry about unknown risks that may be observed in humans, animals, and the environment in the future.

Bacteria, yeast, and fungus are utilized as donors or hosts for genetic modification. Trono in 2019 listed a large number of bioengineered food enzymes which are used in different industries of dairy, meat, cereal, beverage, sweetener, and emulsifier (Trono, 2019). Some approved genetically modified food enzymes are listed as GRAS and are available at GRAS Notice of the US FDA (US FDA, 2020b). It means that those GRAS enzymes can be used in the market without achieving any safety approval by regulatory bodies. Safety considerations of GM enzymes in the US are mainly assessed by the suppliers in collaboration with US FDA.

The new traits improve technical feasibility, such as high-or low-temperature stability, salt tolerance, specificity, and optimized activity under a developed range of pH. For example, bioengineered α-amylase produced by *B. licheniformis* showed high acid resistance by replacing histidine with aspartic and glutamic acid for starch saccharification or high affinity of thermostable lipase to short-chain fatty acids in dairy triglyceride help flavor enhancement. Mesophilic *B. pumilus* was modified to produce a thermostable protease with increased hydrolytic activity at 15 °C (Zhang et al., 2019). However, some indications of negative symptoms from genetically modified foods and enzymes arose. For example, allergenicity symptoms were observed after consumption of StarLink maize engineered by insertion of pesticide gene from *B. thuringinesis* which resulted in an unintended impact on humans. Moreover, the bioengineered soybean by using the gene extracted from Brazil-nut in favor of methionine overexpression induced an allergenicity response (Dadgarnejad et al., 2017). Another example is the observed allergenicity of cooked and uncooked transgene pea following the insertion of the kidney bean gene (Yavari et al., 2016). It might be due to the intrastructure changes in the new protein that made it resistant to high temperature and digestion. Budnik et al. performed IgE diagnostic test in the blood of 813 workers exposed to genetically modified enzymes used in the production of foods, drugs, and chemicals from 2007 to 2013. The results boded that 23% of the all had high level of IgE, most of them sensitized to α-amylase and the least frequency was reported for lipase while the highest blood IgE concentration was diagnosed in those exposed to phytase, xylanase, and glucanase (Budnik et al., 2017).

Importantly, new organisms may produce some enzymes involved in unintended biological mechanisms and produce unintended or harmful chemicals. In addition, the new gene may interfere with the gene integrity and silent or activate the gene toward unwanted metabolic pathways. They may also lead to acquired resistance of pests on the farm by time and propagation of the resistant genes to other violent species. There is a concern regarding the use of antibiotic-resistant genes as a marker in the examination of transgene products by which they remain active in the presence of antibiotics if well affected by the bioengineering process. Researchers worry about the transfer of antibiotic coding genes to pathogenic bacteria and resident flora of human and animal digestive tract (Dadgarnejad et al., 2017). In comparison,

some explanations are presented against the concern. The fans of genetic engineering believe that a chain of reactions should be occurred to transfer the target gene into an unintended organism. They are included in excision of the gene, its resistance within the digestive tract, its insertion into the second organism, its compatibility and stable joining to the host, and active expression within the unwanted cell. In addition, the specialists stated that the normal flora within the digestive tract already has the gene encoding antibiotics and no additional worries would be expected if the known genes are used for this purpose (Dadgarnejad et al., 2017).

It is believed that glycoprotein provokes the immune response directed by immunoglobulin E and glycosylation process is a key factor in vital systems toward the formation of allergenic proteins. Allergenicity of transgenic protein is evaluated by several methods. At first, bioinformatics tests that examine homology of the new protein and the known allergens are conducted (structural information of the allergens is available at databases). Then, the serological tests consisting of immunoglobulin E binding assay are done when more than 35% similarity in a sequence of 80 amino acids in the new protein with the allergens is detected by bioinformatics assay. Pepsin resistance test together with quantity evaluation which is important in risk assessment studies is another way of safety evaluation. In this way, a quantified protein is examined because less allergenic foods may be more harmful than the others due to their high frequency of consumption. Moreover, the heat stability and in vivo trials referred to as animal studies are common examinations. Although, in vivo trials may not be reliable enough because it is species-specific and could not be strictly extrapolated to humans in some cases (Yavari et al., 2016).

A guidance has been presented by EFSA about allergenicity assessment of new proteins stimulated a non-IgE mediated response like what is observed in coeliac patients. It includes finding out the source of protein and the population exposure followed by amino acid sequencing by which its similarity to the known allergens is determined. Performing in silico tests and examining the binding possibility of the protein residues to the receptors overexpressed in coeliac disease (HLA-DQ2- and HLA-DQ8) to evoke immunogenicity is a further step. Finally, in vitro digestibility test is conducted (Naegeli et al., 2017).

Food enzymes that are commonly used by the industry are mainly produced by microorganisms and their full characterization especially on the transgene organisms is done by EFSA. The first step is collecting the taxonomic information up to strain level for both bacteria and fungi. The whole-genome sequencing of chromosomes and plasmid is further required. Third, an antimicrobial resistance test is done on the producing microorganism to avoid propagation of the resistance gene among other viable organisms. If the microbe comprises antimicrobial-resistant gene, the enzyme must be free of genetic materials of the producing organism (Silano et al., 2019). EFSA developed a Qualified Presumption of Safety (QPS) list containing the microorganisms subjected to and passed the safety evaluation for food enzyme production. Filamentous fungi are excluded from the list because of the possible production of toxic secondary metabolites, occasionally (Koutsoumanis et al., 2020). The QPS document also includes a safety assessment approach for enzymes producing microorganisms for additional minor evaluation, if required. Food enzymes

produced by the microorganisms not-listed in the QPS or those genetically modified in the QPS list should be free of viable cells to make sure of safety. In addition, no genetic materials of GM microbes and non-GM microbes carrying antibiotic-resistant genes should be found in the products. Toxicity evaluation of food enzymes by EFSA is done by in vitro genotoxicity assay and micronucleus test via cell culture method and in vivo 90-days trials in rodents via the oral route. Allergenicity assay is done by in vitro gastric simulation test which is a marginal way because it is not reliable enough and should be used in combination with other methods. Food enzymes within a group are assessed separately because those with similar functionality may have different structures leading to different clinical reactions in humans (European Food Safety Authority, 2020). The applicants are required to present a full description of genetic modification including the changed gene, the new trait, and the altered mechanisms in the GM organism (Silano et al., 2019).

Comprehensive data have been released by EFSA about the enzymes produced by genetically modified microorganisms. Estimation of oral toxicity in the form of margin of exposure (MOE) by EFSA is based on the maximum recommended dosage of the enzyme in the industry and European food consumption database which may be overestimated in some cases. Acceptable MOE in Europe with limited variety in the population would be 300 and more compared to the varied geographical countries which may accept MOE 1000 and higher. Therefore, based on the reference points or reference doses, deciding about the safety of foods is hold on the risk managers of each country depending on their local dietary intake (Jamali et al., 2020; Moslemi et al., 2020). EFSA has evaluated the toxicity of GM food enzymes by the reference point of NOAEL (no observed adverse effect level). Some examples of GM food enzymes safety evaluations are as follows. They mainly limited to carbohydrase that are of the most common enzymes in the food industry.

α-amylase produced by GM *Pseudomonas fluorescens* (BASF Enzymes LLC Company) intended for glucose syrup production was free of viable cell and genetic materials. The enzyme was subjected to toxicity assessment within 90 days of trial in rats and a reference point (NOAEL) of 887 mg/kg body weight per day were detected. It was not genotoxic and no similarity to the known allergens was detected. The EFSA scientists believed that induction of adverse reaction by the enzyme might not be avoidable although it was considered rare. Notwithstanding, the GM α-amylase contained antibacterial that will pose the consumers at risk of antibacterial resistance. Therefore, the GM α-amylase was ignored for human dietary consumption (Silano et al., 2020).

α-amylase produced by GM *B. licheniformis* (Novozymes Company) intended for glucose syrup production was introduced as safe by EFSA and no genotoxicity and allergenicity were detected. Toxicological evaluation was not emphasized for the suggested industrial use as more than 99% of the enzyme will be removed at the end of syrup production (Silano et al., 2018g).

α-amylase produced by GM *T. reesei* (Danisco US Inc) for production of distilled alcohol and brewing purposes was studied and no genotoxicity was detected. The enzyme was not similar to the allergenic compounds. Nonetheless, a NOAEL of 230 mg/kg body weight per day was reported which led to MOE of 135 by

calculating the dietary exposure of 1.701 mg/kg body weight per day in Europe. The low MOE might be due to overestimation of dietary exposure as the scientists presumed its intake from all brewing products and extrapolated the data of restricted time to longtime exposure. As a conclusion, they reported that the enzyme can be used but a low concern would not be avoidable under the defined condition of oral consumption if no residue remained in the foods (Silano et al., 2019a).

α-amylase from GM *A. niger* supplied by Novozymes Company was not genotoxic and MOE for bakeries that contain the GM enzyme accounted for 370 in Europe. Two similar segments in amino acid sequences were observed in the allergenicity test which was matched to respiratory, not oral allergens and resulted in a safe status of the GM enzyme for oral intake (Silano et al., 2018).

The enzyme glucan 1,4-α-glucosidase produced by two different GM organisms of *A. niger* and *T. reesei* for conversion of starch to glucose syrup, baking, and brewing purposes was not genotoxic. NOAEL of 1244 and 1149 mg/kg body weight per day was detected for the enzyme produced by recombinant *A. niger* and *T. reesei*, respectively. But, one segment of 80 amino acid in the enzyme of both origins showed more than 35% similarity with a respiratory allergen. Although, the EFSA panel did deny the low safety concern with regard to the oral intake of the enzyme, particularly in those susceptible to the known respiratory allergen, but the enzyme was finally considered safe for oral intake. It is due to the fact that more than 99% of the enzyme is removed in glucose syrup and variable dietary exposure would be expected for dough and beer at different countries (Silano et al., 2018a, 2020a). Same results were observed for glucoamylase produced by GM *A. niger* for conversion of starch to glucose syrup and brewing purposes. A NOAEL of 1360 mg/kg body weight per day and MOE of 375 were determined for European countries. Similarity of the enzyme with the known respiratory allergen was neglected according to the fact mentioned above for glucan 1,4-α-glucosidase. Therefore, GM glucoamylase was considered as safe for dietary consumption (Silano et al., 2018b).

Three domains of GM maltogenic amylase produced by recombinant *B. subtilis* (produced by Novozymes A/S) showed more than 35% similarity with the known respiratory allergens but the GM enzyme was reported as safe by EFSA for bakery, brewing and starch processing because no evidence was found about their allergenicity under dietary exposure. No genotoxicity and acceptable MOE for Europe was also reported (Silano et al., 2018c). In agreement, the same results and conclusion on genotoxicity (negative), oral toxicity (acceptable risk), and allergenicity (three similar domains with the known allergens) were further made by EFSA for maltogenic amylase derived from GM *B. licheniformis* (Danisco US Inc) (Silano et al., 2020b).

Xylanase is a commonly used enzyme in the cereal industry for brewing and starch/gluten production. Its counterpart produced by *T. reesei* (AB Enzymes GmbH) showed no genotoxicity, high MOE in Europe (more than 43,000), and no allergenicity. GM xylanase is removed at the end of grain processing but a trace amount of the enzyme delivered from brewing products might pose the consumer at low risk. However, the EFSA considered it safe under the defined oral condition of

use (Silano et al., 2020c). The enzyme endo-1,4-β-xylanase from GM *B. subtilis* of two different strains of LMG S-27588 and LMG S-24584 (produced by Puratos N. V.) for baking purposes showed no genotoxicity, allergenicity, and high MOE (1363 and 2176 for European countries, respectively). In comparison, a recombinant DNA segment was detected in the food enzyme. Although the last finding was against EFSA requirements, the EFSA panel considered the GM enzyme safe for commercialization under the defined condition of use. It might be due to the amount of MOE far from the danger cut-off point and the no antimicrobial reaction found in the purity test of the GM enzyme (Silano et al., 2018h,d). The enzyme endo-1,4-b-xylanase produced by *T. reesei* strain DP-Nzd22 (DuPont company) was also certified as safe for commercialization by EFSA (Silano et al., 2018e). To the contrary, endo-1,4-β-xylanase from GM *A. niger* was not accepted to be released because of no adequate data achieved by the Ames test and the EFSA panel could not conclude about its genotoxicity (Silano et al., 2017).

Pullulanase produced by GM *B. subtilis* (Novozymes Company) and *B. licheniformis* (Danisco Company) for glucose syrup production from starch and brewing purposes showed no oral toxicity within the exposure range of European and no homology with the known allergens was found. Therefore, the GM enzyme has been considered safe for human consumption under the defined condition (Silano et al., 2017a, 2019b).

β-galactosidase synthesized by GM *E. coli* (supplied by Clasado Ingredients Ltd.) was withdrawn for commercialization despite passing the toxicological and allergenicity experiments due to the inclusion of genetic materials in the food enzyme and no adequate data on their viable cells' containment (Silano et al., 2020d).

Xylose isomerase produced by GM *Streptomyces rubiginosus* supplied by Danisco Company for immobilized isomerization of the sugar into fructose was studied and no genotoxicity and allergenicity were detected in vitro. The GM strain has had antibiotic- resistant gene but no viable cell and DNA fragments were detected in the GM enzyme. Provided that the enzyme will be removed after the process or not entered into the product through immobilization, little risk of dietary exposure and oral toxicity is expected. Thus, the GM enzyme has acquired EFSA acceptance for commercialization in food (Silano et al., 2020e).

Lysophospholipase expressed by GM *A. niger* (produced by Novozymes A/S) usable for starch processing and oil degumming showed no genotoxicity and NOAEL of 1356 mg/kg body weight per day was determined. Nonetheless, no similar sequence of amino acids with the allergens was detected. The scientists reported that there may be a low safety concern regarding the frequent dietary exposure to the enzyme, but the enzyme removal after processing declines any health concern in the final product (Silano et al., 2020f).

Triacylglycerol lipase produced by GM *Ogataea polymorpha* (Danisco US Inc.) showed no genotoxicity, MOE of more than 1280 for Europe, and no similar amino acid segment with the known allergens. No viable cells and DNA residue were observed in the GM enzyme and it was considered safe for use in the food industry by EFSA (Silano et al., 2020g).

The approved acetolactate decarboxylase from GM *B. licheniformis* (produced by Novozymes A/S) for brewing purposes had no genotoxicity, allergenicity, and very high MOE (more than 300,000) (Silano et al., 2018f).

14.8 Conclusion

Enzymes found in nature have been beneficial for the generation of fermented foods for thousands of years. The enzymes producing microorganisms that obtained from natural sources date back to the late-nineteenth century. Furthermore, the progress in molecular genetics and cell biology over the past four decades have altered enzyme generation processes. It is possible to choose and extract enzyme-encoding genes and show them in host microorganisms that are well compatible with large-scale industrial fermentation. In order to utilize effective promoters and introducing variant copies of the enzyme coding genes, enzyme performance can be significantly increased. Also, it became possible to adopt enzyme characteristics to food processing conditions by using recombinant DNA technology. The evolution of metabolic engineering in relation to systems biology in recent years and use them for designation of biological systems promises to produce more sufficient food enzymes in the early future. Currently, USA, EU, and China are the main players in GM foods worldwide. The GM enzymes approval is based on the results of widespread risk assessments. Safety evaluation includes homology assessment of the GM enzymes to the known allergens, immunology evaluation, pepsin resistance, heat resistance, and in vivo trials. With regard to labeling of the GM foods, the US FDA is more lenient than the two others because it believes that the approved GM products do not represent an additional risk to the consumers compared to the traditional foods. However, all GM enzymes should be listed on the labels of food packages in China. Upon the fate of the enzymes under processing, the EU exempts the GM enzymes added as processing aid from labeling while those additives should be mandatorily listed on the labels. Besides the all-positive points addressed about the GM enzymes; the improvements have introduced a new controversial issue in Islamic countries. As a rule, halal food enzymes should be derived from halal sources, produced in environments containing halal ingredients, and exhausted from alcohol up to the acceptable level in the final product. In addition, the gene responsible for encoding recombinant enzymes should be extracted from halal origin.

References

Ab Talib, M. S. (2017). Motivations and benefits of halal food safety certification. *Journal of Islamic Marketing, 8*(4), 605–624.

Abd Latip, M. A., & Hadry Nordin, N. F. (2018). Antarctica as new source for halal industrial enzymes. In *International conference on halal innovations in products and services*, pp. 58–60.

Aboulnaga, E. A. (2019). Cloning and expression of camel pro-chymosin encoding gene in *E. coli* and characterization of the obtained active enzyme. *Journal of Food and Dairy Sciences, 10*(3), 71–78.

Alhariri, M. (2020). Halal and genetically modified ingredients. In Y. R. Al-Teinaz, S. Spear, & I. H. A. Abd El-Rahim (Eds.), *The halal food handbook* (pp. 169–182). Wiley.

Alzeer, J., & Abou Hadeed, K. (2020). Halal certification of food, nutraceuticals, and pharmaceuticals in the Arab world. In I. Laher (Ed.), *Handbook of healthcare in the Arab world* (pp. 1–22). Springer.

Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., et al. (2008a). Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metabolic Engineering, 10*(6), 305–311.

Atsumi, S., Hanai, T., & Liao, J. C. (2008b). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature, 451*(7174), 86–89.

Beilen, J. B. V., & Li, Z. (2002). Enzyme technology: An overview. *Current Opinion in Biotechnology, 13*(4), 338–344.

Boyle, P. M., & Silver, P. A. (2012). Parts plus pipes: Synthetic biology approaches to metabolic engineering. *Metabolic Engineering, 14*(3), 223–232.

Bruetschy, C. (2019). The EU regulatory framework on genetically modified organisms (GMOs). *Transgenic Research, 28*, 169–174.

Budnik, L. T., Scheer, E., Burge, P. S., & Baur, X. (2017). Sensitising effects of genetically modified enzymes used in flavour, fragrance, detergence and pharmaceutical production: Cross-sectional study. *Occupational and Environmental Medicine, 74*, 39–45.

Cameron, D. E., Bashor, C. J., & Collins, J. J. (2014). A brief history of synthetic biology. *Nature Reviews Microbiology, 12*(5), 381–390.

Casini, A., Storch, M., Baldwin, G. S., & Ellis, T. (2015). Bricks and blueprints: Methods and standards for DNA assembly. *Nature Reviews Molecular Cell Biology, 16*(9), 568–576.

Cheraghali, F., Shojaee-Aliabadi, S., Hosseini, S. M., Mirmoghtadaie, L., Mortazavian, A. M., Ghanati, K., Abedi, A. S., & Moslemi, M. (2018). Characterization of microcapsule containing walnut (*Juglansregia* L.) green husk extract as preventive antioxidant and antimicrobial agent. *International Journal of Preventive Medicine, 9*, 101.

Choi, C. W., Choi, Y. H., Cha, M. R., Yoo, D. S., Kim, Y. S., Yon, G. H., et al. (2010). A new glycoside of resveratrol dimer from stem bark of *Vitisvinifera*. *Bulletin of the Korean Chemical Society, 31*(11), 3448–3450.

Choi, O., Lee, J. K., Kang, S. Y., Pandey, R. P., Sohng, J. K., Ahn, J. S., et al. (2014). Construction of artificial biosynthetic pathways for resveratrol glucoside derivatives. *Journal of Microbiology and Biotechnology, 24*(5), 614–618.

Clyne, R. K., & Kelly, T. J. (1997). Identification of autonomously replicating sequence (ARS) elements in eukaryotic cells. *Methods, 13*(3), 221–233.

Craig, W., Vanga, S. R., & Medaglia, J. C. (2012). *Commercialisation of GM crops: Comparison of regulatory frameworks* (pp. 2286–2319). Springer. https://doi.org/10.1007/978-1-4614-5797-8_837

Dadgarnejad, M., Kouser, S., & Moslemi, M. (2017). Genetically modified foods: Promises, challenges and safety assessments. *Applied Food Biotechnology, 4*(4), 193–202.

Deckers, M., Deforce, D., Fraiture, M. A., & Roosens, N. H. (2020). Genetically modified microorganisms for industrial food enzyme production: An overview. *Food, 9*(3), 326.

del Carmen, S., de Moreno de LeBlanc, A., Levit, R., Azevedo, V., Langella, P., Bermudez-Humaran, L. G., & LeBlanc, J. G. (2017). Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model. *International Immunopharmacology, 42*, 122–129.

del Carmen, S., de Moreno de LeBlanc, A., Martin, R., Chain, F., Langella, P., Bermudez-Humaran, L. G., & LeBlanc, J. G. (2014). Genetically engineered immunomodulatory *Streptococcus thermophilus* strains producing antioxidant enzymes exhibit enhanced anti-inflammatory activities. *Applied and Environmental Microbiology, 80*(3), 869–877.

Dhamankar, H., & Prather, K. L. (2011). Microbial chemical factories: Recent advances in pathway engineering for synthesis of value added chemicals. *Current Opinion in Structural Biology, 21*(4), 488–494.

Dijck, P. W. M. V., Selten, G. C. M., & Hempenius, R. A. (2003). On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. *Regulatory Toxicology and Pharmacology, 38*(1), 27–35.

Donohoue, P. D., Barrangou, R., & May, A. P. (2018). Advances in industrial biotechnology using CRISPR-Cas systems. *Trends in Biotechnology, 36*(2), 134–146.

Ermis, E. (2017). Halal status of enzymes used in food industry. *Trends in Food Science & Technology, 64*, 69–73.

Espinoza-Molina, J. A., Acosta-Muniz, C. H., Sepulveda, D. R., Zamudio-Flores, P. B., & Rios-Velasco, C. (2016). Codon optimization of the "*Bos Taurus Chymosin*" gene for the production of recombinant chymosin in *Pichia pastoris*. *Molecular Biotechnology, 58*, 657–664.

Esvelt, K. M., & Wang, H. H. (2013). Genome-scale engineering for systems and synthetic biology. *Molecular Systems Biology, 9*(1), 641.

European Commission. (2003a). *Question and answers on the regulation of GMOs in the EU*. MEMO/02/160REV.

European Commission. (2003b). Regulation no. 1829/2003. Genetically modified food and feed. *Official Journal of the European Union*.

European Commission. (2008). Regulation no. 1332/2008. Food enzymes. *Official Journal of the European Union*.

European Commission. (2014). *Guidance document on criteria for categorisation of food enzymes*, pp. 1–14.

European Commission. (2020a). *Contained use*. Retrieved from https://ec.europa.eu/food/plant/gmo/authorisation/contained_en.

European Commission. (2020b). *Geneticallymodified organisms, community register of GM food and feed*. Retrieved from https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm.

European Food Safety Authority. (2020). *Analysis of JECFA's draft guideline on "Evaluation of enzyme preparations used in the manufacture of foods"*. EFSA Supporting Publication, pp. 1–8.

Feng, J., & Yang, F. (2019). The regulation of genetically modified food in China. *Biotechnology Law Report, 38*(5), 289–293.

Fernandes, P. (2010). Enzymes in food processing: A condensed overview on strategies for better biocatalysts. *Enzyme Research, 2010*, 1–19.

Fernandez-Salguero, J., Prados, F., Calixto, F., Vioque, M., Sampaio, P., & Tejada, L. (2003). Use of recombinant cyprosin in the manufacture of ewe's milk cheese. *Journal of Agricultural and Food Chemistry, 51*, 7426–7430.

Fischer, J. (2011). *The halal frontier, Muslim consumers in a globalized market*. United States, Martin's Press LLC. https://doi.org/10.1007/978-0-230-11978-9

Fitz, E., Wanka, F., & Seiboth, B. (2018). The promoter toolbox for recombinant gene expression in *Trichoderma reesei*. *Frontiers in Bioengineering and Biotechnology, 6*, 135.

Flamm, E. L. (1991). How FDA approved chymosin: A case history. *Nature Biotechnology, 9*(4), 349–351.

Goodwin, K. D., Thompson, L. R., Duarte, B., Kahlke, T., Thompson, A. R., Marques, J. C., et al. (2017). DNA sequencing as a tool to monitor marine ecological status. *Frontiers in Marine Science, 4*, 107.

Halim, M. A. A., & Salleh, M. M. M. (2012). The possibility of uniformity on halal standards in organization of Islamic countries (OIC) country. *World Applied Sciences Journal, 17*(17), 6–10.

Hanzaee, K. H., & Ramezani, M. R. (2011). Intention to halal products in the world markets. *Interdisciplinary Journal of Research in Business, 1*(5), 1–7.

Hui, Y.H., Nip, W.K., Nollet, L.M., Paliyath, G. & Simpson, B.K. (2006). Food biochemistry and food processing. Blackwell Publishing. First edition.

Jacobson, M. (2000). The genetically modified food fight. *Western Journal of Medicine, 172*(4), 220–221.

Jacouton, E., Chain, F., Sokol, H., Langella, P., & Bermudez-Humaran, L. G. (2017). Probiotic strain *Lactobacillus casei* BL23 prevents colitis-associated colorectal cancer. *Frontiers in Immunology, 8*, 1–10.

Jamali, A., Moslemi, M., Akramzadeh, N., Mousavi Khaneghah, A., & Dadgarnejad, M. (2020). Safety factors of oils marketed in Iran and applicable strategies in control of food derived cardiovascular diseases. *Current Nutrition & Food Science, 16*(4), 571–577.

Jian-ping, K., Qiao-ling, T., & Xian-fa, Z. (2015). Agricultural GMO safety administration in China. *Journal of Integrative Agriculture, 14*(11), 2157–2165.

Khattak, J. Z. K., Mir, A., Anwar, Z., Mustatab Wahedi, H., Abbas, G., Khattak, H. Z. K., & Ismatullah, H. (2011). Concept of halal food and biotechnology. *Advance Journal of Food Science and Technology, 3*(5), 385–389.

Khersonsky, O., & Tawfik, D. S. (2010). Enzyme promiscuity: A mechanistic and evolutionary perspective. *Annual Review of Biochemistry, 79*, 471–505.

Kim, G. B., Kim, W. J., Kim, H. U., & Lee, S. Y. (2020). Machine learning applications in systems metabolic engineering. *Current Opinion in Biotechnology, 64*, 1–9.

Koutsoumanis, K., Allende, A., Alvarez-Ordonez, A., Bolton, D., Bover-Cid, S., Chemaly, M., et al. (2020). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 12: Suitability of taxonomic units notified to EFSA until March 2020. *EFSA Journal, 18*(7), 1–42.

Liu, D., Evans, T., & Zhang, F. (2015). Applications and advances of metabolite biosensors for metabolic engineering. *Metabolic Engineering, 31*, 35–43.

Liu, X., & Kokare, C. (2017). Microbial enzymes of use in industry. In G. Brahmachari (Ed.), *Biotechnology of microbial enzymes* (pp. 267–298). Elsevier.

Marchant, G. E., & Cardineau, G. A. (2013). The labeling debate in the United States. *GM Crops & Food: Biotechnology in Agriculture and the Food Chain, 4*(3), 126–134.

Meyer, V. (2008). Genetic engineering of filamentous fungi-progress, obstacles and future trends. *Biotechnology Advances, 26*(2), 177–185.

Moslemi, M., Hosseini, H., Neyestani, T. R., Akramzadeh, N., & Mazaheri Nezhad Fard, R. (2018). Effects of non-digestive polymers used in iron encapsulation on calcium and iron apparent absorption in rats fed by infant formula. *Journal of Trace Elements in Medicine and Biology, 50*, 393–398.

Moslemi, M., Kheirandish, M., Mazaheri, N. F. R., Hosseini, H., Jannat, B., Mofid, V., et al. (2020). National food policies in the Islamic Republic of Iran aimed at prevention of noncommunicable diseases. *Eastern Mediterranean Health Journal, 26*(12), 1556–1564.

Naegeli, H., Birch, A. N., Casacuberta, J., De Schrijver, A., Gralak, M. A., Guerche, P., et al. (2017). Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal, 15*(6), 1–49.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science, 1*, 1806–1815.

Nugraha, W. T., Murti, T. W., Novitasari, I. S., Sari, T. K., Murcita, G., & Wijakangka, G. R. T. (2015). Development of halal goat cheese using rennet like from vegetable source to replace commercial rennet source. In *The 6th international seminar on tropical animal production, integrated approach in developing sustainable tropical animal production*, pp. 733–737.

Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., & DiNovi, M. J. (2006). Food-processing enzymes from recombinant microorganisms—A review. *Regulatory Toxicology and Pharmacology, 45*(2), 144–158.

Oz, B., Unsal, F., & Movassaghi, H. (2017). Consumer attitudes toward genetically modified food in the United States: Are Millennials different? *Journal of Transnational Management*, 1–19. https://doi.org/10.1080/15475778.2017.1373316

Patel, A. K., Singhania, R. R., & Pandey, A. (2017). Production, purification, and application of microbial enzymes. In G. Brahmachari (Ed.), *Biotechnology of microbial enzymes* (pp. 13–41). Elsevier.

Petersen, S. G., Rahbek-Nielsen, H., Hellmuth, K., & Harboe, M. (2006). Increased production of chymosin by glycosylation. *Journal of Biotechnology, 125*(2), 304–310.

Pugh, S., McKenna, R., Moolick, R., & Nielsen, D. R. (2011). Advances and opportunities at the interface between microbial bioenergy and nanotechnology. *The Canadian Journal of Chemical Engineering, 89*(1), 2–12.

Rajamanickam, V., Metzger, K., Schmid, C., & Spadiut, O. (2017). A novel bi-directional promoter system allows tunable recombinant protein production in *Pichia pastoris*. *Microbial Cell Factories, 16*(1), 152.

Riaz, M. N., & Chaudry, M. M. (2019). *Handbook of halal food production*. CRC Press.

Rieder, L., Teuschler, N., Ebner, K., & Glieder, A. (2019). Eukaryotic expression systems for industrial enzymes. *Industrial Enzyme Applications*, 47–69.

Robinson, P. K. (2015). Enzymes: Principles and biotechnological applications. *Essays in Biochemistry, 59*, 1–41.

Schiemann, J., Dietz-Pfeilstetter, A., Hartung, F., Kohl, C., Romeis, J., & Sprink, T. (2019). Risk assessment and regulation of plants modified by modern biotechniques: Current status and future challenges. *Annual Review of Plant Biology, 70*, 699–726.

Scott, S. E., Inbar, Y., & Rozin, P. (2016). Evidence for absolute moral opposition to genetically modified food in the United States. *Perspectives on Psychological Science, 11*(3), 315–324.

Sharma, M., Sangwan, R. S., Khatkar, B. S., & Singh, S. P. (2019). Alginate-pectin co-encapsulation of dextransucrase and dextranase for oligosaccharide production from sucrose feedstocks. *Bioprocess and Biosystems Engineering, 42*, 1681–1693.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018). Safety evaluation of the food enzyme α-amylase from a genetically modified *Aspergillus niger* (strain NZYM-MC). *EFSA Journal, 16*(10), 1–17.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018a). Safety evaluation of the food enzyme glucan1,4-α-glucosidase from a genetically modified *Aspergillus niger* (strain NZYM-BW). *EFSA Journal, 16*(10), 1–13.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018b). Safety of the food enzyme glucoamylase from a genetically modified *Aspergillus niger* (strain NZYM-BF). *EFSA Journal, 16*(10), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018c). Safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SO). *EFSA Journal, 16*(11), 1–17.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018d). Safety evaluation of the food enzyme endo-1,4-β-xylanase from a genetically modified *Bacillus subtilis* (strain LMG S-24584). *EFSA Journal, 16*(10), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018e). Safety evaluation of the food enzyme endo-1,4-β-xylanase from a genetically modified *Trichodermareesei* (strain DP-Nzd22). *EFSA Journal, 16*(11), 1–15.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2018f). Safety evaluation of the food enzyme acetolactate decarboxylase from a genetically modified *Bacillus licheniformis* (strain NZYM-JB). *EFSA Journal, 16*(11), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2019). Characterization of microorganisms used for the production of food enzymes. *EFSA Journal, 17*(6), 1–13.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2019a). Safety evaluation of the food enzyme alpha-amylase from a genetically modified *Trichodermareesei* (strain DP-Nzb48). *EFSA Journal, 17*(1), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Bruschweiler, B. J., Cocconcelli, P. S., Crebelli, R., et al. (2019b). Safety evaluation of the food enzyme pullulanase from a genetically modified *Bacillus licheniformis* (strain DP-Dzp39). *EFSA Journal, 17*(1), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020). Safety evaluation of the food enzyme α-amylase from the genetically modified *Pseudomonas fluorescens* strain BD15754. *EFSA Journal, 18*(3), 1–12.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020a). Safety evaluation of the food enzyme glucan 1,4-alpha-glucosidase from the genetically modified *Trichodermareesei* strain DP-Nzh38. *EFSA Journal, 18*(5), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020b). Safety evaluation of the food enzyme maltogenic amylase from the genetically modified *Bacillus licheniformis* strain DP-Dzr50. *EFSA Journal, 18*(1), 1–17.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020c). Safety evaluation of the food enzyme xylanase from the genetically modified *Trichodermareesei* strain RF5703. *EFSA Journal, 18*(1), 1–16.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020d). Safety evaluation of the food enzyme β-galactosidase from the genetically modified *Escherichia coli* NCIMB 30325. *EFSA Journal, 18*(1), 1–13.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020f). Safety evaluation of the food enzyme lysophospholipase from the genetically modified *Aspergillus niger* strain NZYM-LP. *EFSA Journal, 18*(5), 1–14.

Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., et al. (2020g). Safety evaluation of the food enzyme triacylglycerol lipase from the genetically modified *Ogataeapolymorpha* strain DP-Jzk33. *EFSA Journal, 18*(3), 1–15.

Silano, V., Baviera, J.M.B., Bolognesi, C., Cocconcelli, P.S., Crebelli, R., Gott, DM. et al. (2020e). Safety evaluation of the food enzyme xylose isomerase from the genetically modified *Streptomyces rubiginosus*strain DP-Pz n37. EFSA Journal, 18(1): 1–12.

Silano, V., Bolognesi, C., Castle, L., Chipman, K., Cravedi, J. P., Fowler, P., et al. (2018g). Safety evaluation of the food enzyme alpha-amylase from a genetically modified *Bacillus licheniformis* (strain NZYM-AN). *EFSA Journal, 16*(7), 1–14.

Silano, V., Bolognesi, C., Castle, L., Chipman, K., Cravedi, J. P., Fowler, P., et al. (2018h). Safety evaluation of food enzyme xylanase from a genetically modified *Bacillus subtilis* (strain LMG S-27588). *EFSA Journal, 16*(5), 1–19.

Silano, V., Bolognesi, C., Castle, L., Cravedi, J. P., Fowler, P., Franz, R., et al. (2017). Safety evaluation of the food enzyme endo-1,4-β-xylanase from genetically modified *Aspergillus niger* strain XYL. *EFSA Journal, 15*(5), 1–20.

Silano, V., Bolognesi, C., Castle, L., Cravedi, J. P., Fowler, P., Franz, R., et al. (2017a). Safety evaluation of the food enzyme pullulanase from genetically modified *Bacillus subtilis* strain NZYM-AK. *EFSA Journal, 15*(8), 1–12.

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: Industrial progress in 21st century. *3 Biotech, 6*(2), 174.

Song, R., Zhai, Q., Sun, L., Huang, E., Zhang, Y., Zhu, Y., et al. (2019). CRISPR/Cas9 genome editing technology in filamentous fungi: Progress and perspective. *Applied Microbiology and Biotechnology, 103*(17), 6919–6932.

Stephanopoulos, G. (2012). Synthetic biology and metabolic engineering. *ACS Synthetic Biology, 1*(11), 514–525.

Sun, J. (2019). Genetically modified foods in China: Regulation, deregulation, or governance? In K. C. Liu & U. S. Racherla (Eds.), *Innovation, economic development, and intellectual property in India and China* (pp. 347–366). Springer.

Tieman, M. (2017). Halal Europe: Apremium halal-tayyib brand? *Islam and Civilisational Renewal (ICR), 8*(2), 260–263.

Trono, D. (2019). Recombinant enzymes in the food and pharmaceutical industries. In R. S. Singh, R. R. Singhania, A. Pandey, & C. Larroche (Eds.), *Advances in enzyme technology* (pp. 349–387). Elsevier.

Tseng, H. C., Martin, C. H., Nielsen, D. R., & Prather, K. L. J. (2009). Metabolic engineering of *Escherichia coli* for enhanced production of (R)-and (S)-3-hydroxybutyrate. *Applied and Environmental Microbiology, 75*(10), 3137–3145.

Ujj, O. (2016). European and American views on genetically modified foods. *The New Atlantis*, 77–92.

Ulusu, Y., Senturk, S. B., Kudug, H., & Gokce, I. (2016). Expression, purification, and characterization of bovine chymosin enzyme using an inducible pTOLT system. *Preparative Biochemistry & Biotechnology, 46*(6), 596–601.

US Food and Drug Administration. (2019). *New plant variety consultations*. Retrieved from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=NewPlantVarietyConsultations&sort=fda_letter_dt&order=desc&startrow=151&type=basic&search=.

US Food and Drug Administration. (2020a). *Agricultural biotechnology*. Retrieved from https://www.fda.gov/food/consumers/agricultural-biotechnology.

US Food and Drug Administration. (2020b). *GRAS notices*. Retrieved from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&sort=GRN_No&order=DESC&showAll=true&type=basic&search=.

Uzogara, S. G. (2000). The impact of genetic modification of human foods in the 21st century: A review. *Biotechnology Advances, 18*, 179–206.

Vieille, C., & Zeikus, G. J. (2001). Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews, 65*(1), 1–43.

Vogl, T., Kickenweiz, T., Pitzer, J., Sturmberger, L., Weninger, A., Biggs, B. W., et al. (2018). Engineered bidirectional promoters enable rapid multi-gene co-expression optimization. *Nature Communications, 9*(1), 1–13.

Wang, Y., Halls, C., Zhang, J., Matsuno, M., Zhang, Y., & Yu, O. (2011). Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. *Metabolic Engineering, 13*(5), 455–463.

World Health Organization. (2014). *Frequently asked questions on genetically modified foods*. Retrieved from https://www.who.int/foodsafety/areas_work/food-technology/faq-genetically-modified-food/en/.

Wozniak, C. A., McClung, G., Gagliardi, J., Segal, M., & Matthews, K. (2012). Regulation of genetically engineered microorganisms under FIFRA, FFDCA and TSCA. In C. A. Wozniak & A. McHughen (Eds.), *Regulation of agricultural biotechnology: The United States and Canada* (pp. 57–94). Springer.

Xia, X. X., Qian, Z. G., Ki, C. S., Park, Y. H., Kaplan, D. L., & Lee, S. Y. (2010). Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber. *Proceedings of the National Academy of Sciences, 107*(32), 14059–14063.

Yan, S., & Wu, G. (2017). Bottleneck in secretion of α-amylase in *Bacillussubtilis*. *Microbial Cell Factories, 16*(1), 124.

Yang, S., Sleight, S. C., & Sauro, H. M. (2013). Rationally designed bidirectional promoter improves the evolutionary stability of synthetic genetic circuits. *Nucleic Acids Research, 41*(1), 1–7.

Yavari, B., Sarami, S., Shahgaldi, S., Athari, S. S., & Sharma, A. (2016). If there is really a notable concern about allergenicity of genetically modified foods? *Journal of Food Quality and Hazards Control, 3*, 3–9.

Yuen-Ting Wong, A., & Wai-Kit Chan, A. (2016). Genetically modified foods in China and the United States: A primer of regulation and intellectual property protection. *Food Science and Human Wellness, 5*, 124–140.

Zhang, Y., Geary, T., & Simpson, B. K. (2019). Genetically modified food enzymes: A review. *Current Opinion in Food Science, 25*, 14–18.

Zheng, Z., Gao, Y., Zhang, Y., & Henneberry, S. (2017). Changing attitudes toward genetically modified foods in urban China. *China Agricultural Economic Review, 9*(3), 397–414.

Zou, G., Shi, S., Jiang, Y., den Brink, J. V., de Vries, R. P., Chen, L., & el. (2012). Construction of a cellulase hyper-expression system in *Trichodermareesei* by promoter and enzyme engineering. *Microbial Cell Factories, 11*(1), 1–12.

Enzyme Immobilization and Its Application Strategies in Food Products 15

Nafiseh Sadat Naghavi, Nazanin Sanei, and Martin Koller

Abstract

Enzymes are the dominating class of biocatalysts, which are extensively employed in food industries. Immobilization of enzymes on inactive and not soluble supports practically increases their efficiency owning to their high stability and multiple reuses, while it can negatively impact enzyme activity. The characteristics of immobilized enzymes are based on the procedure of immobilization and achieved beneficial properties, such as biocompatibility, chemical and thermal stability, the impossibility to dissolve (leak) in the reaction liquids, reconstitution, recyclability, and cost efficiency. Various immobilized enzymatic systems, like proteases, amino acylase, glucose isomerase, β-galactosidase, aspartase, lipases, or glucosidase have been shown to be techno-economically used in food industries on a multi-ton's scale per year. This chapter provides a general survey of the benefits and applications of the immobilization enzymes with a major focus on the food industries.

Keywords

Food enzymes · Immobilization · Reuse · Cost efficiency

N. S. Naghavi
Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

N. Sanei
Department of Biology, Faculty of Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran

M. Koller (✉)
Research Management and Service, c/o Institute of Chemistry, NAWI Graz, University of Graz, Graz, Austria

ARENA - Association for Resource Efficient and Sustainable Technologies, Graz, Austria
e-mail: martin.koller@uni-graz.at

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_15

15.1 Introduction

Immobilization is a process used for transformation of biocatalysts (cells and enzymes) or bioactive components from a soluble phase to an insoluble state by using an insoluble carrier or by encapsulating them within a matrix material (Van de Velde et al., 2002; Chen et al., 2016). As a consequence, the properties of the native catalysts may change significantly, and in order to be able to explain the observed reaction behavior the laws of heterogeneous kinetics have to be applied. The immobilization of enzymes has become a major process in manufacture, medication, and biotechnology over the past decades. Scientists have developed many processes based on methods from physical adsorption and covalent binding to encapsulation or entrapment into polymers (Ispas et al., 2009; Alkan et al., 2009; Koszelewski et al., 2010; Klein et al., 2011; Defaei et al., 2018). Already in 2010, the market volume of immobilized enzymes amounted to almost 6×10^9 US-$ with a currently clear upwards trend, with food/beverage production and pharmaceutical applications constituting the major fields of application, each of both occupying about 21% of the entire market for immobilized enzymes (DiCosimo et al., 2013).

In contrast to free, dissolved enzymes, immobilized enzymes are of higher stability and more resilient against inhibition by heavy metals and other chemicals, non-optimal conditions of pH-value, temperature, or salinity in the environment, and turbulent flow regimes. Importantly, the high variety of reported immobilized enzyme systems provides a range of beneficial aspects, such as convenient recovery of enzymes and products, which reduces the number of process steps, recyclability of enzymes, continuous operation mode of enzymatic reactions, high overall productivity, possibility of sudden termination of reaction on demand, contribution to sophisticated development of enhanced bioreactor designs ("immobilized enzyme reactors"), and decreased environmental pollution and economic costs (Sheldon, 2007a; Defaei et al., 2018). Drawbacks of using immobilized enzymes encompass lower activity and reaction rates compared to free enzymes, susceptibility to putrefaction ("fouling"), additional cost for supports (carriers), or the need for disposing spent (exhausted) immobilized enzymes (Basso & Serban, 2019).

To date, various types of matrices and supports have been utilized to immobilize enzymes. Polysaccharides, glass beads, nano-metals, petro-plastics, and biopolymers are the most frequently used matrices. To immobilize enzymes, the binding strength between support and enzyme depends on the type of chemical or physical reaction, which heavily depends on the nature of the support surface, and the interaction between individual enzyme molecules (Kahraman et al., 2007; Asgher et al., 2014; Rana et al., 2014; Reshmi et al., 2006; Dey et al., 2002; Defaei et al., 2018). Especially the immobilization of enzymes on nanosized supports, such as nanofibers, nanobeads, carbon nanotubes (CNTs), and other nanoparticles for different reactions driven by biocatalysts, is nowadays emerging as an innovative research area (Sheldon, 2007a; Klein et al., 2011; Reshmi et al., 2006; Defaei et al., 2018).

Also enzymes of importance for food technology have been immobilized on various matrices. Some examples are given in Fig. 15.1. The immobilized food

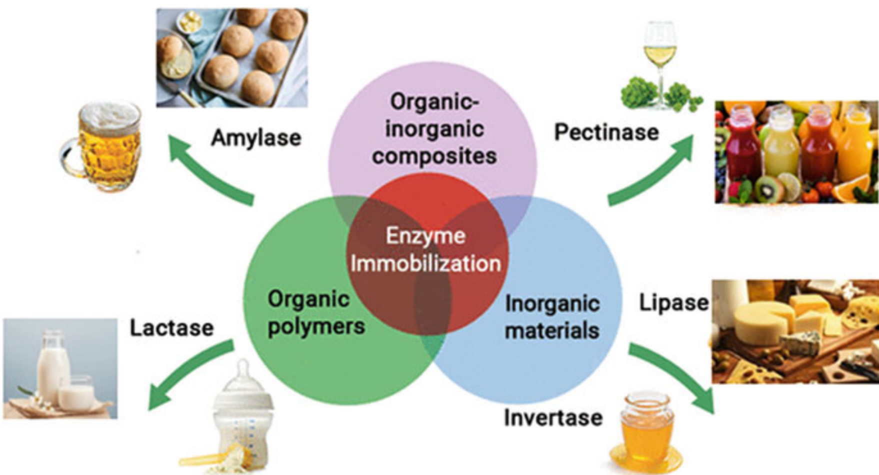


Fig. 15.1 Examples for the applications of different supports for immobilization enzymes used in food processing

enzyme that was the first to be industrially utilized already in the 1970s was amino acylase (EC 3.5.1.14), a hydrolase which was implemented for the production of racemic mixtures of D- and L-amino acids from N-acyl-amino acids as substrates. Industrially, this reaction took and still takes place in columns carrying the immobilized enzyme, while the substrate solution is washed thoroughly. Two other effectually immobilized enzymes were the glycosidase invertase (EC 3.2.1.26), an approved food additive (E 1103) which is used in fructose-rich corn syrup production, and lipases (EC 3.1.1.x), a versatile group of amphiphilic that are applied in the hydrolysis and transesterification of oily products, which, beyond food industry, is also a novel route toward biodiesel production from waste lipids. The advantages of immobilized enzymes in food industries are increased productivity, reduction of product recovery cost, and ultimately increased yields for diverse food products in the future (DiCosimo et al., 2013; Homaei et al., 2013).

15.2 Methods of Enzyme Immobilization

A number of methods can be used to immobilize a given enzyme; these methods encompass simple reversible physical adsorption, formation of ionic bonds between enzymes and supports, and generation of firm covalent linkages (Fig. 15.2). Such immobilization methods are categorized into reversible (adsorption, ionic binding, disulfide formation, affinity binding) and irreversible (covalent binding, cross-linking, physical entrapment [matrix entrapment and encapsulation]) methods. Apart from this classification, immobilization methods can also be classified according to the type of chemical reaction involved in binding as support binding and entrapment methods (Homaei et al., 2013).

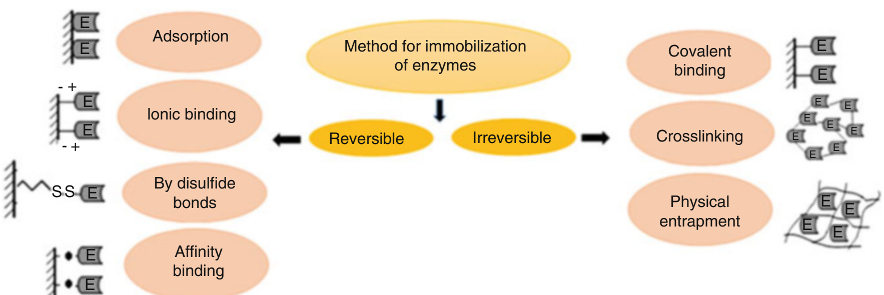


Fig. 15.2 The categories for classification of enzyme immobilization methods

15.2.1 Irreversible Methods

In irreversible enzyme immobilization, the biocatalyst that binds to the matrix cannot be detached without causing an effect on the biological enzyme activity or the structural properties of the support. The most usual methods of irreversible enzyme immobilization encompass covalent binding, cross-linking, and entrapment or encapsulation.

15.2.1.1 Immobilization via Formation of Covalent Binding

This method is widely utilized for enzyme immobilization. Based on this procedure, covalent bonds are created between the chemical groups of the enzyme molecule and the chemical groups present on the support. This method leads to formation of a stable bond between enzyme and support, resulting in the prevention of detachment of enzyme from the support when in use. This covalent bond is typically formed between the side-chain amino acids of biocatalysts, such as aspartic acid, arginine, histidine, and the functional groups, like imidazole, indolyl, phenolic, and hydroxyl present on the support. This method is used when the complete absence of enzyme in the product is desirable. However, it leads to chemical modification of enzymes and, as a result, the activity of the enzyme can get reduced (Homaei et al., 2013). As an option to increase activity of enzymes immobilized via covalent linking, linear spacers are often used to combine enzyme and support, giving the enzyme enough space for mobility and reaction (De Maio et al., 2003). An important example in this context is the carboxyl- and amine-reactive linker 1-ethyl-3-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which can link hydrophobic polymeric supports with enzymes (Mohan et al., 2015).

15.2.1.2 Cross-Linking

Crosslinking resorts to covalent linking between the enzyme and active molecules, and is used to generate biocatalytically active polymeric particles, which can conveniently be used for given reactions. This mechanism is also known as "copolymerization". Enzymes bind to each other with the aid of bi-functional reagents, such as glutaraldehyde, glutardialdehyde, glyoxal, diisocyanates, hexamethylene

diisocyanate, and toluene diisocyanate, which build bridges between individual enzyme molecules. The major drawback of this procedure is that the multifunctional reagents used for cross-linking the enzyme may reconstruct or change the structure of the enzyme, which again causes the loss of catalytic activity (Albayrak & Yang, 2002).

15.2.1.3 Physical Entrapment

This method involves the physical trapping of biocatalysts into a film, gel, fiber, coating, or microencapsulation (Costa et al., 2005). In this procedure, the enzyme or reactive molecule can be mixed with a polymer to attach to it, resulting in creation of a lattice structure that encapsulates/entraps the enzyme. The advantages of this immobilization method include generation of a vast biocatalytically active surface area generated by the substrate and the enzyme, in only a low volume. The most important disadvantages of this method are the probable inactivation of the enzyme during microencapsulation, enzyme leakage into the environment, and the need for typically high concentrations of the enzyme. The most frequently used supports that are used for encapsulation of enzymes are polymers, such as cellulose, collagen, polyacrylamide gel, gelatin, alginate, starch, silicone, and rubber (Nisha et al., 2012).

15.2.2 Reversible Methods

15.2.2.1 Adsorption

The adsorption of enzymes on supports, such as activated charcoal, alumina, and ion exchange resins is among the simplest techniques used to limit enzyme mobility (Brady & Jordaan, 2009). Depending on the nature of amino acids present on the surface of enzymes and the chemical nature of the support, the enzyme is fixed by non-covalent binding trough ionic and hydrophobic interactions, or by formation of hydrogen bonds. This method can be achieved by mixing an aqueous enzyme solution with a matrix for a defined time, followed by a washing step to remove the remaining free enzyme from the immobilization matrix. This method of immobilization is simple and has little effect on enzyme activity and can be repeatedly applied by adding fresh enzyme solution ("recharging" of the support with biocatalyst). However, in this method, shortcomings, like fast enzyme desorption from the support, or loss of activity by changing pH-value, temperature, solvent, and ionic strength of the surrounding environment, can hardly be avoided (Costa et al., 2005).

15.2.2.2 Ionic Bond Formation

Immobilization by generation of ionic bonds is primarily based on the formation of ionic bonds between the enzyme molecules or reactive molecules and a solid matrix that has a charged ionic surface. The main advantage of ionic binding in comparison to physical binding is the strength of generated bonds that is stronger in case of ionic bonds than the power responsible for formation of covalent bonds (Torres et al., 2002). Such non-covalent immobilization assays can be reversed/deactivated by

alterations in the temperature, solvent polarity, and ionic strength conditions (Nisha et al., 2012).

15.2.2.3 Immobilization via Disulfide Bridges

This method involves the configuration of disulfide (-S-S-) bridges between the enzyme and the support. The major benefit of this method is the ability to recover the bonds created between the activated solid surface and the thiol groups on the enzyme, because the leakage of bounded protein leads to the release of high amounts of low-molecular-weight thiol from disulfide bridges. The feasibility of reusing the polymeric matrix after enzyme deactivation may facilitate the actual large-scale immobilization of enzymes in industrial procedures, where their use is currently not economical because of the high cost of support materials (Ovsejevi et al., 2013).

15.2.2.4 Affinity Binding

Affinity immobilization combines the properties of the enzyme to maintain under varied physiological conditions. This can be done via two routes of support pre-junction by an affinity ligand for desired enzyme, or enzyme attachment to the organism that has an affinity toward the support. The benefits of this procedures are that the enzyme is not exposed to any unusual chemical conditions, minimal conformational changes occur during immobilization, and the high activity of immobilized enzyme is maintained (Sardar et al., 2000).

15.3 Classification of Different Support Materials for Immobilization of Food Enzymes

The reaction occurring between the enzyme and a carrier generates an immobilized enzyme with particular structural, biochemical, physical, and kinetic characteristics. Carriers can be divided into different groups based on their appearance or their chemical components. The support can be a synthetic or biological organic polymer, or an inorganic solid. The support must display certain features, like extended surface-to-volume ratio, high permeability (mass transference), acceptable functional groups for enzyme binding under non-denaturing conditions, hydrophilic moieties, insolubility in water, chemical and thermal stability, mechanical strength, high recalcitrance, applicable particle shape, resistance to microbial attack, regenerability, biological safety, and low or acceptable price (Sheldon, 2007b; Garcia-Galan et al., 2011). Furthermore, multi-enzyme biocatalysis on only one support, especially processes resorting to multi-enzyme cascades, is an emerging approach to generate high-value chemicals on an industrial scale (Xu et al., 2020).

15.3.1 Classification of Different Support Materials Based on the Chemical Structure

15.3.1.1 Inorganic Solid Supports

Different inorganic solids, such as zeolites, alumina, silica, and mesoporous silicas, are possible to use for enzyme immobilization. Silica-based supports are the best applicable matrices for immobilization of enzymes in the industrial manufacturing of enzymatically produced products, in addition to research purposes (Vianello et al., 2006; Pierre, 2004; Hudson et al., 2008). In general, the high surface area provided via silica gel matrixes is nonpareil. Moreover, silica gel can be facilely treated to obtain the desired shape, pore size, and microchannels to allow reaction between substrates and ligands. Furthermore, silica gel is mechanically stable and chemically inactive; it is therefore environmentally benign for manufacturing and industrial procedures (Blanco et al., 2004). The simplest and cheapest methods for enzyme immobilizing, namely, attachment on silica, are realized by simple adsorption. For instance, this method is utilized for generation of enzyme formulations in detergent powders that release the enzyme into the washing liquid during the washing process (Deere et al., 2002).

Polymers

A recent method of enzyme immobilization is based on covalent linkage of such enzymes to polymers that undergo significant structural conformation changes in response to even minor environmental changes in terms of pH-value, temperature, and ionic strength (Klouda & Mikos, 2008). A studied sample is poly (N-isopropyl acrylamide) (poly NIPAM), a thermo- and bio-compatible polymer (Klis et al., 2009). Aqueous poly NIPAM has its critical solution temperature (CST) at around 32 °C. Above the CST, it becomes dissolvable because of release of water molecules from the polymer fibers. Thus, the bio-conversion can take place under states that maintain the enzyme solubility, thereby minimizing diffusional restriction. Subsequently, an increase in temperature above the CST leads to detachment of the immobilized enzyme, thus the enzyme is recovered and can be reused (Virtanen & Tenhu, 2000; Virtanen et al., 2000; Lozinsky et al., 2003). Polyurethane has recently been proposed as an entrapping polymer that retains the bioactivity of biocatalysts for long times. The usage of this polymer resulted in a remaining crude oil degradation capacity of 44.31% by a microbial consortium after more than 6 months (Kazemzadeh et al., 2020).

Polymers that possess electrical conductivity have already been successfully synthesized and utilized in different areas, including biotechnology. Recently, a new class of polymers has been proposed as novel electro-active conjugated polymers. This kind of supports exhibit interesting electrical and optical characteristics previously reported only for inorganic systems. Electronically directing polymers are different from all the familiar inorganic crystalline semiconductors, such as silicon. They are molecular in nature and long chains are absent in them. Immobilization of enzymes and biosensor construction are two applications of these polymers (Cirpan et al., 2003). Lots of theoretical models

have also been associated with the electrochemical entrapment of enzymes to evaluate the polymer thickness, enzyme configuration, and the level of enzyme loading in the biosensor design (Gerard et al., 2002).

Nanomaterials

The headway of nanotechnology in the 1990s was preceded by the quick evolvement of nanobiotechnology including the construction of nanobiocatalysts. In the early methods applied in nanobiocatalysis, enzymes were immobilized on various nanostructured materials utilizing conventional procedures, like simple adsorption and covalent linkage. This method attracted attention for immobilizing enzymes on a wide range of nanostructured matrices, like porous nanomaterials, electroconductive nanofibers, and magnetic nanoparticles (MNPs). This large area provides better enzyme loading, which in turn improves the enzyme mobility in comparison to immobilized enzyme systems on conventional matrices. One of the special advantages of nanostructured materials is that the pore size in nanopores, nanofibers, or nanotubes can be controlled at nanometer scale (Homaei et al., 2013).

Recently, nanobiocatalytic methods have evolved from simple strategies for immobilization of enzymes (Homaei et al., 2013). By rapid advancement in nanotechnology, MNPs are presently extremely interesting. The physico-chemical characteristics of MNPs can widely differ from the properties of the bulk material from which the nanoparticle is made; this has attracted attention in these materials also for enzyme immobilization. For instance, magnetizing a particle in a particular direction by magnetic anisotropy is usually done on the surface of a particle (Schellenberger et al., 2002). Nanoparticles constructed by ultra-small superparamagnetic iron oxide (Kooi et al., 2003; Keller et al., 2004), cross-linked iron oxide (CLIO), and mono-crystalline iron oxide (Krause et al., 2004) all were fabricated as imaging elements in magnetic resonance imaging (MRI). Magnetic particles are used for enzyme immobilization for the purpose of increasing the stability of the biocatalyst, to maintain the stability of the catalyst, and, importantly, they can conveniently be separated from the interaction environment and recovered by applying an external magnetic field. (Bilal & Iqbal, 2019). MNPs perform best at typical sizes ranging between 10 and 20 nm, where superparamagnetism emerges (Netto et al., 2013). Such magnetic particles have been suggested for biotechnological applications (Kluchova et al., 2009; Defaei et al., 2018) or for developing analytical systems, like biosensors (Bilal & Iqbal, 2019; Kouassi et al., 2005).

Nanostructured metal oxides (NMOs) recently became of interest in the area of enzyme immobilization because these materials have the best structural revising and high bioactivity, which leads to elevated sensing properties (Antony et al., 2016). As a part of NMOs, MNPs have been widely utilized in enzyme immobilization because of their advantageous properties, such as their size, magnitude, higher safety levels, better reusability, wide surface, and large capacity of enzyme loading. Because they have an inactive surface that limits direct binding to enzymes, protective molecules must be coated on MNPs to supply dynamic functional groups for immobilization of enzymes (Amirbandeh & Taheri-Kafrani, 2016; Mehnati-Najafabadi et al., 2018). With all these benefits in mind, MNPs are highly inhibited in acidic and oxidative

conditions. Therefore, coating of the outer protective surface is so vital to sustain the consistency of MNPs (Landarani-Isfahani et al., 2015). In a recent study, Defaei et al. (2018) immobilized the hydrolase α-amylase (EC 3.2.1.1) onto naringin-functionalized MNPs by ionic reactions. The MNPs were covered with naringin, which is a biocompatible flavonoid. The appearance, structure, and features of functionalized MNPs and the immobilization status of the nanocomposite were determined by analytical instruments, like thermogravimetry (TGA), vibrating sample magnetometer (VSM), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy with energy dispersive X-ray (SEM-EDX), and transmission electron microscopy (TEM). In addition, the optimum conditions of temperature, pH-value, interaction time, and enzyme tendency for better immobilization were evaluated. The results evaluated the optimum conditions for α-amylase immobilization on the synthesized nanocarrier at pH 6.5 and a temperature of 55 °C. Reuse experiments showed high maintenance of immobilized α-amylase activity even after 10 reaction repeats. Furthermore, the storage consistency of enzyme immobilization was repaired by immobilization compared to that of the free enzyme and it retained 60% of its original activity even after 6 weeks of storage at 4 °C. Improving the catalytic properties of enzymes through immobilization has made this nanobiocatalyst a feasible tool in bio-industrial systems.

Another field of application of MNPs as supports for immobilized enzymes is the pharma sector; here, it was shown that the enzyme penicillin G acylase (PGA; EC 3.5.1.11) can be immobilized to functionalized MNPs (epoxy-activated magnetic cellulose beads) due to the cavity and affinity forces in the matrix of activated cellulose, and applied for hydrolytic removal of the side chain of penicillin G molecules, generating 6-aminopenicillanic acid as product. Improved biocatalytic activity and stability of the enzyme were reported for this process in comparison to the use of free enzyme (Luo & Zhang, 2010). In recent years, this process was improved by various research groups globally, such as by Liu and colleagues, who covalently immobilized PGA on hydroxy- and aldehyde-functionalized magnetic Fe_2O_3/Fe_3O_4 nanoparticles (Liu et al., 2020), or Zhaoyu et al., who used novel di-functional magnetic "nanoflowers," equipped with epoxy groups and hydrophilic catechol as well as with phthaloquinone groups enabling the covalent coupling of penicillin G acylase (Zhaoyu et al., 2020).

15.3.1.2 Organic Solid Supports

Different biological polymers, especially hardly water-soluble or de facto water-insoluble polysaccharides, like agarose, cellulose, starch, and carrageenans, have been heavily utilized as matrices for enzyme immobilization. These polymers create highly inactive aqueous gels, with the property of high gel formation even at low concentrations (Van de Velde et al., 2002).

The best described and most widely applied synthetic polymers used to carry the immobilized enzymes are acrylic resins, like sepabeads or eupergit. These macroporous polymers are copolymerized by using N,N′-methylene-bi-(methacrylamide), glycidyl ether, methacrylamide, allyl methacrylate, and glycidyl methacrylate. They are very hydrophilic and highly chemically and mechanically

stable in the entire pH-range of 0 to 14 and are not inactivated even at a sudden and tremendous pH-change. A basic disadvantage of hydrophilic resins are diffusion limitations that have been shown in kinetically monitored procedures. Immobilization by covalent binding to acrylic resins has been successfully utilized for a variety of enzymes in industrial operations (Katchalski-Katzir & Kraemer, 2000; Boller et al., 2002).

Enzymes may also be immobilized in biological or artificial hydro- or cryo-gels in an insoluble environment. For example, poly (vinyl alcohol) (PVA) crucibles made by melting ice are frequently used for whole-cell immobilization (Tripathi et al., 2010). Also, because of their minor size, free enzymes can spread from the gel matrix and get dissolved in an aqueous medium. To trap free enzymes, the enzyme size should be enlarged by mechanisms, such as cross-linking. Another option to increase the biocatalytic enzyme size is to design a composite material with a polyelectrolyte. Because of their ampholytic nature, proteins are released either as polycations or polyanions, based on the environmental pH-value. Therefore, the typically can form complexes with polyelectrolytes of opposite charges (Homaei et al., 2013).

15.4 Multi-Enzyme Immobilization

Multi-enzyme immobilization is a technique that co-establishes more than one enzyme on appropriate supports/carriers, or joins enzymes using a linking agent (cross-linker) with no carriers (Ren et al., 2019). Enzymes will be adjacent to each other, and the total material-transport restriction will be decreased during co-immobilization, which has been shown to boost the activity of enzymes by matrix canaling and raising the consistency and ability to reuse them. As support materials can mainly influence the characteristics of enzymes, selection of support has been mentioned as a hot subject in the majority of reports on enzyme immobilization techniques. To date, different matrices, like CNTs, graphene, metal-organic frameworks (MOFs), DNA nanocomposites, silica, and polymers, have been used for immobilization of multi-enzyme, which can efficiently shelter enzyme activity from biological challenges, such as heavy metals or high temperature (Sheldon & Woodley, 2018; Ren et al., 2019). There are three major types for co-immobilization of enzymes, such as random and positional co-immobilization, and compartmentalization (Hwang & Lee, 2019).

Biocatalytic transformation by a cascade of enzymes ("multi-enzyme catalysis") is an emerging technology to manufacture various industrially valuable compounds; briefly, it mimics the cascadic catalytic steps of pathways in living cells. In this context, positional multi-enzyme immobilization has been demonstrated as a viable tool for effective immobilization of co-enzymes, which can trigger the rates of coenzyme cascade reactions by fine-tuning the sequence of immobilized enzymes, and which, first of all, improves channeling of the substrate flow, and avoids the formation of unwanted side products, thus leading to higher overall yields. Moreover, such positional multi-enzyme immobilization approaches result in high

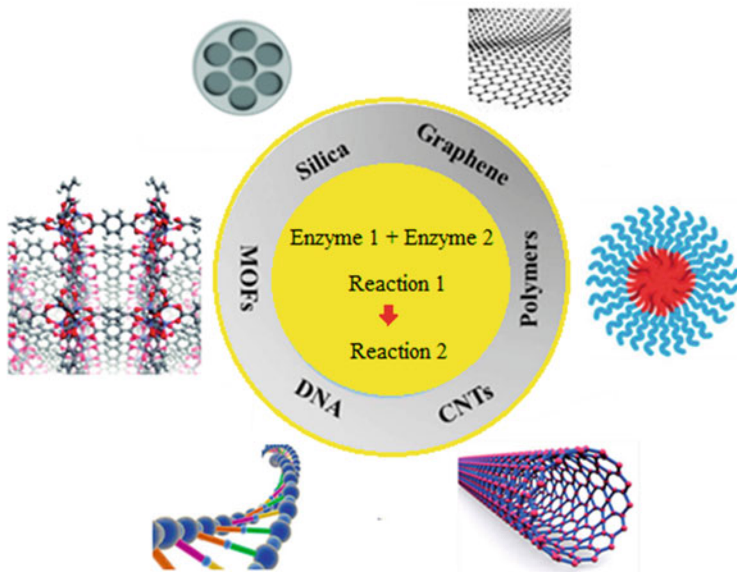


Fig. 15.3 Schematic illustration for multi-enzymes co-immobilization using supports, such as polymers, graphene, silica, MOFs, DNA, and CNTs

stability and reusability of involved enzymes. Polymers, DNA nanostructures, graphene, or CNTs are the most frequently used supports for positional multi-enzyme immobilization grace to their expedient capability to manage the relative positions of enzymes by usual reactions (Xu et al., 2020).

As an imitation of the organization of natural enzymes in cellular environments, compartmentalization can divide enzymes by varied features and ratios spatially, which can inhibit enzymes proteolysis, biological decomposition, or exposure to toxic agents (Marguet et al., 2013). As an example, a compartmentalized co-enzyme operation was created on the base of inorganic nanocrystal–protein complexes via a simple precipitation technique that showed increased overall catalytic performance in comparison to the use of free enzymes. For this purpose, the versatile enzyme horseradish peroxidase (HRP; EC 1.11.1.7), a well-known oxidoreductase, was combined with $CuSO_4$ in aqueous environment in order to form HRP-incorporated complexes; subsequently, glucose oxidase (GOx; EC 1.1.3.4) was attached on the surface of the complexes via the cooperative reaction between Cu^{2+} and protein amino acids (Li et al., 2014). Different organic and inorganic bases can be applied for immobilization of multi-enzymes as described in Fig. 15.3.

DNA nanotechnology has turned out to be a feasible method to construct complex bio-molecular nanocomposites because of the ability of programmed DNA hybridization. For immobilization of multi-enzymes, it is important to decrease the mass transfer consistency by managing the relative location and directions of a variety of enzymes in a limited space. Therefore, DNA nanotechnology has been used as an effective instrument for the multi-enzyme immobilization in

which notably addressable DNA nanocomposites can ease the suitable self-aggregation of varied enzymes and optimize the substrate penetration (Xu et al., 2020).

15.5 Kinetics of Enzyme Immobilization

Compared to measurements of the kinetics of enzymes in absolute solution, the interactions of immobilized enzymes have only scarcely been shown under spatially sole substrate and uniform conditions, such as pH-value. In addition, immobilization can change the innate kinetics of the enzyme by altering the structure of the enzyme and surrounding microenvironment. To diminish the effect of immobilization on the enzyme catalytic activity, the molecular configuration of the enzyme should be considered (Gonzalez-Saiz & Pizarro, 2001).

Immobilization usually enhances enzyme maintenance at the expense of lower catalytic function (Sheldon, 2007a; Garcia-Galan et al., 2011). Nevertheless, the appropriate choice of enzyme immobilization techniques may reduce or increase the enzyme activity (Rodrigues et al., 2013, 2011; Mateo et al., 2007). For example, enzyme immobilization on highly active supports can improve the multipoint covalent linkage and prevent the enzyme from being inactive after immobilization (Pedroche et al., 2007; Mohan et al., 2015). Similarly, the selection of enzyme loading and immobilization matrix may prefer the partition of H^+ or OH^- ions, and change the local pH-value in the support. The changed pH-value may cause the immobilized enzyme to work under conditions close to the pH optimum (Pedroche et al., 2007). The conformation of the enzyme molecule across the immobilization process may also affect its function in the presence of detergents. MCA immobilization of lipases can enhance their activity because the enzyme is immobilized in an active combination (Fernández-Lorente et al., 2006).

Omitting standard metrics for enzymatic activity assessment, such as K_m, V_{max}, and k_{cat}, or extracting separated function metrics from data sets other than interaction progress curves can be prevented using the available literature to lead to rational choose of immobilization schemes (Herzog et al., 2005; Koh & Pishko, 2005). To enhance an acceptable comparison between the studies about kinetics, minimal reporting standards (STRENDA) have been reported that contain features of model election and error diagnosis (Tipton et al., 2014; Gardossi et al., 2010).

15.6 The Usage of Enzyme Immobilization for Production of Different Food Products

Unlike pharmaceutical industries and some chemical industries, the food industry requires the production of vast quantities of commercial products. For this purpose, the cost of the biocatalyst should be lowered, thus, using immobilized enzymes show acceptable operational consistency that allows lots of repetitive production cycles to be carried out. In the food sector, continuous fermentation processes are preferred to

batch processes, especially when large quantities of material are manufactured. Examples of broad-scale usages of immobilized enzymes for production of food products are explained in the subsequent paragraphs of the chapter at hand.

15.6.1 D-Glucose/Xylose Isomerase for Production of High Fructose Corn Syrup

The use of immobilized D-glucose/xylose isomerase (EC 5.3.1.5) in the preparation of highly fructose-rich corn syrup (HFCS) shows a largely applied commercial procedure encompassing an enzyme immobilization step, with a high quantity of enzyme that is used, and expedient product yield (Crabb & Shetty, 1999). Generally, the enzyme, which belongs to the top three industrially used enzymes (others being proteases and amylases), catalyzes the interconversion of D-glucose to D-fructose and D-xylose to D-xylulose, respectively (Gaikwad et al., 1992). HFCS is predominantly used in fructose production that is applied as a sweetener for beverages and foods, or utilized directly as a food and beverage ingredient. While D-xylose is the native substrate for D-glucose/xylose isomerase function, the enzyme has a wide substrate spectrum, and in its industrial application it produces D-fructose from D-glucose in an efficient manner (Bhosale et al., 1996).

Today, HFCS production processes are performed in fixed bed reactors that are ordered in parallel and operated continuously (Nedwin et al., 2014). D-glucose syrup that is stemming from corn is converted into a mixture that is generally named as HFCS-42. This syrup contains approximately 42%, 50%, 6%, and 2% D-fructose, D-glucose, maltose, and maltotriose, respectively, as well as low quantities of other sugars. Higher concentrations of fructose that are obtained by chromatographic enrichment of the 42% syrup to a final 90% D-fructose (HFCS-90) content is used in soft drinks. The two commercially available immobilized D-glucose/xylose isomerase preparations, most commonly used for these processes, are on the basis of inexpensive inorganic supports, like bentonite clay and diatomaceous earth with cross-linking the enzyme by glutaraldehyde. The resulting composite is dewatered and then dried in a fluidized bed dryer. The obtained immobilized D-glucose/xylose isomerase preparations are very consistent, with a half-life of more than 1 year and are used in a packed bed reactor at 60 °C (Basso & Serban, 2019).

15.6.2 Epimerase for Production of Allulose

D-allulose (D-psicose or "pseudofructose") is a low-calorie monosaccharide sweetener recommended due to its sweeting similarity to dextrose, and about 70% of the sweetness of sucrose. D-allulose is the C3-epimer of D-fructose. The difference between allulose and fructose is that allulose is hardly metabolized in the human body and has almost zero calories (only 0.007 kcal/g)). Globally leading food and beverage manufacturers are the main target markets for allulose that replace dextrose, fructose or HFCS in their products, which is important to decrease calories in

parallel with maintenance of the properties obtained by the sugar ingredient, such as browning, bulking, texture, and sweetness. Allulose is also supposed to display potential antihyperglycemic effects, and was shown to prevent postprandial hyperglycemia in humans. More than 0.2 g per day human allulose intake is estimated when consuming naturally found materials, such as processed cane and beet molasses, coffee treated via steam, wheat products, and HFCS. In 2012, allulose was labeled as generally recognized as safe (GRAS) by the FDA and approved as sweetener in food products (not yet approved in, e.g., the European Union!), so it is commercially utilized as food additive in some parts of the world. Moreover, ketose-3-epimerase (EC 5.1.3.31), an isomerase which is found in various microorganisms, can interconvert fructose to allulose and vice versa (Basso & Serban, 2019).

15.6.3 β-Galactosidase for Production of Tagatose

Another emerging sweetener is the ketohexose tagatose, which can be isolated from animal origins. Tagatose is a kind of sweetener with 92% fructose sweetness, but contains only 38% of fructose calories. Its catabolic route is different from sucrose; therefore, it has insignificant effect on insulin and blood glucose levels. Moreover, tagatose is considered a “tooth-friendly” compound for dental care products. Tagatose can be gained from lactose with only 16% of the sweetening of the later. The disaccharide lactose (β-D-galactopyranosyl-(1 → 4)-D-glucose) is a natural sugar found in milk and normally makes up 2–8% of its total mass. The process of tagatose production utilizing the immobilized glucosidase β-galactosidase (3.2.1.23) has been reported in 2014. This process suggests preparation and valorization of lactose present in whey at a concentration of 18 wt.-%. It is possible to obtain tagatose by lactose hydrolysis using immobilized β-galactosidase to produce the monomers glucose and galactose. Then glucose is removed from the mixture by deglycosylation using baker’s yeast; now, tagatose is obtained by epimerization of galactose with aerated $Ca(OH)_2$ (Basso & Serban, 2019). There have also been reports on the utilization of immobilized L-arabinose isomerase (EC 5.3.1.4) to obtain tagatose in stirred tank reactors or continuous flow systems (Lim et al., 2008; Oh, 2007).

15.6.4 Other Sweeteners Produced by Immobilized Enzymes

Another sweetener for food and beverage industry, also accessible by means of biocatalysis, is the canonic amino acid L-aspartate (2-aminobutanedioic acid). Aspartate production is performed by amination of fumaric acid; this reaction is catalyzed by the lyase enzyme aspartase (aspartate ammonia-lyase, EC 4.3.1.1). Already in 1973, Tosa et al. reported the application of aspartase from *Escherichia coli* for aspartate production from ammonium fumarate; these authors immobilized the enzyme by different methods: ionic binding on cellulose derivatives or sephadex,

physical adsorption to silica gel or calcium phosphate gel, covalent binding to cellulose azide or diazonium derivatives of cellulose, or entrapping in polyacrylamide gel lattice. The authors obtained the most active immobilized aspartase by the entrapping process; regarding optimum pH-value, temperature, and ion concentration, no differences were observed between the immobilized and free enzyme in terms of kinetic constants and heat stability. Excellent conversion yields were reported for this process when operated in continuous mode using columns packed with the immobilized aspartase (Tosa et al., 1973). Later, the same group of authors immobilized *E. coli* entrapped in a polyacrylamide gel lattice as whole-cell biocatalyst for continuous aspartate production in a packed column reactor (Tosa et al., 1974). Currently, aspartate is produced on an annual scale of 10^4 tons by cross-linked whole cells or by the isolated enzyme immobilized by different methods (reviewed by DiCosimo et al., 2013).

Moreover, aspartame is another sweetener produced as a dipeptide of L-aspartate and L-phenylalanine. For this purpose, in 1981, Oyama et al. immobilized the hydrolase enzyme thermolysin (EC 3.4.24.4) by different methods: physical adsorption to Amberlite XAD-7 and XAD-8, ionic binding to the ionic ion exchange resin Amberlite IRA-94, adsorption on glass beads, and covalent linking to a hydrophilic ethylenediamine-derivatized polymer gel by glutaraldehyde. The immobilized enzyme assays prepared via these different ways were tested for formation of aspartame by reaction of the precursors N-(benzyloxycarbonyl)-L-aspartic acid with L-phenylalanine methyl ester by incubating the mixture of the substrates in the solvent ethyl acetate, which normally would denature the enzyme. Substrates moved from the organic phase to the aqueous phase in the support, where the reaction took place, and the product (aspartame) diffused back to the organic phase, from which it could be recovered. Among the different tested immobilization strategies, physical adsorption to Amberlite XAD-7 and XAD-8 resulted in the best yields (Oyama et al., 1981). Later, this immobilization technique for thermolysin was successfully used by Miyanaga et al. for continuous production of the aspartame precursor N-(benzyloxycarbony1)-L-aspartyl-L-phenylalanine methyl ester from N-(benzyloxycarbony1)-L-aspartic acid and L-phenylalanine methyl ester in a mixed organic solvent system consisting of *tert*-amyl alcohol and ethyl acetate in a column reactor. Here, excellent conversion yields of 99% were achieved under optimized conditions (Miyanaga et al., 1995).

15.6.5 Enzyme Immobilization for Debittering of Citrus Fruit Juices

The polyphenol naringin, a flavonoid, is responsible for the bitter taste of citrus fruits. There is increased interest in fruit juice industry by using highly efficient immobilized enzymes for debittering of citrus fruit juices (Puri et al., 2008). Already in 1979, Olson and co-workers reported the immobilization of commercially available naringinase (mixture of the hydrolases α-rhamnosidase, EC 3.2.1.40, and β-glucosidase, EC 3.2.1.21; hydrolyzes naringin to naringenin, glucose, and rhamnose) in a reactor system consisting of polysulfone hollow fibers; immobilization

took place by ultrafiltration of the enzymes into the sponge region of the hollow fibers. After 210 min of continuous operation, 50% of naringin contained in grapefruit juice was hydrolyzed at 25 °C and a flow rate of 300 mL/min (Olson et al., 1979). Other approaches to immobilize naringinase encompass entrapping in alginate, which resulted in 60% debittering of kinnow juice after 3 h when using a total enzyme activity of 30 U (Puri et al., 1996), immobilization on electrospun cellulose acetate nanofibers (Huang et al., 2017), on chitin (Tsen, 1984) or chitosan microspheres (Bodakowska-Boczniewicz & Garncarek, 2019) by linking with glutaraldehyde, or adsorption of the enzyme on mesoporous molecular sieves via glutaraldehyde for naringin hydrolysis in white grapefruit juice (Lei et al., 2011). A rather bizarre protocol for naringinase immobilization was developed by Puri and colleagues, who attached the enzyme on chicken egg white beads obtained by cross-linking the protein with glutaraldehyde; debittering of Kinnow juice achieved an efficiency of 68% (Puri et al., 2001). In addition, Busto et al. immobilized thermophilic *Aspergillus niger* naringinase by entrapping it into a PVA hydrogel matrix, which was cryostructured in liquid nitrogen, to generate beads biocatalytically active for naringin hydrolysis. Authors reported high stability of the beads; after storage at 4 °C for 2 months, they retained 75% of initial activity (Busto et al., 2007).

15.6.6 Lipases for Production of Vitamin C Esters and Cocoa Butter Analogs

One of the main water-soluble natural antioxidants is L-ascorbic acid (vitamin C). L-ascorbic acid and its derivatives act as free radical scavengers, reacting with oxygen, and destroying it. Moreover, hydrophobic long-chain fatty acid ester derivatives of L-ascorbic acid are used as antioxidants in fat-rich food because of their higher ability to dissolve in fats in comparison to the typical hydrophilic compound vitamin C, which is insoluble in oils (Burham et al., 2009). In this context, ascorbic palmitate and stearate are currently prepared by reaction between ascorbic acid with sulfuric acid, followed by re-esterification with the corresponding fatty acid; finally, a purification step by re-crystallization is carried out (Ferreira-Dias et al., 2013). In a biocatalytic approach, immobilization of *Candida antarctica* B lipase (CalB) was utilized for generation of ascorbyl esters. The biocatalytic conversion can reach a yield of approximately 95%, depending on process temperature, the level of removal of the side product water, and fatty acid chain length. In spite of the fact that enzymatic synthesis suggests some benefits to the current chemical procedures, such as interaction in the lower temperatures than chemical reactions temperatures, higher material purity, and decreased downstream processing expenditure, many of the manufactures of ascorbyl esters still carry out this synthesis by chemical processes, because of the long interaction time needed by the enzymatic procedure and the high price of the immobilized enzymes in contrast to the chemical catalysts (Villeneuve, 2007).

The source of vegetable oils, such as palm, rapeseed, canola, and sunflower, specifies the physical characteristics of fats and oils present in food products,

because each oil has a different arrangement and type of saturated mono- and polyunsaturated fatty acids in the 1, 2, and 3 locations of triacylglycerides. To obtain the suitable melting properties of fats and oils, especially in the generation of margarine and baking fat, chemical hydrogenation, fractioning, and esterification have been applied. The enzymatic transesterification of food oils and fats is one of the benefits because of the option to better monitor the product composition compared to chemically transesterified products due to the removal of the hydrogenated trans fats that have important health challenges (Marangoni & Rousseau, 1998; Asif, 2011).

Enzymatic transesterification was first investigated to produce an equivalent of cocoa that used the sn-1,3 specificity of different fungal lipases. Cocoa butter homologs are semisolid oils that commonly have a melting temperature of 37 °C. They are obtained from more cost-effective origins than cocoa, like palm, sunflower, or rapeseed oil. A variety of commercial processes have been developed to produce the equivalent of cocoa butter with elevated amount of the demanded triglycerides, 1(3)-palmitoyl-3(1)-stearoyl-2-monooleine, and 1,3-distearoyl-2-monooleine, required for chocolate production. Most systems are made by using fungal lipases immobilized by surface adsorption or encapsulation in liposomes (Basso & Serban, 2019).

15.6.7 β-Galactosidase for Lactose Hydrolysis

Bovine milk contains 4.3–4.5 wt.-% lactose that exposes 38–40% of the whole milk solids. Lactose in milk and milk products is not hydrolyzed in the stomach or in the initial part of the small intestine; it enters to other parts of the intestine and gets hydrolyzed into the monosaccharides D-galactose and D-glucose by the glycosidase β-galactosidase (lactase, EC 3.2.1.23) excreted by the intestinal microflora. About 65% of the entire human population (up to 90% in some Asian countries) are unable to secrete sufficient quantities of β-galactosidase, causing many health disorders. Elimination of lactose from milk and milk products makes them suitable for consumption by people with lactose intolerance (hypolactasia), so the dairy industry has demonstrated great interest to develop advanced lactose hydrolysis processes based on β-galactosidase. Because the sweetening potential of lactose, glucose, and galactose is 20, 70, and 58%, respectively, of sucrose, lactose-hydrolyzed milk is sweeter than pristine milk (Panesar et al., 2010).

The simplest but most expensive solution for this problem is to add free β-galactosidase to whole milk. Enzyme activity is stopped after complete substrate hydrolysis, typically combined with pasteurization. Another procedure is the usage of immobilized β-galactosidase for processing of skimmed milk; after completion of hydrolysis, the fat fraction is added again to the hydrolyzed milk to reassemble its nutritious components. This technique, of course, displays the benefits of recycling and reusing the immobilized enzyme in contrast to adding free enzyme, and the final product is free from additional ingredients, like enzymes or components or the enzyme formulation that can constitute putative allergens.

The techno-economic evaluation of lactose removal by immobilized β-galactosidase from the fungus *Aspergillus niger* dates back as far as to 1990; that time, Axelsson and Zacchi calculated the cost for a tank reactor operated in batch mode, free and immobilized β-galactosidase, a continuously operated stirred tank reactor (CSTR), and for a plug-flow tubular reactor (PFTR). For all cases, the mass transfer behavior and enzyme deactivation were considered. As outcome, the authors concluded that enzyme immobilization indeed is economically more feasible when compared with application of free enzymes, although the high cost for enzyme immobilization itself still constitute an obstacle. The lowest cost of 0.48 Swedish crowns (SEK) per kg lactose to be hydrolyzed, calculated for a half-life time of 80 days, were calculated for immobilized enzyme in the PFTR; however, calculated costs for using immobilized β-galactosidase in a batch reactor were only insignificantly higher with 0.66 SEK/kg lactose. In any case, these two modes of using immobilized enzyme were considerably lower in costs than for the case of free enzyme in a batch reactor (2.10 SEK/kg lactose) (Axelsson & Zacchi, 1990).

In 2003, Roy and Gupta used the commercially available β-galactosidase preparation Lactozym™ (Novozymes, Denmark) from the yeast *Kluyveromyces fragilis*. This preparation is GRAS for hydrolysis of whey to produce lactose-poor milk. The authors immobilized Lactozym™ on cellulose beads via covalent epichlorohydrin coupling. In a column serving as fluidized bed reactor, whey lactose was hydrolyzed by $>90\%$ within 5 h, while the same enzyme, when applied in continuous batch mode, took 48 h for the same hydrolysis outcome. It was possible to reuse the immobilized enzyme three times without decrease in the biocatalytic performance of the fluidized bed reactor column. In the same fluidized bed reactor, also lactose in whole milk was converted to glucose and galactose up to 60% within 5 h (Roy & Gupta, 2003).

A more recent example for the use of an immobilized β-galactosidase is the enzyme isolated from the yeast *Saccharomyces lactis*. This enzyme was immobilized by entrapment in cellulose triacetate fibers. The entrapped enzyme was reused for 50 times with the reduction of enzyme activity less than 9% in a rotary horizontal column reactor; 10 tons milk were processed per day via this process on industrial scale (reviewed by Basso & Serban, 2019). Moreover, *Aspergillus oryzae* β-galactosidase was immobilized by covalently binding the enzyme to an ion exchange resin based on polyphenolic formaldehyde (Hirohara et al., 1981; reviewed by Basso & Serban, 2019).

15.7 The Usage of Immobilized Enzymes in Transforming Food Waste

A notable amount (approximately 40%) of all types of food are disposed as waste (Godfray et al., 2010), and these losses not only lead to environmental pollution but also affect the entire food chain. This amount varies between different geographic regions, and one should differentiate between food waste *sensu stricto* and agricultural waste. These waste streams are responsible for a major global challenge both in

causing environmental pollution and ethical concern considering the huge number of people starving worldwide. With the global population expected to increase to 9.8 billion until 2050, suitable technological solutions should be developed to solve this problem. Some technical proposals are represented at the food processing level. Liquid food processing waste contains numerous organic carbonaceous compounds; therefore, it has high biological oxygen demand (BOD) that causes problems for direct disposal of them to wastewater removal plants. Here, the disposal of about one million liters of lactose-rich whey per day only on the Northern Italian region constitutes a prime example (Koller et al., 2016). Hence, the lipid, carbohydrate, and protein contents of food and agricultural waste liquids are leading to high BOD; however, at the same time, they have the potential to be converted to valuable products, thus upgrading waste liquids into potential recoverable sources. Examples of such conversions include oxidation, hydrolysis, acylation, and phosphorylation of carbohydrates as well as glycosylation and deamination of amino acids, and esterification and hydrogenation of lipids. In particular, esterification processes are widely used for production of different value-added food and agricultural products. Waste oils and animal waste lipids from the slaughtering and rendering industry can be transesterified with alcohol to generate biofuels (Koller et al., 2018). Esterified sugars can be applied as surfactants, and esterified starch may be used as biodegradable plastics and adhesives. Esterification of flavonoids was reported to increase their life time, health, and acceptance characteristics (Walle, 2009). Traditional processes to these transformations require significant amounts of chemical catalysts and energy resources that have limited reactivity, and lead to formation of by-products, especially when done in complex matrices, like food waste liquids (Alissandratos & Halling, 2012; Fang et al., 2002).

15.7.1 Carbohydrate Wastes

Food processing waste streams which are carbohydrate-rich can easily be converted by the enzymatic valorization catalyzed by hydrolases and isomerases into more valuable products, like sweeteners and prebiotics. In fact, some of the best accepted, well-known procedures in food and agricultural systems begin with the use of carbohydrate substrates. In this context, immobilized thermophilic enzymes ("thermozymes"), which have been studied for the production of high fructose corn syrup, could be progressed and used for valorization of food waste liquids that are carbohydrate rich (Andler & Goddard, 2018). Emtiazi et al. (2001) used immobilized cellulase enzymes from *Aspergillus terreus* to decrease chemical oxygen demand (COD) by cellulose removal (40–80%) from pulp manufacturing waste.

15.7.2 Lipid Wastes

According to the significant impact of environmental and economic waste stream valorization, waste oil can be converted to value-added products, such as biodiesel,

surfactants, and lubricants, by the use of enzymes. Lipases, like most other enzymes, can be mined from different microbial sources with different performance properties. For instance, lipases produced by *Thermomyces lanuginosus* and *Candida antarctica* were used for lipid hydrolysis and esterification, respectively, yielding valuable products, such as biofuel, from waste cooking oil. More than 90% conversion was obtained after 10 h of hydrolysis and 10 h of esterification reactions. Noteworthy, after 5 catalysis repeats, the lipase from *C. antarctica* retained its activity, while the lipase from *T. lanuginosus* lost some of its activity after each use (Vescovi et al., 2016). In another study, *Rhizomucor miehei* lipase and *C. antarctica* lipases were immobilized on silica particles that were epoxy-functionalized and used to enhance the performance of biofuel generation from waste cooking oil. A 91.5% conversion rate was achieved during 10 h (Babaki et al., 2017).

Other examples for immobilization of *C. antarctica* lipase for conversion of lipid substrates to value-added products encompass the use of support materials as diverse as core–shell MNPs for conversion of waste cooking oil to biodiesel (Mehrasbi et al., 2017), immobilization by adsorption on poly (styrene) nanoparticles (Miletić et al., 2010), covalent attachment on chitosan-based hydrogels (Silva et al., 2012), or adsorption to green coconut fibers (Brígida et al., 2007).

15.7.3 Proteinaceous Wastes

Proteinaceous food waste may stem from different origins like dairy products (whey retentate), grains (Zhi et al., 2017), oilseeds (Doshi et al., 2014), soybeans (Laskar et al., 2018), eggs (Hong et al., 2019), or even poultry feathers (Pernicova et al., 2019). Proteases are used for hydrolyzing proteins from waste streams and converting them to biological peptides or useful chemicals, such as the monomers that build up polymers. Here, enzymatic pathways are more favorable than chemical reactions, which are not easily controllable, as observed for the degradation of tryptophan via acid hydrolysis (Kumar et al., 2015). Immobilized trypsin has been used for hydrolysis of dairy waste, such as whey protein (retentate fraction remaining after ultrafiltration of full whey), as an alternative to well-established acidic hydrolysis (Koller et al., 2019). Immobilization of bovine pancreas trypsin on porous polymethacrylate with a pore size of 2.1 μm has led to 9.68% hydrolysis. The hydrolysis degree had reached ~6% under the same conditions when using free trypsin, which indicates the need for optimization of the immobilization process. Most significantly, the peptide analysis differed between the immobilized and free trypsin that showed the effect of immobilization methods on enzyme selectivity for hydrolysis of amino acid sequences (Mao et al., 2017). Trypsin has also been immobilized on reusable matrices containing spent grain and lignocellulose for hydrolysis of whey protein (Tavano, 2013; Bassan et al., 2016).

15.8 Commercialization of Immobilized Enzymes for Usage in Food Industries

For successful commercialization of procedures based on immobilized enzymes, the overall process cost is the decisive factor. Some of the major factors determining the cost of immobilization, such as the used matrix (support), the enzyme itself, the chemicals used in the immobilization procedure, and the special equipment that may be needed should be considered. Current requests for immobilized enzymes make up only a small portion of the whole enzyme market (DiCosimo et al., 2013). Different uses of immobilized enzymes may be developed in food manufacturing, medical applications, and food research studies (Homaei et al., 2013). Other examples for commercial usages of immobilized enzymes are synthesis of organic materials on laboratory scale as well as analytical and pharmaceutical usage. Moreover, apart from the food sector, the application of immobilized enzymes for removal of eco-pollutants from aqueous environment might be an important future field of research and development, as it was suggested for the removal of endocrine-disrupting compounds from wastewater by covalently immobilizing HRP on filtration membranes (Mohan et al., 2015; Rathner et al., 2017). For each commercial application, the appropriate selection of a particular immobilized enzyme or strategy of immobilization should be based on the advantages and disadvantages of the performance of free and immobilized enzymes, respectively. This shows that the establishment and use of immobilized enzymes needs profound understanding of affecting functional and economic factors, in-depth kinetic studies, plus the knowledge about the current market requirements and trends (DiCosimo et al., 2013).

15.9 Conclusions

Enzymes usage in soluble form for food processing is well established. Although a high variety of enzymes have already been immobilized and used in different food manufacturing industries, only few procedures have become practical and economical, and succeeded in getting established on the long term. Numerous recent concepts have been attempted or are being used in this field. The future of such applications and operations will depend mainly on their cost and also political decisions. Despite the fact that not all established transformations involved in food processing and food production can to date be replaced by biocatalytic techniques resorting to immobilized enzymes, and although many immobilization processes holding promise in lab-scale experiments are not yet scalable to industrial dimensions, the outlook for immobilized enzymes in food industry is indeed promising considering current food industry trends to become more efficient and sustainable, combined with the rapid progression of immobilized enzyme techniques. In any case, due to the growing human population on Earth and a remarkable decrease in limited natural food resources, the future use of immobilized enzymes may elevate significantly in order to produce higher amounts of food products, and even to unlock alternative food sources for enhanced global food security.

References

Albayrak, N., & Yang, S. T. (2002). Immobilization of β-galactosidase on fibrous matrix by polyethyleneimine for production of galacto-oligosaccharides from lactose. *Biotechnology Progress, 18*(2), 240–251.

Alissandratos, A., & Halling, P. J. (2012). Enzymatic acylation of starch. *Bioresource Technology, 115*, 41–47.

Alkan, S., Gür, A. Y. C. A. N., Ertan, M., Savran, A., Gür, T., & Genel, Y. (2009). Immobilization of catalase via adsorption into natural and modified active carbon obtained from walnut in various methods. *African Journal of Biotechnology, 8*(11).

Amirbandeh, M., & Taheri-Kafrani, A. (2016). Immobilization of glucoamylase on triazine-functionalized Fe3O4/graphene oxide nanocomposite: Improved stability and reusability. *International Journal of Biological Macromolecules, 93*, 1183–1191.

Andler, S. M., & Goddard, J. M. (2018). Transforming food waste: How immobilized enzymes can valorize waste streams into revenue streams. *NPJ Science of Food, 2*(1), 1–11.

Antony, N., Balachandran, S., & Mohanan, P. V. (2016). Immobilization of diastase α-amylase on nano zinc oxide. *Food Chemistry, 211*, 624–630.

Asgher, M., Shahid, M., Kamal, S., & Iqbal, H. M. N. (2014). Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. *Journal of Molecular Catalysis B: Enzymatic, 101*, 56–66.

Asif, M. (2011). Process advantages and product benefits of interesterification in oils and fats. *International Journal of Nutrition, Pharmacology, Neurological Diseases, 1*(2), 134.

Axelsson, A., & Zacchi, G. (1990). Economic evaluation of the hydrolysis of lactose using immobilized β-galactosidase. *Applied Biochemistry and Biotechnology, 24*(1), 679.

Babaki, M., Yousefi, M., Habibi, Z., & Mohammadi, M. (2017). Process optimization for biodiesel production from waste cooking oil using multi-enzyme systems through response surface methodology. *Renewable Energy, 105*, 465–472.

Bassan, J. C., de Souza Bezerra, T. M., Peixoto, G., Da Cruz, C. Z. P., Galán, J. P. M., Vaz, A. B. D. S., et al. (2016). Immobilization of trypsin in lignocellulosic waste material to produce peptides with bioactive potential from whey protein. *Materials, 9*(5), 357.

Basso, A., & Serban, S. (2019). Industrial applications of immobilized enzymes—A review. *Molecular Catalysis, 479*, 110607.

Bhosale, S. H., Rao, M. B., & Deshpande, V. V. (1996). Molecular and industrial aspects of glucose isomerase. *Microbiological Reviews, 60*(2), 280–300.

Bilal, M., & Iqbal, H. M. (2019). Sustainable bioconversion of food waste into high-value products by immobilized enzymes to meet bio-economy challenges and opportunities–A review. *Food Research International, 123*, 226–240.

Blanco, R. M., Terreros, P., Fernández-Pérez, M., Otero, C., & Díaz-González, G. (2004). Functionalization of mesoporous silica for lipase immobilization: Characterization of the support and the catalysts. *Journal of Molecular Catalysis B: Enzymatic, 30*(2), 83–93.

Bodakowska-Boczniewicz, J., & Garncarek, Z. (2019). Immobilization of naringinase from *Penicillium decumbens* on chitosan microspheres for debittering grapefruit juice. *Molecules, 24*(23), 4234.

Boller, T., Meier, C., & Menzler, S. (2002). Eupergit oxirane acrylic beads: How to make enzymes fit for biocatalysis. *Organic Process Research & Development, 6*(4), 509–519.

Brady, D., & Jordaan, J. (2009). Advances in enzyme immobilization. *Biotechnology Letters, 31*(11), 1639.

Brígida, A. I., Pinheiro, Á. D., Ferreira, A. L., & Gonçalves, L. R. (2007). Immobilization of *Candida antarctica* lipase B by adsorption to green coconut fiber. In *Biotechnology for fuels and chemicals* (pp. 293–307). Humana Press.

Burham, H., Rasheed, R. A. G. A., Noor, N. M., Badruddin, S., & Sidek, H. (2009). Enzymatic synthesis of palm-based ascorbyl esters. *Journal of Molecular Catalysis B: Enzymatic, 58*(1–4), 153–157.

Busto, M. D., Meza, V., Ortega, N., & Perez-Mateos, M. (2007). Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly (vinyl alcohol) cryogels for the debittering of juices. *Food Chemistry, 104*(3), 1177–1182.

Chen, Y., Yu, B., Lin, J., Naidu, R., & Chen, Z. (2016). Simultaneous adsorption and biodegradation (SAB) of diesel oil using immobilized *Acinetobacter venetianus* on porous material. *Chemical Engineering Journal, 289*, 463–470.

Cirpan, A., Alkan, S., Toppare, L. E. V. E. N. T., Hepuzer, Y., & Yagci, Y. (2003). Immobilization of invertase in conducting copolymers of 3-methylthienyl methacrylate. *Bioelectrochemistry, 59*(1–2), 29–33.

Costa, S. A., Azevedo, H. S., & Reis, R. L. (2005). Enzyme immobilization in biodegradable polymers for biomedical applications. In R. L. Reis & J. S. Roma (Eds.), *Biodegradable systems in tissue engineering and regenerative medicine* (pp. 301–323). CRC Press, LLC.

Crabb, W. D., & Shetty, J. K. (1999). Commodity scale production of sugars from starches. *Current Opinion in Microbiology, 2*(3), 252–256.

Deere, J., Magner, E., Wall, J. G., & Hodnett, B. K. (2002). Mechanistic and structural features of protein adsorption onto mesoporous silicates. *The Journal of Physical Chemistry B, 106*(29), 7340–7347.

Defaei, M., Taheri-Kafrani, A., Miroliaei, M., & Yaghmaei, P. (2018). Improvement of stability and reusability of α-amylase immobilized on naringin functionalized magnetic nanoparticles: A robust nanobiocatalyst. *International Journal of Biological Macromolecules, 113*, 354–360.

De Maio, A., El-Masry, M. M., Portaccio, M., Diano, N., Di Martino, S., Mattei, A., et al. (2003). Influence of the spacer length on the activity of enzymes immobilised on nylon/polyGMA membranes: Part 1. Isothermal conditions. *Journal of Molecular Catalysis B: Enzymatic, 21*(4–6), 239–252.

Dey, G., Nagpal, V., & Banerjee, R. (2002). Immobilization of α-amylase from Bacillus circulans GRS 313 on coconut fiber. *Applied Biochemistry and Biotechnology, 102*(1–6), 303–313.

DiCosimo, R., McAuliffe, J., Poulose, A. J., & Bohlmann, G. (2013). Industrial use of immobilized enzymes. *Chemical Society Reviews, 42*(15), 6437–6474.

Doshi, P., Srivastava, G., Pathak, G., & Dikshit, M. (2014). Physicochemical and thermal characterization of nonedible oilseed residual waste as sustainable solid biofuel. *Waste Management, 34*(10), 1836–1846.

Emtiazi, G., Naghavi, N., & Bordbar, A. (2001). Biodegradation of lignocellulosic waste by Aspergillus terreus. *Biodegradation, 12*(4), 257–261.

Fang, J. M., Fowler, P. A., Tomkinson, J., & Hill, C. A. S. (2002). The preparation and characterisation of a series of chemically modified potato starches. *Carbohydrate Polymers, 47*(3), 245–252.

Fernández-Lorente, G., Palomo, J. M., Mateo, C., Munilla, R., Ortiz, C., Cabrera, Z., et al. (2006). Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance. *Biomacromolecules, 7*(9), 2610–2615.

Ferreira-Dias, S., Sandoval, G., Plou, F., & Valero, F. (2013). The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries. *Electronic Journal of Biotechnology, 16*(3), 12–12.

Gaikwad, S. M., Rao, M. B., & Deshpande, V. V. (1992). d-Glucose/xylose isomerase from Streptomyces: Differential roles of magnesium and cobalt ions. *Enzyme and Microbial Technology, 14*(4), 317–320.

Gardossi, L., Poulsen, P. B., Ballesteros, A., Hult, K., Švedas, V. K., Vasić-Rački, Đ., et al. (2010). Guidelines for reporting of biocatalytic reactions. *Trends in Biotechnology, 28*(4), 171–180.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis & Catalysis, 353*(16), 2885–2904.

Gerard, M., Chaubey, A., & Malhotra, B. D. (2002). Application of conducting polymers to biosensors. *Biosensors and Bioelectronics, 17*(5), 345–359.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., et al. (2010). Food security: The challenge of feeding 9 billion people. *Science, 327*(5967), 812–818.

Gonzalez-Saiz, J. M., & Pizarro, C. (2001). Polyacrylamide gels as support for enzyme immobilization by entrapment. Effect of polyelectrolyte carrier, pH and temperature on enzyme action and kinetics parameters. *European Polymer Journal, 37*(3), 435–444.

Herzog, G., Gorgy, K., Gulon, T., & Cosnier, S. (2005). Electrogeneration and characterization of photoactivable films and their application for enzyme grafting. *Electrochemistry Communications, 7*(8), 808–814.

Hirohara, H., Yamamoto, H., Kawano, E., & Nabeshima, S. (1981) *Immobilized lactase, its preparation and use*. EP 0037667B1.

Homaei, A. A., Sariri, R., Vianello, F., & Stevanato, R. (2013). Enzyme immobilization: An update. *Journal of Chemical Biology, 6*(4), 185–205.

Hong, Y. G., Moon, Y. M., Hong, J. W., Choi, T. R., Jung, H. R., Yang, S. Y., Jang, D.-W., Park, Y.-R., Brigham, C. J., Kim, J.-S., Lee, Y.-K., & Lee, Y. K. (2019). Discarded egg yolk as an alternate source of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate). *Journal of Microbiology and Biotechnology, 29*(3), 382–391.

Huang, W., Zhan, Y., Shi, X., Chen, J., Deng, H., & Du, Y. (2017). Controllable immobilization of naringinase on electrospun cellulose acetate nanofibers and their application to juice debittering. *International Journal of Biological Macromolecules, 98*, 630–636.

Hudson, S., Cooney, J., & Magner, E. (2008). Proteins in mesoporous silicates. *Angewandte Chemie International Edition, 47*(45), 8582–8594.

Hwang, E. T., & Lee, S. (2019). Multienzymatic cascade reactions via enzyme complex by immobilization. *ACS Catalysis, 9*(5), 4402–4425.

Ispas, C., Sokolov, I., & Andreescu, S. (2009). Enzyme-functionalized mesoporous silica for bioanalytical applications. *Analytical and Bioanalytical Chemistry, 393*(2), 543–554.

Kahraman, M. V., Bayramoğlu, G., Kayaman-Apohan, N., & Güngör, A. (2007). α-Amylase immobilization on functionalized glass beads by covalent attachment. *Food Chemistry, 104*(4), 1385–1392.

Katchalski-Katzir, E., & Kraemer, D. M. (2000). Eupergit® C, a carrier for immobilization of enzymes of industrial potential. *Journal of Molecular Catalysis B: Enzymatic, 10*(1–3), 157–176.

Kazemzadeh, S., Naghavi, N. S., Emami-Karvani, Z., Fouladgar, M., & Emtiazi, G. (2020). Gas chromatography-mass spectrometry analyses of crude oil bioremediation by the novel *Klebsiella variicola* SKV2 immobilized in polyurethane polymer scaffold and two-layer microcapsulation. *Bioremediation Journal, 24*(2–3), 129–149.

Keller, T. M., Michel, S. C., Fröhlich, J., Fink, D., Caduff, R., Marincek, B., & Kubik-Huch, R. A. (2004). USPIO-enhanced MRI for preoperative staging of gynecological pelvic tumors: Preliminary results. *European Radiology, 14*(6), 937–944.

Klein, M. P., Scheeren, C. W., Lorenzoni, A. S. G., Dupont, J., Frazzon, J., & Hertz, P. F. (2011). Ionic liquid-cellulose film for enzyme immobilization. *Process Biochemistry, 46*(6), 1375–1379.

Klis, M., Karbarz, M., Stojek, Z., Rogalski, J., & Bilewicz, R. (2009). Thermoresponsive poly (N-isopropylacrylamide) gel for immobilization of laccase on indium tin oxide electrodes. *The Journal of Physical Chemistry B, 113*(17), 6062–6067.

Klouda, L., & Mikos, A. G. (2008). Thermoresponsive hydrogels in biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics, 68*(1), 34–45.

Kluchova, K., Zboril, R., Tucek, J., Pecova, M., Zajoncova, L., Safarik, I., et al. (2009). Superparamagnetic maghemite nanoparticles from solid-state synthesis–Their functionalization towards peroral MRI contrast agent and magnetic carrier for trypsin immobilization. *Biomaterials, 30*(15), 2855–2863.

Koh, W. G., & Pishko, M. (2005). Immobilization of multi-enzyme microreactors inside microfluidic devices. *Sensors and Actuators B: Chemical, 106*(1), 335–342.

Koller, M., Hesse, P., & Braunegg, G. (2019). Application of whey retentate as complex nitrogen source for growth of the polyhydroxyalkanoate producer *Hydrogenophaga pseudoflava* strain DSM1023. *The EuroBiotech Journal, 3*(2), 78–89.

Koller, M., Marsalek, L., & Braunegg, G. (2016). PHA Biopolyester production from surplus whey: Microbiological and engineering aspects. *Recent Advances in Biotechnology, 1*, 100–172.

Koller, M., Shahzad, K., & Braunegg, G. (2018). Waste streams of the animal-processing industry as feedstocks to produce polyhydroxyalkanoate biopolyesters. *Applied Food Biotechnology, 5*(4), 193–203.

Kooi, M. E., Cappendijk, V. C., Cleutjens, K. B. J. M., Kessels, A. G. H., Kitslaar, P. J. E. H. M., Borgers, M., et al. (2003). Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging. *Circulation, 107*(19), 2453–2458.

Koszelewski, D., Müller, N., Schrittwieser, J. H., Faber, K., & Kroutil, W. (2010). Immobilization of ω-transaminases by encapsulation in a sol–gel/celite matrix. *Journal of Molecular Catalysis B: Enzymatic, 63*(1–2), 39–44.

Kouassi, G. K., Irudayaraj, J., & McCarty, G. (2005). Activity of glucose oxidase functionalized onto magnetic nanoparticles. *BioMagnetic Research and Technology, 3*(1), 1.

Krause, M. H., Kwong, K. K., Gragoudas, E. S., & Young, L. H. (2004). MRI of blood volume with superparamagnetic iron in choroidal melanoma treated with thermotherapy. *Magnetic Resonance Imaging, 22*(6), 779–787.

Kumar, M. B., Gao, Y., Shen, W., & He, L. (2015). Valorisation of protein waste: An enzymatic approach to make commodity chemicals. *Frontiers of Chemical Science and Engineering, 9*(3), 295–307.

Landarani-Isfahani, A., Taheri-Kafrani, A., Amini, M., Mirkhani, V., Moghadam, M., Soozanipour, A., & Razmjou, A. (2015). Xylanase immobilized on novel multifunctional hyperbranched polyglycerol-grafted magnetic nanoparticles: An efficient and robust biocatalyst. *Langmuir, 31*(33), 9219–9227.

Laskar, I. B., Rajkumari, K., Gupta, R., Chatterjee, S., Paul, B., & Rokhum, L. (2018). Waste snail shell derived heterogeneous catalyst for biodiesel production by the transesterification of soybean oil. *RSC Advances, 8*(36), 20131–20142.

Lei, S., Xu, Y., Fan, G., Xiao, M., & Pan, S. (2011). Immobilization of naringinase on mesoporous molecular sieve MCM-41 and its application to debittering of white grapefruit. *Applied Surface Science, 257*(9), 4096–4099.

Li, Z., Zhang, Y., Su, Y., Ouyang, P., Ge, J., & Liu, Z. (2014). Spatial co-localization of multienzymes by inorganic nanocrystal–protein complexes. *Chemical Communications, 50*(83), 12465–12468.

Lim, B. C., Kim, H. J., & Oh, D. K. (2008). Tagatose production with pH control in a stirred tank reactor containing immobilized L-arabinose isomerase from Thermotoga neapolitana. *Applied Biochemistry and Biotechnology, 149*(3), 245–253.

Liu, R., Huang, W., Pan, S., Li, Y., Yu, L., & He, D. (2020). Covalent immobilization and characterization of penicillin G acylase on magnetic Fe2O3/Fe3O4 heterostructure nanoparticles prepared via a novel solution combustion and gel calcination process. *International Journal of Biological Macromolecules, 162*, 1587–1596.

Lozinsky, V. I., Simenel, I. A., Kulakova, V. K., Kurskaya, E. A., Babushkina, T. A., Klimova, T. P., et al. (2003). Synthesis and studies of N-vinylcaprolactam/N-vinylimidazole copolymers that exhibit the "proteinlike" behavior in aqueous media. *Macromolecules, 36*(19), 7308–7323.

Luo, X., & Zhang, L. (2010). Immobilization of penicillin G acylase in epoxy-activated magnetic cellulose microspheres for improvement of biocatalytic stability and activities. *Biomacromolecules, 11*(11), 2896–2903.

Marangoni, A. G., & Rousseau, D. (1998). Chemical and enzymatic modification of butterfat and butterfat-canola oil blends. *Food Research International, 31*(8), 595–599.

Marguet, M., Bonduelle, C., & Lecommandoux, S. (2013). Multicompartmentalized polymeric systems: Towards biomimetic cellular structure and function. *Chemical Society Reviews, 42*(2), 512–529.

Mehnati-Najafabadi, V., Taheri-Kafrani, A., & Bordbar, A. K. (2018). Xylanase immobilization on modified superparamagnetic graphene oxide nanocomposite: Effect of PEGylation on activity and stability. *International Journal of Biological Macromolecules, 107*, 418–425.

Mao, Y., Černigoj, U., Zalokar, V., Štrancar, A., & Kulozik, U. (2017). Production of β-lactoglobulin hydrolysates by monolith based immobilized trypsin reactors. *Electrophoresis, 38*(22–23), 2947–2956.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology, 40*(6), 1451–1463.

Mehrasbi, M. R., Mohammadi, J., Peyda, M., & Mohammadi, M. (2017). Covalent immobilization of *Candida antarctica* lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil. *Renewable Energy, 101*, 593–602.

Miletić, N., Abetz, V., Ebert, K., & Loos, K. (2010). Immobilization of *Candida antarctica* lipase B on polystyrene nanoparticles. *Macromolecular Rapid Communications, 31*(1), 71–74.

Miyanaga, M., Tanaka, T., Sakiyama, T., & Nakanishi, K. (1995). Synthesis of aspartame precursor with an immobilized thermolysin in mixed organic solvents. *Biotechnology and Bioengineering, 46*(6), 631–635.

Mohan, T., Rathner, R., Reishofer, D., Koller, M., Elschner, T., Spirk, S., et al. (2015). Designing hydrophobically modified polysaccharide derivatives for highly efficient enzyme immobilization. *Biomacromolecules, 16*(8), 2403–2411.

Nedwin, G. E., Sharma, V., & Shetty, J. K. (2014). *Alpha-amylase blend for starch processing and method of use thereof*. US 2014/0087429 A1, 2014.

Netto, C. G., Toma, H. E., & Andrade, L. H. (2013). Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes. *Journal of Molecular Catalysis B: Enzymatic, 85*, 71–92.

Nisha, S., Karthick, S. A., & Gobi, N. (2012). A review on methods, application and properties of immobilized enzyme. *Chemical Science Review and Letters, 1*(3), 148–155.

Oh, D. K. (2007). Tagatose: Properties, applications, and biotechnological processes. *Applied Microbiology and Biotechnology, 76*(1), 1.

Olson, A. C., Gray, G. M., & Guadagni, D. G. (1979). Naringin bitterness of grapefruit juice debittered with naringinase immobilized in a hollow fiber. *Journal of Food Science, 44*(5), 1358–1361.

Ovsejevi, K., Manta, C., & Batista-Viera, F. (2013). Reversible covalent immobilization of enzymes via disulfide bonds. In *Immobilization of enzymes and cells* (pp. 89–116). Humana Press.

Oyama, K., Nishimura, S., Nonaka, Y., Kihara, K., & Hashimoto, T. (1981). Synthesis of an aspartame precursor by immobilized thermolysin in an organic solvent. *The Journal of Organic Chemistry, 46*(25), 5241–5242.

Panesar, P. S., Kumari, S., & Panesar, R. (2010). Potential applications of immobilized β-galactosidase in food processing industries. *Enzyme Research, 2010*, 473137.

Pedroche, J., del Mar Yust, M., Mateo, C., Fernández-Lafuente, R., Girón-Calle, J., Alaiz, M., et al. (2007). Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme–support linkages and thermal stability. *Enzyme and Microbial Technology, 40*(5), 1160–1166.

Pernicova, I., Enev, V., Marova, I., & Obruca, S. (2019). Interconnection of waste chicken feather biodegradation and keratinase and mcl-PHA production employing *Pseudomonas putida* KT2440. *Applied Food Biotechnology, 6*(1), 83–90.

Pierre, A. C. (2004). The sol-gel encapsulation of enzymes. *Biocatalysis and Biotransformation, 22*(3), 145–170.

Puri, M., Kaur, A., Singh, R., & Kanwar, J. (2008). *Immobilized enzyme technology for debittering citrus fruit juices* (pp. 91–103). Application of new technologies.

Puri, M., Marwaha, S. S., & Kothari, R. M. (1996). Studies on the applicability of alginate-entrapped naringiase for the debittering of kinnow juice. *Enzyme and Microbial Technology, 18*(4), 281–285.

Puri, M., Seth, M., Marwaha, S. S., & Kothari, R. M. (2001). Debittering of kinnow mandarin juice by covalently bound naringinase on hen egg white. *Food Biotechnology, 15*(1), 13–23.

Rana, M., Kumari, A., Chauhan, G. S., & Chauhan, K. (2014). Modified chitosan microspheres in non-aggregated amylase immobilization. *International Journal of Biological Macromolecules, 66*, 46–51.

Rathner, R., Petz, S., Tasnádi, G., Koller, M., & Ribitsch, V. (2017). Monitoring the kinetics of biocatalytic removal of the endocrine disrupting compound 17α-ethinylestradiol from differently polluted wastewater bodies. *Journal of Environmental Chemical Engineering, 5*(2), 1920–1926.

Ren, S., Li, C., Jiao, X., Jia, S., Jiang, Y., Bilal, M., & Cui, J. (2019). Recent progress in multienzymes co-immobilization and multienzyme system applications. *Chemical Engineering Journal, 373*, 1254–1278.

Reshmi, R., Sanjay, G., & Sugunan, S. (2006). Enhanced activity and stability of α-amylase immobilized on alumina. *Catalysis Communications, 7*(7), 460–465.

Roy, I., & Gupta, M. N. (2003). Lactose hydrolysis by Lactozym™ immobilized on cellulose beads in batch and fluidized bed modes. *Process Biochemistry, 39*(3), 325–332.

Sardar, M., Roy, I., & Gupta, M. N. (2000). Simultaneous purification and immobilization of Aspergillus niger xylanase on the reversibly soluble polymer EudragitTM L-100. *Enzyme and Microbial Technology, 27*(9), 672–679.

Schellenberger, E. A., Bogdanov Jr, A., Högemann, D., Tait, J., Weissleder, R., & Josephson, L. (2002). Annexin V–CLIO: A nanoparticle for detecting apoptosis by MRI. Molecular Imaging, 1(2), 15353500200202103.

Sheldon, R. A. (2007a). Cross-linked enzyme aggregates (CLEA® s): Stable and recyclable biocatalysts. *Biochemical Society Transactions, 35*(6), 1583–1587.

Sheldon, R. A. (2007b). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis, 349*(8–9), 1289–1307.

Sheldon, R. A., & Woodley, J. M. (2018). Role of biocatalysis in sustainable chemistry. *Chemical Reviews, 118*(2), 801–838.

Silva, J. A., Macedo, G. P., Rodrigues, D. S., Giordano, R. L. C., & Gonçalves, L. R. B. (2012). Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. *Biochemical Engineering Journal, 60*, 16–24.

Rodrigues, R. C., Berenguer-Murcia, Á., & Fernandez-Lafuente, R. (2011). Coupling chemical modification and immobilization to improve the catalytic performance of enzymes. *Advanced Synthesis & Catalysis, 353*(13), 2216–2238.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews, 42*(15), 6290–6307.

Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic, 90*, 1–11.

Tipton, K. F., Armstrong, R. N., Bakker, B. M., Bairoch, A., Cornish-Bowden, A., Halling, P. J., et al. (2014). Standards for reporting enzyme data: The STRENDA consortium: What it aims to do and why it should be helpful. *Perspectives in Science, 1*(1–6), 131–137.

Torres, R., Mateo, C., Fuentes, M., Palomo, J. M., Ortiz, C., Fernández-Lafuente, R., & Guisan, J. M. (2002). Reversible immobilization of invertase on Sepabeads coated with polyethyleneimine: Optimization of the biocatalyst's stability. *Biotechnology Progress, 18*(6), 1221–1226.

Tosa, T., Sato, T., Mori, T., & Chibata, I. (1974). Basic studies for continuous production of L-aspartic acid by immobilized *Escherichia coli* cells. *Applied Microbiology, 27*(5), 886–889.

Tosa, T., Sato, T., Mori, T., Matuo, Y., & Chibata, I. (1973). Continuous production of L-aspartic acid by immobilized aspartase. *Biotechnology and Bioengineering, 15*(1), 69–84.

Tripathi, A., Sami, H., Jain, S. R., Viloria-Cols, M., Zhuravleva, N., Nilsson, G., Jungvid, H., & Kumar, A. (2010). Improved bio-catalytic conversion by novel immobilization process using cryogel beads to increase solvent production. *Enzyme and Microbial Technology, 47*(1–2), 44–51.

Tsen, H. Y. (1984). Factors affecting the inactivation of naringinase immobilized on chitin during debittering of fruit juice. *Journal of Fermentation Technology, 62*(3), 263–267.

Van de Velde, F., Lourenço, N. D., Pinheiro, H. M., & Bakker, M. (2002). Carrageenan: A food-grade and biocompatible support for immobilisation techniques. *Advanced Synthesis & Catalysis, 344*(8), 815–835.

Vescovi, V., Rojas, M. J., Baraldo, A., Botta, D. C., Santana, F. A. M., Costa, J. P., et al. (2016). Lipase-catalyzed production of biodiesel by hydrolysis of waste cooking oil followed by esterification of free fatty acids. *Journal of the American Oil Chemists' Society, 93*(12), 1615–1624.

Vianello, F., Ragusa, S., Cambria, M. T., & Rigo, A. (2006). A high sensitivity amperometric biosensor using laccase as biorecognition element. *Biosensors and Bioelectronics, 21*(11), 2155–2160.

Villeneuve, P. (2007). Lipases in lipophilization reactions. *Biotechnology Advances, 25*(6), 515–536.

Virtanen, J., Baron, C., & Tenhu, H. (2000). Grafting of poly (N-isopropylacrylamide) with poly (ethylene oxide) under various reaction conditions. *Macromolecules, 33*(2), 336–341.

Virtanen, J., & Tenhu, H. (2000). Thermal properties of poly (N-isopropylacrylamide)-g-poly (ethylene oxide) in aqueous solutions: Influence of the number and distribution of the grafts. *Macromolecules, 33*(16), 5970–5975.

Walle, T. (2009). Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. *International Journal of Molecular Sciences, 10*(11), 5002–5019.

Xu, K., Chen, X., Zheng, R., & Zheng, Y. (2020). Immobilization of multi-enzymes on support materials for efficient biocatalysis. *Frontiers in Bioengineering and Biotechnology, 8*.

Zhaoyu, Z., Ping, X., Keren, S., Weiwei, Z., Chunmiao, H., & Peng, L. (2020). Di-functional magnetic nanoflowers: A highly efficient support for immobilizing penicillin G acylase. *Journal of the Chinese Chemical Society, 67*, 1591–1601.

Zhi, Y., Wu, Q., & Xu, Y. (2017). Production of surfactin from waste distillers' grains by co-culture fermentation of two *Bacillus amyloliquefaciens* strains. *Bioresource Technology, 235*, 96–103.

16 Prospects and Challenges in Food-Grade Enzymes Industrial Production

Musliu Olushola Sunmonu and Mayowa Saheed Sanusi

Abstract

Food-grade enzymes are biocatalyst in industrial production of food and they have numerous advantages over conventional chemical processes due to their ability to achieve efficient and sustainable processes and products. It has found extensive application in bakery, beverages, meat, cheese, alcoholic and juice industries. Commercial food-grade enzymes have been preferred to be sourced from microorganisms (bacteria, fungi, yeast, mold) rather than from plants and animals because of their high production yield and controlled conditions. The relevance, applications and challenges of some novel technologies; enzymes screening, enzymes immobilization, solid-state fermentation, recombinant DNA, protein engineering and bioinformatics as techniques for producing food-grade enzymes were discussed. The sources and the industrial applications of some selected food-grade enzymes, such as amylase, proteases, lipase, rennet, cellulase, lactase, invertase, glucose oxidase, glucose isomerase and catalase in the food industry, and food-grade enzymes safety issues, were also discussed.

Keywords

Industrial application · Food-grade enzymes · Food industry · Novel technologies · Safety

M. O. Sunmonu (✉) · M. S. Sanusi
Department of Food Engineering, Faculty of Technology and Engineering, University of Ilorin, Ilorin, Nigeria

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022
A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_16

16.1 Introduction

The emergence of the food-grade enzyme for industrial production can be tagged as green revolution approach due to its catalytic biological nature in transforming food into various value-added products in different food industries, such as in beverages and juices, baking, development of functional foods, meat, wine, dairy, fats and oils. The food-grade enzyme has been projected as a potential replacement for chemical additives or harsh physical conditions (temperature, pressure, pH) used in industrial production, therefore guarantees sustainable production for safe and nutritious food supply. Also, with an increase in the awareness on the need to consume a healthier diet which has increased consumers' preference for new and convenient food products manufactured from natural food additives has further increased the use of food-grade enzymes. Presently, food-grade enzymes are being used in the food industry to increase diversity, quality and varieties of food produced. The food-grade enzymes can be generated from the plants, animals and microbial sources. However, out of the entire sources used for enzymes generation, microbial is the most preferred choice in the food industry. Enzymes from microbial sources can be produced in control conditions at a faster rate with minimal harmful by-products and in larger quantities. Large-scale Industrial production has explored the use of *Saccharomyces cerevisiae*, *Aspergillus oryzae*, Fungi *Aspergillus niger* and *Bacillus subtilis* bacteria as sources for microbial enzymes production. Some of the commercially important food-grade enzymes produced from this sources are proteases, rennet, pectinases, invertases, cellulases, lipases, amylases, catalase, lactase, raffinase, pullulanase, glucose oxidase, etc. Most of naturally occurring enzymes from plants, animals or microbial sources are not often suitable for economical production processes and sustainability. Therefore, there is a need to adopt novel technological approaches that can be used to improve the industrial production of food-grade enzymes. Uzuner and Cekmecelioglu (2019) reported that industrial enzymes market is competitive with minimal profit margin and the enzymes are currently manufactured by few companies in the United States, Denmark and Germany with approximately twenty (20) enzymes being commercially produced at present. Hydrolase represents 75% of food-grade enzymes in the market. Carbohydrase (i.e. tannase, phytases, amylases, pectinases, cellulases, β-galactosidases), lipase and proteases are family of hydrolase usually employed as food-grade additives or processing aids in the food industry. This chapter provides an overview of the prospect and challenges of a novel application of techniques for improving the industrial production of food-grade enzymes through screening, enzyme immobilization, solid-state fermentation, recombinant DNA technology, protein engineering, and use of bioinformatics tool in enzyme engineering. The potential application of some selected food-grade enzymes used for industrial production and the safety of food-grade enzyme were discussed.

16.2 Food-Grade Enzymes Screening

Screening technique is used for isolating enzymes by judiciously selecting plant, animal and the species and strains of microorganism or cells. Maximum volumetric productivity is used as an objective function for selecting strains or original cultures with the capacity to produce the preferred enzyme of high productivity rate (Ramos et al., 2011). Also, screening is used in finding alternative strains with improved possibility of cloning proposed enzyme. The process of isolating enzymes from extremophile source using screening techniques is useful in stabilizing, showing high activity and turnover numbers, and specificity of enzymes when operated under severe environmental conditions for industrial application (Synowiecki et al., 2006). For instance, there is a rising interest in the sourcing of enzymes from isolations of bacteria from the marine environment, such as proteases from several genera esterase from *Vibrio fischeri*, inulinase from *Cryptococcus aureus*, amylolytic enzymes, glucosidases and invertase/inulinase from *Thermotoga neapolitana DSM 4359* (Dipasquale et al., 2009; Rastogi & Bhatia, 2019). In the meat and dairy industry, cryophiles are needed potential microbes for screening. Since the industry operates under room-temperature lactase, protease and pectinase can be obtained for the production of lactose-free milk, milk coagulation and cheese maturing, fruit juice or oligosaccharide production, respectively (Nakagawa et al., 2004, 2006; Wang et al., 2008; Mao et al., 2010). In the screening technique, the major challenge is to cultivate the screened and identified microbes in laboratory conditions most especially the extremophile. However, if the extremophiles are expressed in mesophiles using genetic engineering, they can be easily cultured in the laboratory with easier protein extraction and purification (Rastogi & Bhatia, 2019).

16.3 Food-Grade Enzyme Immobilization

Food-grade enzyme immobilization simply means confining enzymes in a given portion of space with the use of microencapsulation or by the use of solid matrix, such as cross-linking, ion exchange, adsorption and covalent binding, so that the enzyme can be physically separated from the reaction medium. The prospect of the industrial application of food-grade enzyme immobilization is that it permits the reuse of the enzymes and also extends its usefulness to be incorporated in the food products or food processing to improve productivity. Among the food-grade supports used of immobilization are alginate, carrageenan, agar, glass beads, polyurethane foam, polyacrylamide, metal surfaces and vermiculite (Ramos et al., 2011). Ramos et al. (2011) reported that for protease enzymes, vermiculite which is less-expensive support using adsorption had been used for immobilization. It is a must to consider food-grade support for any food-grade enzymes immobilization. According to Sumantha et al. (2006), immobilization of microorganism during the production of the enzyme has been reported to produce higher enzyme yields than non-immobilized cells of bacteria or mycelia. Some of the immobilized enzymes that had been applied in the food industry are immobilized glucose isomerase for

manufacturing high-fructose corn syrup, immobilized aminoacylase for manufacturing amino acids, immobilized lactase for producing tagatose, immobilized invertase for producing inverted sugar, immobilized lipases for producing trans-free oils (cocoa butter equivalents, and for modification of triacylglycerols), immobilized β-fructofuranosidase for producing fructooligosaccharides and immobilized isomaltulose synthase for producing isomaltulose, respectively (Rastogi & Bhatia, 2019). From an economic perspective, it is advisable to immobilize expensive enzymes to have a less-expensive process (Fernandes, 2010). The challenges associated with immobilization are that if the procedure of immobilization is not properly done, it might lead to the loss of enzyme activity which might be as a result of mechanical and chemical stress (Rastogi & Bhatia, 2019).

16.4 Solid-State Fermentation

Fermentation is a technique used for the synthesis of food-grade enzymes. The conventional fermentation method of synthesizing enzymes is through submerged fermentation. The type of substrate used in the submerge fermentation is a determinant in obtaining either high or low enzyme activities. For instance, complex substrate suspended or dissolved in an aqueous medium is expected to give enzymes with high activities as compared with a simple substrate in submerged fermentation. Aside from the type of substrate, the continuous operation has been reported to favour enzyme yield as compared with the batch operation process which is simple to handle and also versatile (Ramos et al., 2011). Optimization of submerge fermentation parameters through the use of advance experimental design, such as response surface methodology, artificial neural network and radial basis function, had been reported as tools that can be used to obtain optimum parameters combination aimed at given maximum volumetric productivity of the enzymes during fermentation (Sumantha et al., 2006). However, solid-state fermentation is gaining greater interest in recent years than the conventional submerged fermentation. The reason is that in solid-state fermentation, cultures are grown of moist solid rather than in aqueous liquid as in submerge fermentation. Also, the culture media are not usually complex; the substrate media for solid-state fermentation can be enriched with nutrient. The enzyme of interest in solid-state fermentation is not diluted, thus paves the way for easy purification after synthesis. There is also low effluent generation, catabolic repression hardly affects the synthesis of enzymes and higher inoculum and relatively low humidity content used in solid-state fermentation reduces extraneous microbial contamination (Ramos et al., 2011). The wide use of agricultural waste as a substrate in solid-state fermentation is another advantage of this technique over submerge fermentation as it aids in reducing the cost associated with enzyme synthesis (Aguilar et al., 2008). Sumantha et al. (2006) reported that the use of wheat bran as a substrate for solid-state fermentation gives a higher yield of proteases than in submerge fermentation using *Aspergillus spp.* and *Mucor pusillus*.

The use of two or more substrates has been reported as a way of increasing the yield of the enzymes in solid-state fermentation (Aguilar et al., 2008).

16.5 Recombinant DNA/Genetic Engineering

According to the Food Drug Administration (FDA) in the USA, Escherichia coli K-12 also known as bovine chymosin is the first recombinant enzyme. The recombinant deoxyribonucleic acid (rDNA) techniques or genetic engineering has now been used to produce chymosin by transforming microorganisms with bovine prochymosin gene. At least there are thirty-six (36) enzymes which are genetically modified microorganisms (GMOs) among the one hundred and sixty (160) enzymes produced for use in the food industry based on Association of Manufacturers and Formulators of Enzyme Products (AMFEP) enzymes lists. To achieve the synthesis of efficient enzymes that is free of undesirable enzymes or metabolites of microbes using rDNA, appropriate selection of host microorganisms as recombinant strains with potential DNA sequences that can code for the defined enzyme amino acid sequence is needed. For instance, engineering microbial host stains were applied to some fungi strain to manipulate or eliminate their capacity to release metabolites that are toxic to the formulation of the synthesis enzyme (Olempska-Beer et al., 2006). The application of rDNA has immensely increased the availability of food-grade enzymes with distinct properties to meet the food process and food matrices requirement. Also, it has assisted in synthesizing enzymes that have desirable sensitivity and specificity at a low cost of production.

Rastogi and Bhatia (2019) stated that the application of rDNA can be implemented in batch or continuous operations using cheaper raw material, therefore lessens the criteria for enzyme extraction and purification. The use of food-grade enzymes from genetic engineering has minimized the cost connected with upstream product processing and also increases productivity (Panesar, 2010; Rastogi & Bhatia, 2019). α-amylases, proteases, lipases and pullulanases used in food applications, such as in hydrolysis of starch, are produced by using improve strains (Ramos et al., 2011; Rastogi & Bhatia, 2019). Despite this entire prospect, the main challenge of genetically improved strain is that is not yet certified safe for consumption by the FDA and European Union due to the rise in public consciousness regarding the non-consumption of genetic modified processed foods. Also, the lack of standard in its production and purification procedures has caused a restriction in its application food technology.

16.6 Food-Grade Enzymes by Protein Engineering

The improvement of the food-grade enzyme in terms of stability and tolerance to severe temperature, pH and organic solvents can be achieved through protein engineering where the catalytic rate is improved and amino acids are modified for enhancing substrate specificity. Rational protein design and direct evolution are two

approaches used by protein engineering either exclusively or alongside each other (Jackel et al., 2008; Tiwari et al., 2012; Rastogi & Bhatia, 2019). According to Singhania et al. (2010), previous knowledge of the function and structure of the protein to introduce the desirable traits using sited mutagenesis is required for rational protein design approach, while for direct evolution, information of the protein is not compulsory and random mutagenesis is performed by error-prone PCR or chemical mediation. In order to forecast the overall structure of enzyme using rational protein design, multiple sequence alignment technique, homology modelling and protein treading methods are used (Gulati & Poluri, 2016). Rational design is easier to improve properties during screening due to an infinite number of protein variants generated (Singhania et al., 2010). Quax et al. (1991) used rational design to generate mutant glucose isomerase from *Actinoplanes missouriensis* with enzyme activity that is thermal and pH stable as against the parental enzyme. Double mutant isomerase (G138P, G247D) generated using rational design showed 2.5-fold increases in half-life and 45% specificity increment as compared with the wild type (Zhu et al., 1999). A similar approach was also reported by Lin et al. (2008) for generating amylase mutants from the strain of TS-23 *Bacillus sp.*to enhance the thermal stability of the enzyme. However, some of the biocatalysts generated by direct evolution are glucoamylases, cellulases, α-amylases, lipases and proteinases (Singhania et al., 2010).

16.7 Application of Bioinformatics Technique in Enzyme Engineering

In order to analyse the information in enzymes data within a limited time either online or off the network, the novel application of bioinformatics technique in enzyme engineering is the recent trend useful in performing sophisticated computations analysis (Alderson et al., 2012). The enormous of structural data and sequencing coming out of nuclear magnetic resonance spectroscopy, X-ray crystallography, mass spectrometry and NGS next-generation sequencing (NGS) for enzyme informatics are now better understood by the application of bioinformatics technique (Rastogi & Bhatia, 2019).

16.8 Application of Food-Grade Enzymes for Industrial Production

The use of food-grade enzymes for industrial production has widened their application in the development of different food products. The application of some selected food-grade enzymes for industrial production of food are as follows.

16.8.1 Application of Food-Grade Amylase Enzymes

α-amylase and β-amylase have been used over years for the industrial production of maltose and glucose by applying the enzyme on starch. The application of α-amylase has paved way for the production of glucose syrup and has replaced the chemical hydrolysis of starch in most starch manufacturing companies (Reddy et al., 2003). The sources used for commercial production of α-amylase are some selected strains of bacteria (*Bacillus* spp.: *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, *B. cereus*, *B. stearothermophilus*, *B. licheniformis; halophilic bacteria: Chromohalobacter sp.*, *Halobacillus sp.*, *Haloarcula hispanica*, *Halomonas meridiana;* fungi: *Aspergillus* species and few species of *Penicillium*: *P. brunneum*, *P. fellutanum*) (Konsoula & Liakopoulou-Kyriakides, 2007; Erdal & Taskin, 2010). α-amylase had been used in the baking industry to play an important role in enhancing the quantity, aroma and taste of baked products. Jegannathan and Nielsen (2013) reported that the addition of amylase and lipase enzymes in bread helps in extending its shelf life and reduces crystallization. Antistaling in bread and improved softness can also be achieved by the addition of α-amylase (Gupta et al., 2008). However, more research still needs to be done on the application of α-amylase in the baking industry as a small overdose of α-amylase can result in sticky bread (Singh et al., 2019). For the fermentation of raw starch into ethanol, a combination of α-amylase produced from *Streptococcus bovis* and glucoamylase from *Rhizopus oryzae* has been reported to improve the ethanol production (Singh e*t al.*, 2019).

16.8.2 Application of Food-Grade Protease Enzymes

Protease enzymes can be the source from plant, animal, humans and microorganisms (bacteria, fungi and virus) and are known to hydrolyse peptide bones of proteins. Protease enzymes produced from microorganisms' source are the most preferred for industrial production due to ease of genetic manipulation and extensive diversity, respectively. They are also known to favour low manufacturing cost, extensive chemical and physical characteristics, lack of seasonal variation, large production in industrial fermentators and quick culture development (Singh e*t al.*, 2019). Protease enzymes of a wide variety have been exploited for industrial production in food and pharmaceutical industries because of their specificity in action (Kalpana et al., 2008). Almost 60% of the total food-grade enzymes in the market fall under the category of protease enzymes (Mala et al., 1998). Alkaline and neutral proteases produced for commercial purpose are usually generated from *Bacillus* genus. Powdered bacteria proteases derived from *Bacillus* can be stable and remain 95% active for a year. In the food industry, proteases can be applied in cheese production, baked products making, soy hydrolysates production and meat tenderization. Proteases from *A. oryzae* can improve bread colour and texture when mixed with flour dough. More so it also aids in the reduction of mixing time. Heredia-Sandoval et al. (2016) stated that the gluten content of bakery products can be decreased and degraded

using proteases. In the dairy industry during cheese manufacturing, vegetable rennet, animal rennet, genetically engineered chymosin and microbial milk coagulants are milk coagulating enzymes used. Paracasein and micropeptides are generated from the hydrolysis of peptide bone using protease enzymes in cheese making. However, chymosin is good for generating casein. In beer production, application of protease and amylase enzymes have been used as an efficient alternative way of achieving malting process, thus saves energy and agricultural land that would have been used if the conventional malting that involves drying was to be used (Jegannathan & Nielsen, 2013). Microbial proteases have been reported to hydrolyse proteinaceous in beer that causes haze formation, while production of wort can be achieved by the use of *B. subtilis* proteases to solubilize proteins from barley adjuncts (Singh e*t al.*, 2019). For commercial purpose, improved tenderness of meat which is the organoleptic quality of meat as desired by the consumers and for easy marketability can be achieved by using thermophile protease derived from *Bacillus* strain, caldolysin from *Thermus* strain, collagenase from *Clostridium histolyticum*, and aspartic protease from *A. oryzae* (Bekhit et al., 2014). For industrial production of aspartame which is known as nutrasweet (zero-calorie sweetener), enzymatic synthesis of aspartame has been preferred over chemical method due to their ability to maintain stereospecificity. Proteases have been reported to be used to synthesize aspartame by catalysing the reverse reaction in two amino acids, thus maintain stereospecificity and reduce the cost of production (Singh e*t al.*, 2019).

16.8.3 Application of Food-Grade Lipase Enzymes

Lipases are important food-grade enzymes due to their biocatalysts activities in breaking down fats and oils, esterification, hydrolysis, interesterification, acidolysis, alcoholysis and aminolysis. Lipases can be sourced from the plant (barley, corn and cotton), animal (adipose tissues, blood, gastric juices, intestinal juices and pancreatic secretion), yeast (*Candida* and *Torulopsis*), filamentous fungi (*Geotrichum*, *Humicola* and *Rhizopus*), bacterial species (*B. subtilis*, *P. fragi*, *B. megaterium*, *S. aureus*, *Burkholderia cepacia*, *P. aeruginosa*, P. pseudoalcaligenes) and fungi species (*Helvinal anuginosa*, *Rhizopus delemar*, *Eurotium herbariorum*, *A. niger*, *M. circinelloides and Penicillium citrinum*) (Hasan et al., 2006; Sachan & Singh, 2015; Singh e*t al.*, 2019). However, the microbial source is mostly used for commercial production of lipase due to their ability to reduce the cost of production, greater widespread ability in animal and plant lipases, and greater stability (Singh e*t al.*, 2019). Hasan et al. (2006) and Treichel et al. (2009) noted that lipase enzymes have found application in food processing, pharmaceuticals, agrochemical, detergents, paper and cosmetic industries. The first commercial lipase from recombinant technique was isolated from *Thermomyces lanuginosus* which is a fungus and since then many other recombinant lipases have been produced for commercial purpose (Singh e*t al.*, 2019).

Lipases have been applied extensively in the dairy industries for hydrolysis of milk fat, lipolysis of cream, producing cheese products and in enhancing the flavour

of cheeses in order to accelerate cheese ripening (Aravindan et al., 2007). In the bakery industry, lipolytic enzymes have been used to produce emulsifying liquids in situ by degrading wheat lipids instead of using traditional emulsifiers. Also, the application of lipolytic enzymes helped in the release of short-chain fatty acids through esterification, thereby aids in improving the flavour of baked products flavour. Apple wine has been produced by the Japanese company Tanabe Seiyaku with improved alcoholic content and aroma by isolating lipase enzyme from *R. delemar* or a *Candida* species during fermentation (Singh e*t al.*, 2019). Lipases have also been used to improve the quality and texture of mayonnaise, whipping and dressing products. In the meat and fish processing, lipases are used to enhance and remove fat. Ramarethinam et al. (2002) reported that volatile flavours and the aroma of black tea were increased using lipase secreted by *Rhizomucor miehei*. Lastly, in the fat and oil industry, cheap oils have been converted into nutritional-rich oils, oleic acids oils, low calorie triacylglycerols and polyunsaturated fatty acid using microbial lipases (Gupta et al., 2003). Besides commercial production in fats and oils modification, low energy consumption and high level of specificity can be achieved using lipase enzyme modification when compared with chemical modification.

16.8.4 Application of Food-Grade Rennet Enzymes

Rennet is enzyme made up of chymosin, lipase and pepsin and it is usually referred to as complex enzymes. Rennet can be generated from the plant, animal and microbial sources. Rennet has been extensively applied in the commercial production of different types of cheese. However, rennet from the microbial source is mostly used for cheese production worldwide. For instance, *R. miehei*, *Irpex lactis*, *A. oryzae and R. pusillus* are the good source of microorganism that can be used to generate rennet for cheese production (Neelakantan et al., 1999; Singh e*t al.*, 2019). Also, microbial rennet has served as a replacement for mammalian rennet used in cheese production due to consumers' demand of non-animal derivative. Unlimited availability, low cost of production, bulk production and no risk of disease transmission are some of the advantages of microbial rennet over animal rennet (Singh e*t al.*, 2019). The application of rennet enzyme is one of the most important uses of enzymes in the food industry.

16.8.5 Application of Food-Grade Catalase Enzymes

Catalase enzymes also known as hydroperoxidases and is popularly known commercially for converting hydrogen peroxidase to water and oxygen. According to Singh et al. (2019) one molecule of catalase can transform millions of hydrogen peroxide to water and oxygen per second. Catalase can be found in all living organism but mostly source from microorganisms and bovine liver (Chelikani et al., 2004; Tukel & Alptekin, 2004). Plant leaves with high antioxidant properties are *M. sylvestris* L., black gram (*Vigna mungo*) seeds, cotton, sunflower and

pumpkin (Eising et al., 1990; Kandukuri et al., 2012). Catalase found industrial application in the removal of hydrogen peroxide used in sterilizing, oxidizing and bleaching agents (Ertaş et al., 2000). The combination of catalase and glucose oxidase has been used for the preservation of some selected foods. The enzyme can also be used in egg processing but has limited usage in cheese production.

16.8.6 Application of Food-Grade Lactase Enzymes

This enzyme is very important in the dairy industry, most especially in the production of low-lactose or lactose-free food, such as flavoured milk drinks, ice cream, yoghurt and frozen dessert. Lactase can be the source and isolate from animal organs, plants, yeasts, bacteria or molds. For industrial production of lactase, it is usually source from *A. niger*, *A. oryzae* and *Kluyveromyces lactis* due to their safety of use history and the numerous safety tests they undergo before use. The source used for generating the lactase and the method of preparation determines its properties, such as the temperature and pH. For optimal performance of immobilized lactases, the procedure of immobilization and the type of carrier are important. Acidic pH of 2.5 to 4.5 is favourable for fungi lactase production, while neural region pH is favourable for bacterial and yeast lactase with ranges of 6.5–7.5 and 6–7, respectively. The prospects in the utilization of lactase, hydrolase and β-galactosidase have been extensive research due to the discovery of enzymes immobilization techniques (Mehaia & Cheryan, 1987). The application of lactase in milk has assisted in preventing diarrhea and severe tissue dehydration which affect lactose intolerance people. It has also been reported to increase digestibility, sweetness and scoop and creaminess of ice cream, yoghurt and frozen dessert. Increase in sweetness and sugar addition level reduction has also been ascribed as another advantage of lactase-treated milk in the production of flavoured milk drinks.

16.8.7 Application of Food-Grade Cellulase Enzymes

Cellulases are enzymes used for extracting constituents from soy protein, essential oils, aromatic products, green tea and sweet potato in the food industry. Dillon et al. (2004) described cellulase enzymes as enzymes that break the glucosidic bond of cellulose microfibrils and release oligosaccharides, cellobiose and glucose. Cellulases can be produced from filamentous fungi (*Aspergillus*, *Chaetomium*, *Fusarium*, *Penicillium*, *Phoma* and *Trichoderma*), anaerobic bacteria (*Acetovibrio*, *Bacteroides*, *Butyrivibrio*, *Caldocellum*, *Clostridium*, *Eubacterium*, *Erwinia*, *Pseudonocardia*, *Ruminococcus* and *Thermoanaerobacter*) and aerobic bacteria (*Acidothermus*, *Bacillus*, *Cellvibrio*, *Pseudomonas*, *Streptomyces*, *Staphylococcus*, and *Xanthomonas*) (Soares et al., 2012; Singh e*t al.*, 2019). Among all the sources of producing cellulolytic enzymes, *Aspergillus* has been reported to be the most outstanding (Singh e*t al.*, 2019). Combination of cellulases, hemicellulase and

pectinase is currently being used in the food industry to extract and clarify fruits juice. Cellulase can also be used to degrade solid phase, thus enhances liquefaction.

16.8.8 Application of Food-Grade Invertase Enzymes

This enzyme has a great commercial significance in the production of inverted sugar and is known for its sweetness effect. Invertase can catalyse sucrose to glucose and fructose through hydrolysis. Temperature and pH within the range of 40–60 °C and 3–5, respectively, are more favourable for more active invertase. At the industrial level, invertase is mostly isolated from yeast, such as *S. cerevisiae*, and there is ongoing research for the use of high yielding filamentous fungi for production of invertase. Examples of the filamentous fungi are *Rhizopus* sp. And *Aspergillus casiellus*. The filamentous fungi *Rhizopus sp.* was cultivated in the medium of wheat bran to obtain invertase (Soares et al., 2012); *Aspergillus casiellus* was inoculated in a medium of soybean meal and the invertase was isolated after 72 h, while in yeast, invertase (S-b D-fructofuranosidase) is isolated from *S. cerevisiae* (Singh e*t al.*, 2019).

16.8.9 Application of Food-Grade Glucose Isomerase Enzymes

In the food industry glucose isomerase has found extensive application in the production of fructose-rich corn syrup. The enzyme is also known as d-xylose ketol isomerase which aids in catalysing reversible isomerase from d-glucose and d-xylose into d-xylulose and d-fructose, respectively. Protein engineering technique is being explored to generate thermostable glucose isomerase enzymes (Hartley et al., 2000). Glucose isomerase has also been used for bioconversion of hemicellulose into ethanol (Singh e*t al.*, 2019). For commercialization of glucose isomerase, the application of biotechnology in isolating mutants of promising prospect is important.

16.8.10 Application of Food-Grade Glucose Oxidase Enzymes

Glucose oxidase is majorly used in the food industry to eliminate harmful oxygen in food product containing probiotic microorganisms. The enzyme allows catalysing oxidation–reductions reaction through the prosthetic group flavin adenine dinucleotide (FAD). High level of glucose oxidase can be isolated from filamentous fungi, such as *Aspergillus versicolor* and *Rhizopus stolonifera* (Guimarães et al., 2006). Singh e*t al.* (2019) suggested that the use of glucose oxidase through biotechnology can be used to improve the stability of probiotic bacteria in yoghurt without adding chemical additives.

16.9 Safety of Food-Grade Enzyme

Industrial food-grade enzymes are generally considered safe and are known as food additives, while some considered them as food processing aids. The safety regulation on the use of food-grade industrial enzymes differs among countries, premarket approval of the enzymes and the information provided by the manufacturer. The prominent occupational food safety risks that have been associated with the use of industrial food enzymes are mainly allergic reaction (in the respiratory tract), irritation risks, residual microbiological activity, chemical toxicity and oral toxicity among consumers (Spök, 2006; Ramos et al., 2011; Singh et al., 2019). Ramos et al. (2011) stated that enzymes are sensitizers and can cause allergic reaction in the respiratory tract (asthma) upon inhalation and also irritation upon contact with the skin. However, aside from the occupational risk, the final consumers of food products containing food-grade enzymes rarely report allergic reaction because low levels of the enzymes are usually used in food formulation which would have eventually be deactivated upon processing and before reaching the consumers. The future challenges that might be associated with the safety concern of food-grade enzymes are toxic substance arising from the by-product of contaminants present in the preparation of the enzyme. Adequate attention must be paid towards fermentation broth contaminate or environmental conditions of some phylogenetical strains used in enzyme production. Although with the advent of genetic engineering technique, toxicity problems can easily be eliminated. Food industry and national or European regulatory bodies must develop strategies to harmonize and enforce legislation on the available food enzymes that are categorized as a novel or those with unusual properties and their food safety assessment practice to eradicate occupational risks that may arise from their use. One of the current issues related to food enzymes legislation is that in the USA, Japan and Canada food-grade enzymes are considered as food additives, while Australia regards food enzymes as food processing aids. WHO/FAO, joint committee of food additives, does not differentiate food-grade enzymes either as a food processing aids or food additives (Ramos et al., 2011). However, EU food legislation considered more than 160 food-grade enzymes as food processing aids, while invertase and lysozyme enzymes are the only two food-grade enzymes considered as food additives. National legislation of France, Hungary, Denmark and Poland do subject food-grade enzymes that are referred to as food processing aids to authorization before use, while in the UK the authorization is voluntary (Ramos et al., 2011). Therefore, it is imperative for legislation of each country worldwide to define the ranges of the permitted enzymes and their applications.

16.10 Conclusions

For the future purpose, there is need to explore the benefits of novel approaches, such as recombinant DNA technology, screening, protein engineering, enzyme immobilization, solid-state fermentation and use of bioinformatics tool in enzyme

engineering in generating food-grade enzymes, and to adapt the enzymes for sustainable and economical industrial production processes. Understanding the novel approaches can aid the industrial production of food-grade enzymes which can be useful in improving food product quality and consistency, preventing potential harmful by-products in the food, reducing raw material dependence during processing, replacing chemical additives, reducing process time, increasing shelf life and creating more opportunities for enhancing the aromatic, structural and textural properties of food. In addition, application of food-grade enzymes for industrial processes should be able to lead to the development of new or improve healthy food products and food properties within the food processing industry; bakery, dairy, beverages, juice, alcoholic, meat, fats and oils, vegetables and other industries. Proper selection of enzymes source, enzymes isolating method and purification technique that is safe and non-toxic must be carefully selected as they influence the cost of production of food-grade enzymes and yield. Solid-state fermentation produces higher enzymes than submerged fermentation at a lower cost. National legislation of countries should make provision for permitting and regulating products from food-grade enzymes and their applications. More research should be channel towards the application of enzymes technologies in food processing to broaden our knowledge and future use because consumers are now demanding for food products or additives that are free of chemicals.

References

Alderson, R. G., Ferrari, L. D., Mavridis, L., McDonagh, J. L., Mitchell, J. B. O., & Nath, N. (2012). Enzyme informatics. *Current Topics in Medicinal Chemistry, 12*(17), 1911–1923.

Aguilar, C. N., Gutiérrez-Sánchez, G., & Rado-Barragán, P. A. (2008). Perspectives of solid state fermentation for production of food enzymes. *American Journal of Biochemistry and Biotechnology, 4*, 354–366.

Aravindan, R., Anbumathi, P., & Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology (IJBT), 6*(02), 141–158.

Bekhit, A. A., Hopkins, D. L., Geesink, G., Bekhit, A., & Franks, P. (2014). Exogenous proteases for meat tenderization. *Critical Reviews in Food Science and Nutrition, 54*(8), 1012–1031.

Chelikani, P., Fita, I., & Loewen, P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences, 61*(2), 192–208.

Dillon, A., Said, S., & Pietro, R. C. L. (2004). Celulases. In L. Summa (Ed.), *Enzimas como agentes biotecnológicos* (pp. 243–270). Legis Summa.

Dipasquale, L., Gambacorta, A., Siciliano, R. A., Mazzeo, M. F., & Lama, L. (2009). Purification and biochemical characterization of a native invertase from the hydrogen-producing Thermotoga neapolitana (DSM 4359). *Extremophiles, 13*(2), 345–354.

Eising, R., Trelease, R. N., & Ni, W. (1990). Biogenesis of catalase in glyoxysomes and leaf-type peroxisomes of sunflower cotyledons. *Archives of Biochemistry and Biophysics, 278*(1), 258–264.

Erdal, S., & Taskin, M. (2010). Production of alpha-amylase by Penicillium expansum MT-1 in solid-state fermentation using waste Loquat (Eriobotrya japonica Lindley) kernels as substrate. *Romanian Biotechnological Letters, 15*(3), 5342–5350.

Ertaş, N., Timur, S., Akyılmaz, E., & Dinfikaya, E. (2000). Specific determination of hydrogen peroxide with a catalase biosensor based on mercury thin film electrodes. *Turkish Journal of Chemistry, 24*(1), 95–99.

Guimarães, L. H. S., Peixoto-Nogueira, S. C., Michelin, M., Rizzatti, A. C. S., Sandrim, V. C., & Zanoelo, F. F. (2006). Screening of filamentous fungi for production of enzymes of biotechnological interest. *Brazilian Journal of Microbiology, 37*, 474–480.

Gulati, K., & Poluri, K. M. (2016). Mechanistic and therapeutic overview of glycosaminoglycans: The unsung heroes of biomolecular signaling. *Glycoconjugate Journal, 33*(1), 1–17.

Gupta, A., Gupta, V. K., Modi, D. R., & Yadava, L. P. (2008). Production and characterization of a-amylase from *Aspergillus niger*. *Biotechnology, 7*(3), 551–556.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry, 38*(11), 1599–1616.

Hartley, B. S., Hanlon, N., Robin, J., Rangarajan, J., & Ragaranjan, M. (2000). Glucose isomerase: Insights into protein engineering for increased thermostability. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1543*(2), 294–335.

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology, 39*, 235–251.

Heredia-Sandoval, N. G., Valencia-Tapia, M. Y., Calderón de la Barca, A. M., & Islas-Rubio, A. R. (2016). Microbial proteases in baked goods: Modification of gluten and effects on immunogenicity and product quality. *Food, 5*(3), 59.

Jackel, C., Kast, P., & Hilvert, D. (2008). Protein design by directed evolution. *Annual Review of Biophysics, 37*, 153–173.

Jegannathan, K. R., & Nielsen, P. H. (2013). Environmental assessment of enzyme use in industrial production—A literature review. *Journal of Cleaner Production, 42*, 228–240.

Kalpana, D. M., Banu, R. A., Gnanaprabhal, G. R., Pradeep, B. V., & Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. *Indian Journal of Science and Technology, 1*, 1–6.

Kandukuri, S. S., Noor, A., Ranjini, S. S., & Vijayalakshm, M. A. (2012). Purification and characterization of catalase from sprouted black gram (Vignamungo) seeds. *Journal of Chromatography B, 889-890*, 50–54.

Konsoula, Z., & Liakopoulou-Kyriakides, M. (2007). Co-production of alpha-amylase and beta-galactosidase by *Bacillus subtilis* in complex organic substrates. *Bioresource Technology, 98*(1), 150–157.

Lin, L.-L., Liu, J.-S., Wang, W.-C., Chen, S.-H., Huang, C.-C., & Lo, H.-F. (2008). Glutamic acid 219 is critical for the thermostability of a truncated α-amylase from alkaliphilic and thermophilic Bacillus sp. strain TS-23. *World Journal of Microbiology and Biotechnology, 24*(5), 619–626.

Mao, X., Hong, Y., Shao, Z., Zhao, Y., & Liu, Z. (2010). A novel cold-active and alkali-stable beta-glucosidase gene isolated from the marine bacterium Martelella mediterranea. *Applied Biochemistry and Biotechnology, 162*(8), 2136–2148.

Mala, R. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews, 62*(3), 597–635.

Mehaia, M. A., & Cheryan, M. (1987). Production of lactic acid from sweet whey permeates concentrates. *Process Biochemistry, 22*(6), 185–188.

Nakagawa, T., Nagaoka, T., Taniguchi, S., Miyaji, T., & Tomizuka, N. (2004). Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes. *Letters in Applied Microbiology, 38*(5), 383–387.

Nakagawa, T., Ikehata, R., Uchino, M., Miyaji, T., Takano, K., & Tomizuka, N. (2006). Cold-active acid β-galactosidase activity of isolated psychrophilic-basidiomycetous yeast *Guehomyces pullulans*. *Microbiological Research, 161*(1), 75–79.

Neelakantan, S., Mohanty, A. K., & Kaushik, J. K. (1999). Production and use of microbial enzymes for dairy processing. *Current Science, 77*(1), 143–148.

Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., & DiNovi, M. J. (2006). Food-processing enzymes from recombinant microorganisms—A review. *Regulatory Toxicology and Pharmacology, 45*(2), 144–158.

Panesar, P. S. (2010). *Enzymes in food processing: Fundamentals and potential applications*. IK International Pvt Ltd..

Quax, W. J., Mrabet, N. T., Luiten, R. G., Schuurhuizen, P. W., Stanssens, P., & Lasters, I. (1991). Enhancing the thermostability of glucose isomerase by protein engineering. *Biotechnology (N Y), 9*(8), 738–742.

Rastogi, H., & Bhatia, S. (2019). Future prospectives for enzyme technologies in the food industry. In *Enzymes in food biotechnology* (pp. 845–860). Academic Press.

Ramarethinam, S., Latha, K., & Rajalakshmi, N. (2002). Use of a fungal lipase for enhancement of aroma in black tea. *Food Science and Technology Research, 8*(4), 328–332.

Ramos, Malcata, O. S., & Malcata, F. X. (2011). *3.48 Food-grade enzymes* (pp. 555–569). Elsevier.

Reddy, N. S., Nimmagadda, A., & Sambasivarao, K. R. S. (2003). An overview of the microbial α-amylase family. *African Journal of Biotechnology, 2*(12), 645–648.

Synowiecki, J., Grzybowska, B., & Zdziebło, A. (2006). Sources, properties and suitability of new thermostable enzymes in food processing. *Critical Reviews in Food Science and Nutrition, 46*(3), 197–205.

Uzuner, S., & Cekmecelioglu, D. (2019). Enzymes in the beverage industry. In *Enzymes in food biotechnology* (pp. 29–43). Academic Press.

Wang, Q.-F., Hou, Y.-H., Xu, Z., Miao, J.-L., & Li, G.-Y. (2008). Purification and properties of an extracellular cold-active protease from the psychrophilic bacterium *Pseudoalteromonas* sp. NJ276. *Biochemical Engineering Journal, 38*(3), 362–368.

Fernandes, P. (2010). Enzymes in food processing: A condensed overview on strategies for better biocatalysts. *Enzyme Research, 2010*, 862537. https://doi.org/10.4061/2010/862537

Sachan, S., & Singh, A. (2015). Lipase enzyme and its diverse role in food processing industry. *Everyman's Science, 4*, 214–218. (A publication of Indian Science Congress Association, Kolkata).

Singhania, R. R., Patel, A. K., & Pandey, A. (2010). The industrial production of enzymes. In *Industrial biotechnology* (pp. 207–225). Wiley-VCH Verlag GmbH & KGaA.

Singh, R. S., Singh, T., & Pandey, A. (2019). Microbial enzymes—An overview. In *Advances in enzyme technology* (pp. 1–40). Elsevier.

Soares, I., Tavora, Z., Barcelos, R. P., & Baroni, S. (2012). Microorganism-produced enzymes in the food industry. In B. Valdez (Ed.), *Scientific, health and social aspects of the food industry*. Intech. ISBN 978-953-307-916-5.

Spök, A. (2006). Safety regulations of food enzymes. *Food Technology and Biotechnology, 44*, 197–209.

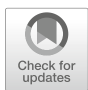
Sumantha, A., Larroche, C., & Pandey, A. (2006). Microbiology and industrial biotechnology of food grade proteases: A perspective. *Food Technology and Biotechnology, 44*, 211–220.

Tiwari, M. K., Singh, R., Singh, R. K., Kim, I.-W., & Lee, J.-K. (2012). Computational approaches for rational design of proteins with novel functionalities. *Computational and Structural Biotechnology Journal, 2*, e201209002. https://doi.org/10.5936/csbj.201209002

Treichel, H., de Oliveira, D., Mazutti, M., Di Luccio, M., & Oliveira, J. (2009). A review on microbial lipases production. *Food and Bioprocess Technology, 3*, 182–196. https://doi.org/10.1007/s11947-009-0202-2

Tukel, S. S., & Alptekin, O. (2004). Immobilization and kinetics of catalase onto magnesium silicate. *Process Biochemistry, 39*(12), 2149–2155.

Zhu, Y. L., Pilon-Smits, E. A., Tarun, A. S., Weber, S. U., Jouanin, L., & Terry, N. (1999). Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. *Plant Physiology, 121*(4), 1169–1177.



Nanotechnology and Food Grade Enzymes 17

Zahra Beig Mohammadi, Khadijeh Khoshtinat, Sanaz Ghasemi, and Zahra Ahmadi

Abstract

Public concerns about food safety and its health, on the one hand, and the rapid advancement in emerging technologies and the green ones, on the other hand, have led to less interest in chemical interactions occurring in the food industry. Enzymes are proteins and consequently sensitive to environmental effects such as pH and temperature. Additionally, they are less likely to be reused and the cost of their recovery is exorbitant. Therefore, their application as natural catalysts has been limited. Having tackled the problems associated with free enzymes, enzyme immobilization made it possible to reuse and store enzymes. Then, nanotechnology implemented this technique to end in nanobiocatalysis which merges nanotechnology and biotechnology and stabilizes enzymes accordingly. Nano carriers improve enzymes' activity and efficiency, stability, and storage ability as they possess unique features such as nanoscale size, high surface/volume ratio, and variety in composition and structure. Nanobiosensors are another breakthrough in bio and nanotechnologies which can substitute chemical and electrochemical

Z. B. Mohammadi (✉)
Department of Food Science and Technology, North Tehran Branch, Islamic Azad University, Tehran, Iran
e-mail: z.beigmohammadi@iau-tnb.ac.ir

K. Khoshtinat
Institute of Nutrition and Food Technology Research Institute, Nutrition and Food Technology Faculty, Shahid Beheshti Medical Science of University, Tehran, Iran

S. Ghasemi
Faculty of Food Science and Technology, Isfahan University of Technology, Isfahan, Iran

Z. Ahmadi
Food Hydrocolloids Research Center, Department of Food Science and Technology, Ferdowsi University of Mashhad (FUM), Mashhad, Iran

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022

A. Dutt Tripathi et al. (eds.), *Novel Food Grade Enzymes*,
https://doi.org/10.1007/978-981-19-1288-7_17

sensors for analysis, quality control, and food safety measurement. Recently, nanomaterials-based biosensors have acted as the focal point in food contamination detection, namely pesticides, antibiotics, and heavy metals, due to their variety, highly specialized activity, and high precision. In this chapter, we investigate nanotechnology and food enzymes, probable risks, and legal aspects of nanomaterial application in food bioprocess.

Keywords

Nanotechnology · Enzyme · Nanobiocatalysis · Nanobiosensor · Food analysis · Food safety

17.1 Introduction

The prefix "nano" comes from the Greek word for dwarf which means "one-billionth" and the nanometer is equal to one-billionth of a meter that is extremely small to see even by a conventional light microscope (Ravichandran, 2010; Sundarraj, 2019). The late Norio Taniguchi initially applied the prefix "nanotechnology" in 1974 and Richard Feynman first gave its concepts in 1959 (Handford et al., 2014; He & Hwang, 2016).

Nanomaterials are tiny particles at least ranging from 1 to 100 nm in size in one dimension, bio-persistent or insoluble in nature, synthesized through numerous ways. Nanotechnology is a branch of science that manipulates the materials at nanoscale which are synthesized in various routes, and are used in different fields including electronics, medicine, agriculture, and food industries (He & Hwang, 2016; Ghasemi et al., 2017).

This reduction in size results in highly impressive features and functions. Nanomaterials are able to be applied in different forms such as nanotubes, nano rods, nanoparticles, nano sheets, and nanofibers in various technologies and industries of food, electronics, catalysts, energy, and agriculture (Jafarizadeh-Malmiri et al., 2019; Handford et al., 2014). Comparing nanomaterials and materials in bulk form, it can be concluded that changing the particle sizes of materials into nanoscale increases the ratio of surface area to volume, leading to unique physical, mechanical, chemical, optical, and biological properties such as solubility, absorption of the cells, delivery properties, and the residence time in the cells of body. On the other hand, it affects the bioavailability, safety, nutritional value, and efficiency properties (McClements & Xiao, 2017). This factor plays a critical role in creating unique features, so engineered nanomaterials have raised more attention in the industries of agro-food, medicine, sewage water treatments, and others (Haroon & Ghazanfar, 2016).

Recently, the industries of food and agricultural have had significant investments in nanotechnology which enhance the quality of product and reduce total prices (Yu et al., 2018a). Firstly, Pasteur introduced nanotechnology by using it in pasteurization process in food industries to omit the bacteria of spoilage (1000 nm) which

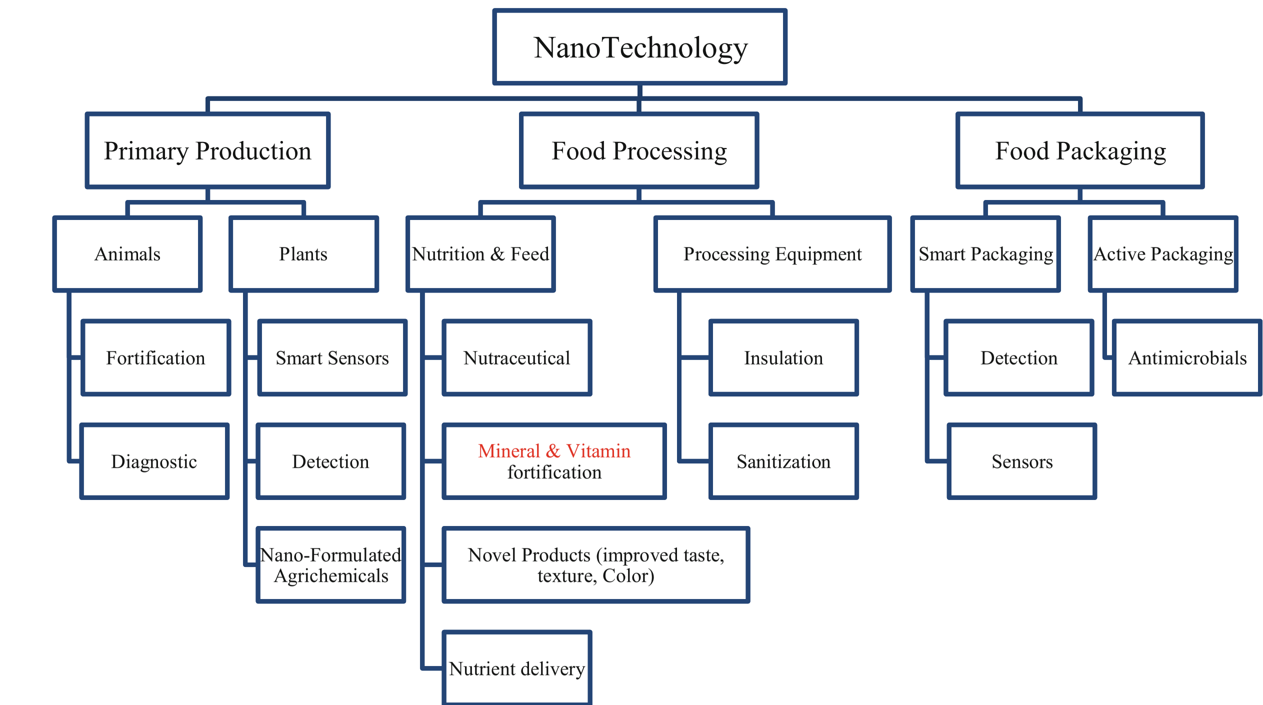
made the first step to transform the food processing and improve food quality. Watson and Crick first built a model for the structure of DNA at almost 2.5 nm which enabled the applications in biomedicine, biotechnology, production processes, and agriculture (Hansen et al., 2013). Nanotechnology has immense potential in the postharvest food processing. The main applications of nanotechnology in the food and agriculture structures are food processing and security improvements, production, packaging of food which enhances its functionality, food bioavailability, nutrition and flavor, pathogen detection, protection of the environment, nutrients absorption of plants, and the cost-effectiveness of storage and distribution. It also modifies the particle size, size distribution, surface charge, possible cluster formation, and delivery methods (Rashidi & Khosravi-Darani, 2011; Ghasemi et al., 2018). Study in nanotechnology has high potential to advantage humanity in food-related industries (Fig. 17.1).

Generally the potential of applying nanotechnology in food industries has been in nanocarriers (such as nanoparticles, nanoemulsion, nanocomposites, nanobiocomposites, and nanolaminates), nanosensors and nanobiosensors (for the control of safety and quality of the food), processing (such as nanofiltration, nanoscale enzymatic reactor, heat and mass transfer, nanofabrication and nanocapsules for modification of absorption), delivery, packaging, formulation, DNA recombinant technology, evaluation, etc. (Ghasemi et al., 2018; Jafarizadeh-Malmiri et al., 2019), but the main nanotechnology applications in food industries are food nanostructured components and food nano sensing to increase the safety and quality of food. Improvements in the production of inorganic nanoparticle have permitted the preparation of effective nanosensors to identify pathogens or pesticides rapidly in food (Neethirajan & Jayas, 2011; Nile et al., 2020).

Our main goal in this chapter was to provide a brief overview of the use of nanotechnology and nanobiotechnology, namely enzymes, in/on nanocarriers on the food industry improvements especially food packaging. Also, the potential applications and future perspectives of nanobiotechnology on food safety were discussed. Also this chapter overviews the information usually accessible about nanomaterials' risk assessments, marketing concerns, safety and toxicity aspects, and regulatory aspects.

17.2 Overview of Nanotechnology and Nanobiotechnology Applications in Food Industry

Request for extra innovative improvements and technologies in valuable tools and materials for the life study are supported by the technologies of nanotechnology and biotechnology which are among the promising technologies in the twenty-first century (McClements & Xiao, 2017). Nanotechnology is a technology that uses materials and devices possessing at least one dimension sized in nanoscale, which plays an essential role in the food and agriculture parts, contributes to improve the crop, progresses the food quality and safety, and supports the health of humans through innovative and novel approaches (Jafarizadeh-Malmiri et al., 2019).



accessible about nanomaterials' risk assessments, marketing concerns, safety and toxicity aspects, and regulatory aspects.

Fig. 17.1 The nanotechnology applications in food areas

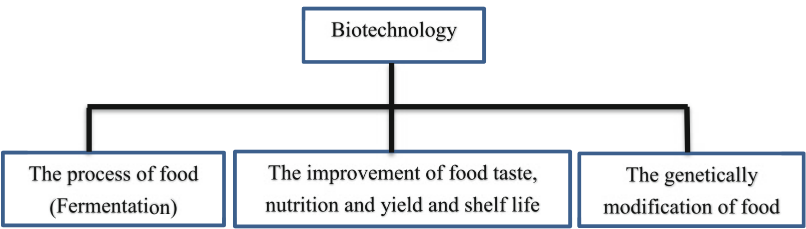


Fig. 17.2 The applications of biotechnology in food industries

Generally, biotechnology contains physiological processes of biological agents such as microorganisms, enzymes, and living cells. Actually, the techniques and knowledge of biology in biotechnology have been used to manage the cellular, genetic, and molecular processes for developing services and products in different fields such as food and agriculture and medicine (Faramarzi et al., 2020; Jafarizadeh-Malmiri et al., 2019). Biotechnology is helpful in the processing of foods (the processes involved enzymes and fermentation) and in removing malnutrition, hunger, and illnesses from all the countries (Haroon & Ghazanfar, 2016). The most important biotechnology applications in industries of food are categorized into three groups which are shown in Fig. 17.2.

In food industries biotechnology is applied to food processing as shown in Fig. 17.1 by using microorganisms and their enzymes to improve characteristics including the aroma and taste, texture, nutritional values, and shelf life in foods which is known as fermentation (Haroon & Ghazanfar, 2016; Ruane & Sonnino, 2011). In other words, in the process of fermentation the microorganisms break and catabolize the organic compounds under aerobic and anaerobic circumstances for manufacturing the ultimate products. Also biotechnological tools can be applied for modifying food genetically, which means that genetically engineered microorganisms are applied in order to produce organic acids, vitamins, sweeteners, amino acids, nutritional supplements, and edible oils. This process can be developed from the insertion of functional gene on DNA into a host bacteria (such as lactic acid bacteria) (Haroon & Ghazanfar, 2016; Jafarizadeh-Malmiri et al., 2019).

Nanobiotechnology has obtained more attention nowadays because of its varied applications in the industries of food. Progress in nanobiotechnology leads to a better viewpoint in enzyme technology and has more importance toward commercialization of enzymes, as well as a further development in catalytic performance (Jafarizadeh-Malmiri et al., 2019).

Nanobiotechnology is the integration of nano-engineering and molecular biology which combines the design of novel materials in nanoscale and devices with the wonderful characteristics for cells, biological molecules, and enzymes to find the solution for the most important difficulties in biology (Ghouri et al., 2020; Jafarizadeh-Malmiri et al., 2019).

The investigation in the field of nanobiotechnology in the food industries mainly contains adding biosensors, antimicrobial, antioxidants, and further nanomaterials in the form of nanoparticles, edible nanocomposite films, and nanobiosensor at

packaging. In recent years, Nanobiotechnology has been a center of attention in food packaging. Innovative food packaging has significant results on preserving safety and quality of foods, during storage and transportation to customers. Actually, packaging can be able to extend the shelf life of foods by controlling disagreeable conditions and factors such as microorganisms, moisture, enzyme activities, and chemical contaminants. Many studies have been done on the application of nanobiotechnology in packaging, which are described in Table 17.1.

As shown in Table 17.1, the merging of the materials for food packaging and active ingredients is a novel way for surface microbial contamination control in foods. Several nanomaterials such as silver exhibit antimicrobial influences. The potential perspectives of nanocomposites for the applications of packaging for foods based on nano and bio ingredients, including biodegradable and edible nanocomposite films, have achieved consideration because the coatings on food products have significant effects in preservation, distribution process, and selling various foods (Morais et al., 2014).

Nanosensors can identify particular indicators of the metabolism of pathogens or can inform the customers of the light, temperature of product, or changes of humidity in the environment during storing. This can omit the need for expiration date in various occurrences and causes the consumers know the precise state of spoilage in food and develop the products' shelf life (Jafarizadeh-Malmiri et al., 2019). The sensor which is applied for the biological organisms is known as biosensor. Biosensors are used for the detection of biomolecule such as several microorganisms and pathogens, glucose, and urea and as a complementary tool to classical analytical methods such as high-performance liquid chromatography which is required for food analyses. It is clear that the applications of nanomaterial in biosensors prepare chances to produce a novel aspect for biosensor technologies for applying them in food analysis (Jafarizadeh-Malmiri et al., 2019; Sundarraj, 2019).

Nanobiosensors gained their specificity from the reaction of biological binding, which includes enzyme/substrate/cofactor, antigen/antibody, nucleic acid hybridization, chemical interactions, and ligand/receptor in combination with different transducers. Nowadays enzymes are applied as nanobiosensors much more than the others (Thakur & Ragavan, 2013).

Today, enzymes can improve texture, nutritional value, and appearance and may produce desirable aromas and flavors in a wide range of applications such as processing of the cheese and starch, bakery, and producing the various fruit juices and further drinks. Food enzymes sometimes are originated from plants or animals (such as amylase, an enzyme for starch digestion), but most of them come from a range of useful microorganisms (Fathi et al., 2018).

Enzymes are biological catalysts which can lower activation energy due to accelerating the chemical reactions (Jafarizadeh-Malmiri et al., 2019). Due to their excellent properties such as specificity, selectivity, and high activity, enzymes have various applications in food productions such as yogurt, bread, cheese, and fermented beverages (Sheldon, 2007; Jafarizadeh-Malmiri et al., 2019). But use of native enzymes in food have following limitations which are effective keys in enzymatic production and their commercialization (James et al., 1996):

Table 17.1 Nanobiotechnology applications in food industries

Method of nanobiotechnology	Type of nanomaterial	Matrix	Application	Reference
Nanoparticles	Silver	Fresh-cut melon, poultry meat, asparagus, orange juice, beef meat exudates	Delays the growth of aerobic psychrotrophics, yeasts, and molds; effective as an antimicrobial material in killing *Staphylococcus aureus* and *Escherichia coli*	An et al. (2008), Fernández et al. (2009), Fernández et al. (2010), Fernandez et al. (2010), Emamifar et al. (2010)
	Titanium oxide	Chinese Strawberry, jujube	Decreasing browning, slow-senescence, down ripening, and decay	Li et al. (2009)
	Zinc oxide	Liquid egg albumen, orange juice	Decreased *Lactobacillus plantarum*, yeast, Salmonella, and mold counts with no changes in the parameters of quality	Emamifar et al. (2010), Emamifar et al. (2012), Jin and Gurtler (2011)
	Silver oxide	Apple slice	Delays microbial spoilage	Zhou et al. (2011)
Edible nanocomposite film	Silica in situ improved the biodegradable films of PVA/chitosan (CS)	Cherries	Significant reduction in the permeability of moisture and oxygen by 10.2% and 25.6%, respectively, and extending the preservation time of cherries	Yu et al. (2018b)
	Edible film with chitosan	Ready-to-eat (RTE) roast beef	Chitosan exhibited antimicrobial activity against *Listeria monocytogenes*	Beverlya et al. (2008)
Nanosensors	Cadmium telluride quantum dot nanoparticle (CdTe QD)	2,4-D (herbicide)	The pesticide of 2,4dichlorophenoxyacetic acid was detected	Vinayaka et al. (2009)
	Hetero-structured silicon/gold nanorod	Suspension of salmonella bacterial in 1% BSA solution	Au/Si nanorods based biosensor could detect Salmonella	Park et al. (2007)

- High prices
- The need of various enzymes
- Low stability of the enzyme
- High residual enzyme remained in the product and difficulties for their recovery and reuse
- Incompatibility of the continuous process
- Low activity
- Higher labor costs

Therefore, immobilization appeared as a new and powerful method to overcome these limitations and achieve enzyme separation, recovery, lifetime cycle rate, and good stability. By enzyme immobilization, the enzyme mobility artificially becomes restricted that leads to change of its properties, structure, and activity. On the other hand, this process is based on absorption of enzymes onto nanocarriers known as nanobiocatalysis (Jafarizadeh-Malmiri et al., 2019). The methods and conditions for immobilizing the enzymes are explained in Chap. 14 completely. Application of this nanotechnology as a novel method can improve the material properties such as remote addressability, multifunctionality, multi-compartmentalization, and stability (Cushen et al., 2012). So use of carriers for enzymes in nanoscale can improve the enzyme characteristics such as maximum surface area per unit mass, diffusional limitation, the loading of enzyme, the activity of enzyme, the possibility of magnetic separation, resistance to proteolytic digestion, and better rate of electron transfer which causes more applications for nanobiocatalyst in food industries (Faramarzi et al., 2020).

The nanoencapsulated enzymes, antigens, antibodies, glycol-proteins, and polysaccharides can be recommended as effective nanobiosensors in food packaging, which can detect numerous chemical or microbial spoilage of foods and eliminate pathogenic microorganisms, thus reducing diseases in people as a consequence of food consumption (Bajpai et al., 2018; He & Hwang, 2016). Immobilization of enzymes in/on the nanocarriers is a critical and essential step for the design of nanobiosensors. Nanobiosensors were advanced for being sensitive, small, portable, very exact, quantifiable, reproducible, stable, and trustworthy (Kumar & Neelam, 2016). So in this chapter the use of enzymes as nanobiosensors was discussed completely.

17.3 Application of Nanotechnology with Food Enzymes

Recent advances in biotechnology have shown increasing attention to the use of modern technologies for the development and growth of green and sustainable biotechnology using enzymes. Immobilization of suitable enzymes along with the use of biodegradable has gained currency as reduced chemical consumption has received much attention for many attractive applications, including biocatalysts and biosensors. The main challenge of using bioprocesses with enzyme catalysts is high operating costs due to low stability and inability to reuse enzymes on large-scale

industrial processes. Enzyme immobilization has been growing rapidly in recent years. Immobilization of enzymes means that they are physically restricted to a specific area by contact with the support, while trying to keep their catalytic activity at a maximum level. This technology is usually able to protect enzymes against adverse chemical and environmental conditions such as changes in temperature and pH level. Importantly, immobilized enzymes can be recovered and reused on a large-scale continuous industrial scale. However, immobilization may lead to a series of structural changes that may lead to serious changes in the properties, activity, and performance of enzymes relative to their natural state. To solve these problems, the process of immobilization of enzymes in various methods and various carriers is optimized to consider the best option with the highest efficiency for the intended purpose (Jafari, 2019; Netto et al., 2013).

Nanotechnology as a new and advanced technology offers more efficient methods than previous conventional methods to overcome technological barriers in many areas, especially for food production (Jafari et al., 2015). Many researches have been conducted on the use of nanotechnology in food for the production, preservation, packaging, and delivery of biologically sensitive food and pharmaceutical compounds. Nanoscale food, compared to micro type, leads to increased solubility, more bioavailability and more accurate and controllable release. Nanomaterials (NMs) have been highlighted in technological advances due to their adjustable physical, chemical, and biological properties with better performance than their bulky types. NMs are classified based on their origin, composition, size, and shape. The ability to predict the unique characteristics of NMs increases the value of each classification for their industrial applications. In the following section, more attention will be paid to the different nanostructures and their applications for food enzyme immobilization (Shahiri Tabarestani & Jafari, 2019).

17.3.1 Enzyme Immobilization in/on Nanocarriers

The protein and sensitive nature of enzymes justifies the need to select appropriate immobilization methods to maintain their structure and activity. Significant advances in the recognition of enzymes and their immobilization have led to the use of NMs in various structures and compounds as a support to immobilize enzymes. Enzyme carriers, in general, should have a large surface area, functional groups, as well as biodegradability. NMs have a much higher surface-to-volume ratio than conventional carriers and can provide more and better enzymatic immobilization (Jia et al., 2014; Saallah et al., 2016).

Enzyme immobilization methods are generally divided into three main categories: entrapment or encapsulation, binding to a carrier or matrix, and cross-linking. Immobilization may be reversible or irreversible, involving direct adsorption to the surface. Different bonds on the surface of enzymes lead to various interactions, such as hydrophobic, electrostatic, and van der Waals forces and hydrogen bonds. Types of irreversible immobilization include immobilizing the enzyme by covalent bonding to the carrier surface, entrapment in the matrix, or

encapsulating the enzyme. In this case, it prevents the enzyme from being washed during the reaction, but these supports cannot be reused after finishing the work and reducing the activity of the enzyme and should be discarded. In contrast, reversible immobilization allows recovery of enzymes after inactivation of the enzyme, but some of the enzyme may be lost during washing. Cross-linking agents (such as glutaraldehyde) are commonly used in cross-linking method. These agents have very low molecular weight relative to the enzyme, so in this method the enzyme remains almost unchanged and is also called a carrier-free biocatalyst with 100% activity. It is undeniable that in order to achieve optimal immobilization, a proper understanding of support and enzyme properties is required. On the one hand, changes in the three-dimensional structure of enzymes and the amphiphilic nature of proteins (hydrophobic inner part and hydrophilic outer part), the functional groups of supports, and the type of bonds they can form should be carefully considered (Netto et al., 2013).

Advanced developments in nanotechnology have created different modes for enzyme immobility in different nanomaterials (Husain, 2010). The combination of enzymes with nanomaterials as nanobiocatalysts (NBs) provides a better option than a variety of chemical catalysts that can be referred to green technology. Enzyme immobilization has been used by various nanostructures such as nanofibers, nanotubes, and nanoparticles. These supports are preferred because of their large specific surface areas (great surface area-to-volume ratios) which can provide extreme immobilization enzymes (Agustian et al., 2016; Beig Mohammadi et al., 2016). However, some nanostructured materials also have their own disadvantages. For example, mesoporous silica typically binds enzyme molecules to its inner surface, which restricts the diffusion of the substrate to the enzyme and reduces activity. Nanoparticles and nanotubes also dramatically reduce mass transfer limitations, while their dispersing and recycling is more challenging. Conversely, electrospun nanofibers have a high ability to solve these problems and can be favorable supports for enzyme immobilization (Beig Mohammadi et al., 2016).

17.3.1.1 Nanofibers

Nanofiber membranes with special properties such as continuous, porous, and nano-open structure allow easy access of the substrate to the active sites of enzyme molecules. Therefore, compared to other nanostructures, substrate can more easily diffuse in this continuous porous space and perform the necessary reactions. In general, nanocarriers for enzyme immobilization can be solid or liquid, spherical or non-spherical, fibers, tubes, and network characteristics (Wen et al., 2017). During the last few years, materials based on one-dimensional nanostructures (nanofibers and nanotubes) have created a subject of substantial interest due to their unique properties. Nanoelectrospun fibers, for instance, have achieved more attention compared to other thicker fibers made of the equal material, and their applications in different industries are relatively new. Immobilization of enzymes and encapsulation of nutraceutical compounds or probiotics in/on nanofibers for maintaining the quality and effectivity of foods have gained due to the specific properties of nanofibers. In addition, nanofibers produced by electrospinning method have many

advantages over their counterparts by traditional methods, such as drawing, template synthesis, self-assembly, phase separation, and melt-blowing techniques (Neo et al., 2018).

Nanofibers are continuous filaments with diameters ranging from a few nanometers to 1 μm. In general, nanofibers are produced by electrospinning process with different apparatus programs. Due to its unique properties (high surface-to-volume or surface-to-mass ratio, high porosity, and easy to use for fabricating various structures), nanofibers are potentially useful for different fields in food industries such as enzyme immobilization, nutrient delivery, food packaging, food indicator, and biosensing (Ghosal et al., 2018).

Electrospun nanofibers are formed by various materials such as synthetic and natural polymers alone or in combination with repulsion of electrostatic charges and elongation of the solution. Electrospun technique began in 1897 and it was revitalized during the 1960s by Taylor. Finally, in the 1990s, with the advent of nanotechnology, much attention was received. In recent years, this technology has been developed due to valuable features such as high efficiency, good mechanical properties, low-diameter distributions, and usability for many natural or synthetic polymers or their combinations (Neo et al., 2018).

The electrospinning mechanism is considered as the combined effects of electrostatic repulsions by the charges collected on the exterior layer of the polymer solution and the Coulombic forces exerted by an external electric field. A remarkable degree of sufficient molecular interaction and cohesion to resist electrical tension leads to the formation of a fiber, and this process is referred to as electrospinning. Generally electrospinning does not alter the structure of systems, but reduction of material to the nano size may lead to change some of their properties (Ghorani et al., 2017; Alehosseini et al., 2017).

The desired polymer is prepared to produce nanofibers in a suitable solvent. Solvents are evaporated using high-pressure evaporative electric fields. Finally, ultrathin polymer structures as continuous fiber structures are collected dry by the collector plate. This mechanism is visible in Fig. 17.3.

- All ingredients must be nontoxic.
- Their preparation is cost-effective and affordable.
- They must be resistant during production (heating, freezing, drying, and mechanical stresses), storage, and transportation.
- No adverse effects on organoleptic properties of food products must be observed (appearance, texture, aroma, and taste of the mouthfeeling).
- This not only must not reduce the bioavailability of enzymes and nutrients but also it must have the ability to increase them (McClements et al., 2009).

Enzymes are actually biocatalysts that have many applications in the commercial and industrial regions of food processing, bioreactors, and biosensors. Commercial and recyclable use of biocatalysts depends on the degree of efficiency of the immobilization technique and the residual activity of the enzymes. Methods of enzyme immobilization are divided into different categories: carrier-free.

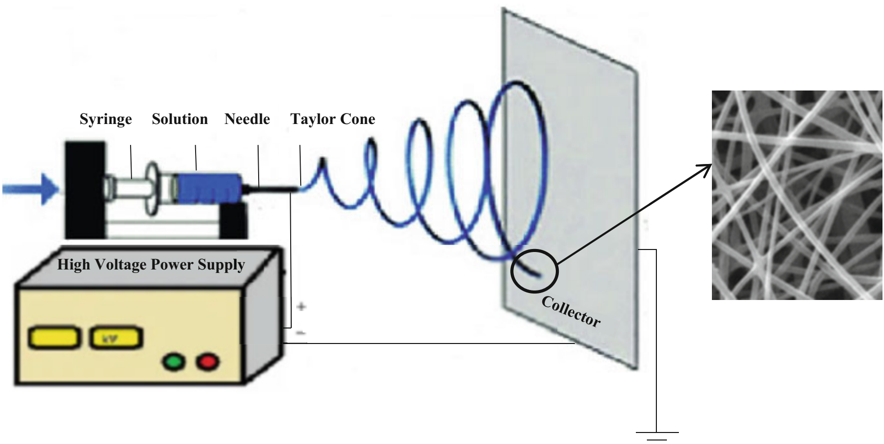


Fig. 17.3 Schematic of the electrospinning process and nanofibers production

Enzyme immobilization on these nanofibers can enhance and retain their activity and binding of enzymes to the nanofibers as well as enzyme separation for recycling. Enzyme encapsulation in nanofibers can also be used as an efficient method to preserve the third structure and activity of the enzyme. The main limitation for immobilized enzymes on nanoelectrospun fibers is the nature of a liquid system for dissolving enzymes and water-insoluble polymers that can remain resistant in a variety of environments and are easily separated. Fiber materials are generally the most desirable supports due to their inherent properties such as good mechanical strength, high specific surface area, and inter-fiber porosity (Gabrielczyk et al., 2018; Rojas-Mercado et al., 2018).

Enzymes can be immobilized after electrospinning by covalent or noncovalent bonding on the fibers, or encapsulated coelectrospinning methods within polymer fibers. Encapsulation of ficin and papain enzymes in nanofiber polyvinyl alcohol (PVA) electrospun and glutaraldehyde (GA) as cross-linking agents were studied. The results showed phycin and papain remained stable 92% (after nine consecutive use periods) and 40% (after four consecutive use periods) of their initial activity, respectively (Rojas-Mercado et al., 2018).

Phospholipase A1 was successfully immobilized on modified surface (MS) chitosan:polyethylene oxide (CS:PEO; 90:10) nanofibrous mat (NFM) with atmospheric plasma (AP) at different times (2, 6 and 10 min). Scanning electron microscopy (SEM) images showed that the membrane structures maintained the nanofiber porous structures and uniformity before and after AP treatments. PLA1 was successfully immobilized by covalent bonding with functional groups of chitosan NFM. The results showed 80% of initial activity of immobilized PLA1 on APSM CS: PEO NFM was still maintained after ten consecutive use periods (Beig Mohammadi et al., 2016).

17.3.1.2 Nanoparticles

Nanoparticles are generally unique and discrete particles with three dimensions with a size of 100 nm or less and have very different properties from bulk materials. Magnetic nanoparticles are composed of various elements, especially iron, nickel, cobalt, and their various chemical compounds. In the food industry, due to the existing safety limitations, nontoxicity of good biocompatibility, nanoparticles commonly used include iron oxides such as Fe_3O_4 magnetic nanoparticles.

Like other nanoscale materials, they perform better than bulk magnetic materials in sizes of 10–20 nm. Magnetic nanoparticles are very efficient as they can be easily separated and recovered using a magnetic field during the process. Since surface energies play an important role, surface interactions between magnetic particles and proteins must be carefully considered. Bates noted that the adsorption capacity of proteins is highly correlated with the activation of chemical bonds on the particle and the protein surface. The absorption capacity of proteins is highly related to the activation of chemical bonds on the particle and the surface of the protein. Nanoporous materials have a lower enzyme load as support but no restriction on substrate diffusion. In contrast, porous materials have a lower restriction of substrate diffusion while showing a high enzymatic load (Netto et al., 2013). Immobilization of α-amylase on carboxylated magnetic nanoparticles, lipase on magnetic Fe3O4 chitosan to synthesize ascorbyl palmitate, invertase on silica-modified magnetic nanoparticles in order to hydrolyze the potato starch to glucose and fructose are successful examples of the application of nanoparticle-immobilized enzymes in the food industry (Cao et al., 2016; Wang et al., 2015; Bayramoglu et al., 2017).

17.3.1.3 Nanosheets

The carbon family is one of the most widely used materials to immobilize enzymes. For this reason, different methods of producing carbon-derived nanomaterials have been studied. Among these materials, graphene is used in the immobilization of enzymes due to its remarkable optical, thermal, electronic, and mechanical properties. It is composed of carbon atoms with a dense two-dimensional structure similar to a honeycomb network. Graphene, in addition to successful drug and gene delivery, has been able to immobilize enzymes due to maintaining the natural protein structure and activity of enzymes (Singh et al., 2014).

Immobilization of Laccase obtained from *Aspergillus oryzae* (for ethanol production, clarification of wine and tea) and α-galactosidase (for hydrolysis of raffinose oligosaccharides (RFOs) cause of flatulence in soybeans) on graphene nanosheets are significant examples of the application of nanosheets in food industries (Singh et al., 2014; Skoronski et al., 2017).

17.3.1.4 Nanotubes

In recent years, carbon nanotubes (CNTs) have been considered for enzyme immobilization due to their special surface properties along with their desirable structural and mechanical properties as well as their very specific surface. The main types of CNT, including single-walled (SWCNTs) and multiwalled (MWCNTs), are used to immobilize enzymes which provide higher enzyme loading and lower mass transfer

resistance. MWCNTs are generally composed of multiple layers of graphite that surrounded a central pipe, which are less expensive and more stable in harsh thermal and mechanical conditions. Both covalent and noncovalent methods could be carried out for binding enzymes to nanotubes (Homaei & Samari, 2017).

Mukhopadhyay et al. (2015) immobilized pectate lyase by entrapment in SWCNTs. The results showed enzymatic behavior and its stability boosted at different temperatures, namely 4 °C and 80 °C and repeated freeze-thaw cycles. In other study enzyme activity of Laccase immobilized on MWCNTs maintained was more stable than the free one after nine cycles and storage time (Tavares et al., 2015).

17.3.2 Application of Nanobiocatalyst in Food Industry

17.3.2.1 Food Products

Different immobilized enzymes have been used by different nanostructured supports in different food industry processes, some of which are discussed in this section (Table 17.2).

Mosafa et al. (2013) immobilized papain onto silica-coated magnetic nanoparticles for applying in pomegranate juice clarification. The immobilized papain, compared with the free papain, improved storage stability and exhibited enhanced enzyme activity, good reusability, and well tolerance to different pH and temperature of the medium. Both the immobilized and free enzymes were influenced for the pomegranate juice clarification (Mosafa et al., 2013).

Sojitra et al. (2016) prepared nanobiocatalyst of magnetic tri-enzyme by co-immobilizing three enzymes simultaneously, pectinase, cellulase, and α-amylase, onto nanoparticle of amino-functionalized magnetic for clarification of juice. The results showed that up to 75% of residual activity of the immobilized enzymes was retained even after reusing eight cycles consecutively. Finally, the clarification of grapes, pineapple, and apple juices using magnetic tri-enzyme was obtained, with 46, 53, and 41% reduction in turbidity, respectively, during 150 min treatment (Sojitra et al., 2016).

Shahrestani et al. (2016) successfully immobilized xylanase onto 1,3,5-triazinefunctionalized silica encapsulated magnetic nanoparticles (MNPs) for clarification of fruit juice. The results showed that the immobilized xylanase on functionalized MNPs (Xy-MNP) had excellent catalytic activity at 65 °C and pH 6.5. Also, Xy-MNPs had a significant influence on juice clarity and showed quite impressive stability so that up to 55% of the initial activity of the immobilized enzymes were retained even after 10 reaction cycles consecutively in the enrichment of the juices (Shahrestani et al., 2016).

Patel et al., (2018) immobilized Smt3-D-psicose 3-epimerase onto nanoparticles of the functionalized iron oxide magnetic for the clarification of wash from the pomace of kinnow and apple fruits. The results showed that the immobilized enzyme had excellent storage stability by missing only 20% activity during 60 days of storage at 4 °C. Up to 90% of initial activity for the immobilized enzyme retained even after reacting 10 cycles of catalyzing D-fructose epimerization consecutively.

Table 17.2 Application of nanobiocatalyst in food industry

Product	Types of nanocarriers	Type of enzyme	Results	References
Soybean oil	Superparamagnetic Fe_3O_4 nanoparticles	Lipase	Immobilizing lipase improved storage stability, exhibited enhanced enzyme activity until 70% initial activity, 90% soybean oil transesterification	Xie and Ma (2010)
Pomegranate juice	Silica-coated magnetic nanoparticles	Papain	Immobilizing papain improved storage stability and exhibited enhanced enzyme activity, good reusability, and well tolerance to different pH and temperature of the medium. Immobilized enzymes could be influenced for the pomegranate juice clarification	Mosafa et al. (2013)
α-Amylase	Magnetic nanoparticles		Up to 70% of the initial activity of the immobilized enzymes was remained. Simply recovered	Baskar et al. (2015)
Fruit juice	1,3,5-triazinefunctionalized silica encapsulated	Xylanase	Up to 55% of the initial activity of the immobilized enzymes were retained even after 10 reaction cycles consecutively	Shahrestani et al. (2016)
Grapes, pineapple, and apple juices	Nanoparticle of amino-functionalized magnetic	Pectinase, cellulase and α-amylase	Up to 75% of residual activity of the immobilized enzymes were retained even after reusing eight cycles	Sojitra et al. (2016)
Wash from the pomace of kinnow and apple fruits	Functionalized iron oxide magnetic nanoparticles	Smt3-D-psicose 3 epimerase	Excellent storage stability by missing only 20% activity during 60 days up to 90% of initial activity for the immobilized enzyme	Patel et al. (2018)

(continued)

Table 17.2 (continued)

Product	Types of nanocarriers	Type of enzyme	Results	References
			retained even after reacting 10 cycles of catalyzing D-fructose epimerization consecutively	
Soybean oil	Atmospheric plasma surface modified chitosan:polyethylene oxide (90:10) nanofibers	PLA_1	78.50% Immobilization efficiency retained 80% of its initial activity after 10 time of recycling decreased phospholipids to 8.6 mg/kg after 6 h	Beig Mohammadi et al. (2020)
Pineapple juice	Polyethylene glycol grafted magnetic nanoparticles	Pectinase	Up to 59% turbidity reduction occurred in treated pineapple juice Up to 55% of the initial activity of the immobilized enzymes was retained after 10 repeated uses	Kharazmi et al. (2020)

Immobilizing made significant improvements in storage stability of the enzyme, its recycling efficiency, and thermal tolerance (Patel et al., 2018).

Kharazmi et al. (2020) immobilized pectinase covalently onto nanoparticles of polyethylene glycol grafted magnetic via trichlorotriazine for fruit juice clarification. The immobilized pectinase exhibited improved operational stability, enhanced catalytic activity, and simple reusability. Up to 59% turbidity reduction occurred in treated pineapple juice with immobilized pectinase. In addition, it exhibited high operational stability of immobilized pectinase and the enhanced reusability, and up to 55 and 94% of the initial activity of the immobilized enzymes was retained after 10 repeated uses for clarification of the pineapple juice and 125 days storage at 25 °C, respectively. It can be suggested for juice and food processing industries (Kharazmi et al., 2020).

Lipase was immobilized onto superparamagnetic Fe3O4 nanoparticles by covalent bonding for soybean oil transesterification. The results demonstrated the residual activity to be almost 70% of its initial activity after four cycles (Xie & Ma, 2010).

The immobilization of PLA_1 on APSM CS:PEO NM by Beig Mohammadi et al. (2020) for soybean oil degumming was carried out. The result showed the immobilized PLA_1 can be reused 10 times without a significant loss in the enzyme activity. In addition, high enzyme loading, effective catalytic activity, and easy

recovery reusability were observed. The residual phosphorus content by immobilized PLA_1 decreased to less than 10 mg/kg after 6 h, which is acceptable according to the standard for the physical refining of soybean oil (Beig Mohammadi et al., 2020).

The magnetic nanoparticles were used as a carrier for immobilized fungal α-amylase. This nanobiocatalyst is used for the production of glucose from starch. The results showed the potato starch had more affinity toward immobilized α-amylase than free type. In addition, the α-amylase simply recovered after each progression (Baskar et al., 2015).

17.3.2.2 Application of Nanotechnology with Food Enzymes in Food Packaging

Materials used in food packaging should improve mechanical, barrier, and antimicrobial properties, as well as imbedded nanosensors to monitor packaged food during transportation and storage. Nanofibers amplify mechanical property to protect foods with brittle texture; adding green tea and oregano extracts to edible films prepare antioxidant activities for packaging materials. Monitoring of changes of food quality during storage could be possible by smart packaging, such as application of multiple pH dyes in fibers used as packaging materials. Nanofibers, as a product of nanotechnology, as well as antimicrobial nanoparticles with controlled release properties, and bioactive components could have significant role in food industries. The structured polymeric films containing nanofibers make great surface area and mass transfer rates, they could be used to detect pathogens in food.

There have been many efforts to innovate nanostructure functional food system, in order to control food safety by nanosensors and nanobiosensors; make tools for objective transfer of nutrients for their nanotherapy; extend controlled release system of nanoencapsulated materials; and form enzymatic reactor in nano size for extension fortification of food with valuable ingredients such as essential fatty acids.

Bionanocomposites, such as polymer nanocomposites, with hybrid nanostructure can improve gas barrier and thermal and mechanical features. Biodegradable types could be environmentally friendly, because of reducing the need to use plastics. These materials need not only inorganic particles to create biopolymer structure but also some additives such as surfactants.

Nanotechnology-derived food products are significantly expected to be reachable for people, because of development of some new innovations such as electrospun nanofibers as a food packaging material or to encapsulate valuable components (Dwivedi et al., 2018).

Nanoparticles, nanoemulsions, nanocomposites, and nanostructured materials are used for food processing, delivery, formulation, and packaging, which are very important for food safety and biosecurity. Application of nanosensors in food packaging has many advantages, and there are some examples including:

- Palladium, platinum, and gold based nanosensors to detect any sort of color changes in food, any gases produced by spoilage, any changes in light, heat,

humidity, and chemicals into electrical signals, and toxins such as aflatoxin in milk.

- Single-walled carbon nanotubes and DNA to monitor soil condition needed to crop growth and detect pesticides on fruit and vegetable surface.
- Carbon black and polyaniline to detect carcinogens and pathogens in food.
- Array biosensors, electronic noses, nano-test strips, and nanocantilevers to monitor color changes resulting from food spoilage.
- Nano-smart dust in order to detect any type of environmental pollution.
- Nanobarcodes to detect agricultural products quality.
- Nanobiosensors in order to detect viruses and bacteria.
- Biomimetic sensors (protein and biomimetic membranes) and smart biosensors to determine mycotoxins and some toxins, act as pseudo cell surfaces to assist pathogens detection and removal.
- Surface plasmon-coupled emission biosensors (containing gold) to detect pathogens.
- Cerium oxide immunosensors and chitosan-based nanocomposites to detect some toxins like ochratoxin A.
- Carbon nanotubes and silicon nanowire transistors to detect staphylococcal enterotoxin B and cholera toxin.
- iSTrip of time-temperature indicator/integrator to detect the spoilage of food based on the history of temperature.
- Abuse indicators to detect desired temperature has been achieved or not.
- Partial temperature history indicator, when the temperature exceeds a certain pre-determined value.
- Full-temperature history indicator to register continuous temperature changes considering time and detect frozen food temperature changes.
- Reflective interferometry in order to detect *E. coli* contamination in packaged foods (Pradhan et al., 2015).

To keep food quality and safety during storage and transport, food packaging plays a key role, by extending the shelf life. Therefore, the type of food packaging materials is very important, as well as being biodegradable and using natural polymer instead of petroleum-based plastics. Some biodegradable polymers used in food packaging are:

- Polysaccharide-based polymers, such as alginate, cellulose, chitin, chitosan, gellan, pectin, starch, and xanthan.
- Protein-based polymers, such as collagen, gelatin, whey protein, soy protein, and zein.
- Aliphatic polymers, such as polylactic acid and polyhydroxybutyrate.

Today, in order to control microbial contamination on surface of foods, active components are to be combined with edible films. The application of biopolymer needs using some additives to make better their mechanical and thermal properties as well as retarding oxidation, controlling of respiration rate, migration of moisture, and

microbial growth. In active packaging, shelf life of foods would be prolonged by using antioxidant and antimicrobial agents in formulation of packaging materials. Organic acids, bacteriocins, and enzymes are some examples of active components used for the formulation of packaging with antimicrobial property.

Because of some specific features of bionanomaterials, like improvement of sustainability and also biological activity, they might be used to encapsulate and deliver some micronutrients. Chitosan, a biopolymer having particular properties, which has been extracted from shells of shrimp and crab, is nontoxic and biodegradable and possesses film forming and antimicrobial properties. Nanoparticles of chitosan have more surface area-to-volume ratio and more effectiveness compared to bulk ones, which make them appropriate for formulation of food packaging materials.

Metal nanomaterials, such as silver and gold, have excellent antimicrobial activity and can show inactivation of microorganisms. As silver nanoparticles could absorb and break down ethylene, they are able to prolong the senescence of fruits and vegetables in packages with films containing them. Also, it is shown that they have good effectiveness for strains such as *E. coli,* which are resistant to silver (Jafarizadeh-Malmiri et al., 2019).

In order to detect *E. coli* O157:H7AQ19 -the pathogen separates by immunomagnetic method- after hydrolyzing of p-nitrophenyl phosphate by alkaline phosphatase the absorbance of p-nitrophenol will be red. Another example of enzyme application for meat freshness monitoring is using yeast sensor (Trichosporon cutaneum), in which signals of flow-injection analysis will show polyamines and amino acids producing by meat, and increasing of bacteria count during its storage.

Most sensors for quality control of meat packaging, which have been introduced by researchers, are not commercialized and should be developed for releasing to market. The application of antimicrobial ingredients in coating in smart packaging by nanomaterials can be a promising impact on food packaging. Of course, fast monitoring methods are needed to check a lot of food packages during storage and shipping for a long period of time (Fatima Mustafa & Andreescu, 2018).

Biocatalysts can have many advantages as an aid in bioprocessing and bioactive packaging, because these components or any reaction adjusted by enzymes help bioactive materials to bring a substrate to react. In fact, an enzyme is a specific catalyst that can increase product quality by producing useful ingredients or decomposing unpleasant ones. Immobilized enzymes not only can be simply recovered, and used again, but also have a low cost for environment, used materials, if the suitable method for immobilizing of enzyme be used. By using nanomaterials for applying immobilized enzymes in food packaging, increasing the ratio of surface area to volume, production scaling up, and mechanical resistance will happen (Wong, 2016).

The intelligent packaging using nanosensor will have a lot of advantages in food industries. Very small chips will be put in the food packages, which act as an electronic bar code, in which food would be monitored in all steps including process,

storage, and even consumption. Developing of wireless nanosensor networks is necessary to cover limitations of nanosensors.

In future, intelligent food packaging using nanotechnology could be a necessity for food safety and quality control by precise and fast methods. These new packaging tools need innovation and advancement of nanosensors as well as lowering the cost (Fuertes et al., 2016).

Time temperature integrators (TTIs) are one of the newest commercialized methods for monitoring fresh foods, which need cold chain during distribution and handling. TTIs functions are based on some reactions, such as enzymatic, microbial, and photochemical, which are dependent on temperature, and reaction speed increases by temperature enhancement.

In a kind of TTIs, lipase shows color changes which are dependent on pH changes. As the spoilage of meat usually is made by microbial growth and enzymatic autolysis, TTIs using enzymatic biological reaction are very effective tool as a freshness indicator and fresh and frozen meat spoilage trend. TTIs can simply be applied into packaging material formulation for frozen fish, fruits and vegetables, and dairy products. Available commercialized TTIs demonstrate changes of color which are simultaneously dependent on temperature and time.

As in meat spoilage, most bacteria use glucose; predicting of meat shelf life can be possible by glucose measuring. The color of some indicators which work on chemical reaction will change.

In rigor mortis, glucose changes to lactic acid and protons in muscles, by anaerobic fermentation. This happens in cold packages of meat as well as freeze-thawed meat products. Lactic acid will simply be detected by pH indicator dyes. In order to control the quality and freshness of food products, in commercialized glucose sensors, glucose oxidases is immobilized on the electrodes surface and ferrocene acts as a mediator.

Electrochemical biosensors are used to monitor food quality in smart packaging. Two main electrochemical biosensors are biocatalytic devices and affinity sensors. The first one redox enzymes, whole cells, or tissue slices would produce electrical signal, and in the second type, antibodies, antibody fragments, or aptamers are targets. In another kind of electrochemical biosensors, enzymes are used to recognize analytes. These are very specific, not expensive, and could simply be suited to materials of packaging (Park et al., 2015).

In active packaging, silver nanoparticles are investigated by many researchers due to their antimicrobial property against foodborne pathogens, and inhibition of fungi and some viruses. Because of silver bactericide effect, binding to bacteria DNA, proteins, and enzymes will happen. Nanoparticles of silver can destroy outer and cytoplasmic membranes of bacteria by inhibition of their respiratory chain enzymes and be effective against *E. coli,* when used in agar film formulation.

The antibacterial activity of nano titanium dioxide particles (nano-TiO2) is also known, although it be limited by exploring UV irradiation. DNA damage through hydroxyl radicals and attack to outer and inner membrane of bacteria are two proposed mechanisms for their bactericide effects. It is shown that plastic films

coated by TiO2 nanoparticles could stop *Penicillium expansum* spoilage in apple, tomato, and lemons, because of their photocatalytic activities when exposed to light.

Also, it is observed the effectiveness of lactase or cholesterol reductase to increase food quality, when are enclosed by packaging materials, as well as adsorption into nanoclays incorporation by polymers (Sharma et al., 2017).

Some sensors utilize enzymes like laccase or tyrosinase for biocatalytic conversion of phenolic antioxidants to their quinones, which will be measured by electrochemical reduction at a low applied potential.

Antimicrobial packaging by inhibition of microbial growth will extend shelf life of food products. In this packaging, active agent, such as organic acids, enzymes will be coated on inner layer or enclosed by packaging material. Silver nanoparticles have been used in biodegradable film, and their antimicrobial activity against *E. coli* and *Staphylococcus aureus* and increasing cherry tomatoes packages shelf life was shown. It is suggested to study on the toxicity of AgNPs, and their potential migration from packaging into food, before commercialization (Mustafa & Andreescu, 2020).

17.3.3 Application of Immobilized Enzyme on/in Nanocarriers as Biosensors in Food Analysis and Safety Aspects

Biosensing is a way for detection, in which combination of biological components and physicochemical methods are used. By increasing of food adulteration, the application of nanobiosensors in safety analysis has got an important potential as high sensitive and selective detection methods. Better identify of target, fast recognition by enhancement of signal production, boost of selectivity and sensitivity, and reduction of time analysis are some of the advantages of biosensors. Various nanomaterials have had effectiveness as biosensors, which can be classified into metallic nanoparticles, metal compound nanomaterials, carbon materials, non-metallic nanomaterials, nanostructures, and composite nanomaterials. Traditional and time-consuming methods to detect microorganisms, as well as increasing demands for quick tests, without culture, led to find the other selective and sensitive techniques. Initial screening of food bacteria could be one of the nanobiosensing advantages. Trace quantity of pathogens' DNA could be detected by nanomaterials and amplification methods such as polymerase chain reaction (PCR), or recognize by antibodies and or aptamer (antibodies as a part of a sensing). Inappropriate storage might make contamination of animal feed and agricultural products with mycotoxins or bacteria toxins; therefore, the detection of toxins is very important in point of food safety. Detection by nanomaterials in biosensing technique could be particularly way as well as fast, susceptible, compared with ordinary methods. For example, aflatoxin B1 has been detected by nanoparticles based biosensors, using antibody-functionalized mesoporous carbon (MSC). Also, fluorescent nitrogen-doped carbon dots on gold nanoparticles (AuNPs) have been used to detect ochratoxin A, an aptamer-based assay method, which is highly selective for other toxins, such as deoxynivalenol, fumonisin B1, and zearalenone. Gold nanoparticles doped

ferrosoferric oxide nanoparticles, cadmium telluride quantum dots-glucose oxidase, and silver nanoclusters are some of metal compound nanomaterials used for toxins detections. Application of nano-extraction using mass spectrometry to extract aflatoxins from liquid samples by an absorbant (polydopamine-coated on nanoparticle) has been reported, as well as to quantify, that was coupled with HPLC-MS/MS.

Pesticides are another harmful components, if accumulate in humans and animals. Therefore, to control the Maximum Residue Levels, using suitable determination methods are necessary. Inhibition of acetylcholine esterase by pesticide is a fast and widely used method, in which organophosphorus compounds and carbamates specifically are inhibited by the enzyme. By nanobiosensing assay, thiocholine generation by acetylcholine esterase catalysis would cause the accumulation of gold nanoparticles, and better their recovery. Another method is direct detection by organophosphorus hydrolase, in which ordered mesoporous carbons are used to immobilize cell surface-displayed enzyme, and could detect paraoxon, parathion, and methyl parathion. Some pesticides would be detected by nanobiosensors based on their electrochemical activities, such as malathion and lindane. The most prevalent method to detect pesticides in food is an immune test by nanobiosensors. Prevention of photochemical property of nanomaterial by pesticides would be the principle of antibodies reorganization, for example, blocking of gold nanoclusters/grapheme hybrid's electrochemiluminescence by pentachlorophenol.

The uncontrolled usage of some antibiotics in animal feed to raise the growth would result in the increase of antibiotic residues in their meat and milk. Therefore, some recognizing assays based on aptamer, antibody, and liposome are used to detect antibiotic residues in food, by using some nanomaterials like glucose oxidase and nanoparticles of gold.

Detection of heavy metals, as hazardous elements, can be done by recognizing some biomolecules such as nucleotides (with colorimetric assay for Hg2+ using gold nanoparticles), DNAzyme (for lead ions with DNA-stabilized AgNCs), amino acid (cadmium and leads ions with graphene-enhanced electrochemical signals), and antibodies (for cadmium biosensing based on coreshell of bimetallic nanoparticles of gold and silver enhanced Raman scattering).

Existence of some components could be recognized by biosensors, like melamine, as a food adulteration (to increase protein content milk), by aptamer of triplex molecular beacon and fluorescent nanocluster of silver or nitrite, as a hazardous additive, by electrochemistry of myoglobin on a reduced glucose oxidase-multiwalled carbon nanotubes-platinum nanoparticles.

Biosensor is an apparatus or tool which contains a biological sensing material composed of a chemical or physical transducer, to convert chemical or biological signals into electrical ones (Fig. 17.4).

The potential of a biosensor will be increased by application of nanomaterials such as carbon nanotubes, nanowires, magnetic nanoparticle, and nanorod. These materials increase sensitivity and decrease limits of detection.

However, formal methods for detecting foodborne microorganisms, like enzyme-linked immunosorbent assay, polymerase chain reaction, and the application of fast, reliable, specified, and low-cost analysis assays, such as electrochemical biosensors,

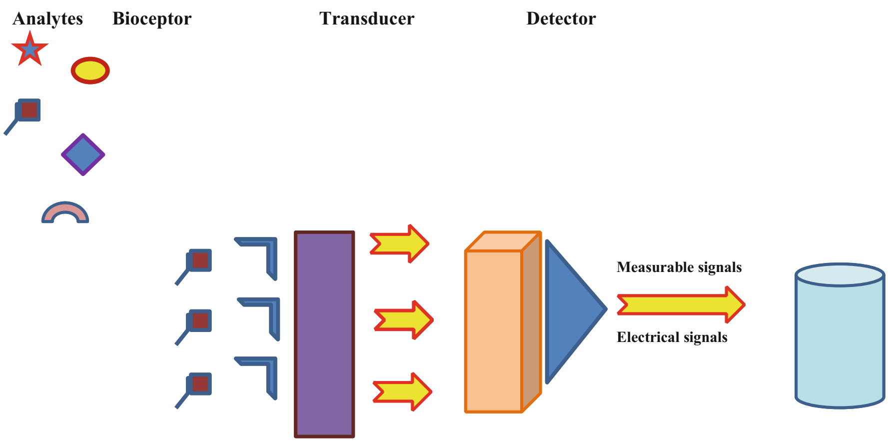


Fig. 17.4 A schematic biosensor

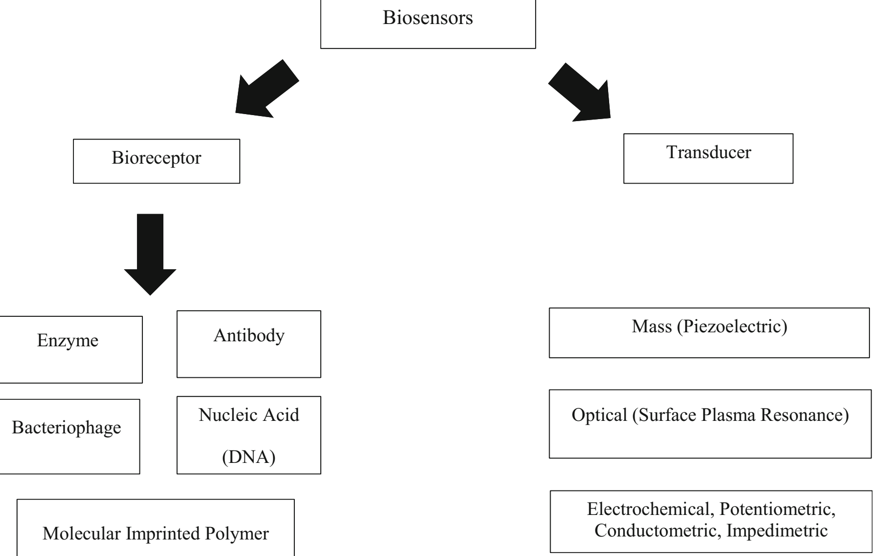


Fig. 17.5 Biosensor classifications and methods

have their own importance. E. coli and Salmonella are examples which have been detected by amperometric and electrochemical DNA biosensors. Classifications and methods of biosensors can be seen in Fig. 17.5.

Transducers, the main part of biosensors, cause physical responses, which may include heat absorption (calorimetric biosensor); variation of charge distribution (potentiometric biosensor); motion of electrons made by a redox reaction (amperometric biosensor); or total effects of reactants (piezo-electric biosensor).

Three creations of biosensors are included: ordinary one, an electrical reply of converter resulted by product reaction; the next one set by particular mediator among convertor and reaction for better reply; in the newest one reaction makes reply without involving product or mediator, straightly.

The main features of a biosensor are having high particularly for the reason of analyses, stability in ordinary storage and a lots of experiments; precise and reproducible reactions with no affected by physical variables such as temperature; no toxic affect; low price, small size, and user-friendly application.

Raghu et al. (2014) immobilized acetylcholinesterase on carbon paste electrode by silica sol–gel method, which worked as an electrode to monitor Malathion and Acephate in foods. The detection and quantification of these two pesticides were done directly, without extraction and pre-concentration as well as less quantity of enzyme. They demonstrated that this immobilized enzyme can work as a biosensor with high sensitivity and low cost.

Biosensors are made of three parts: biological recognition segment, transducer, and a signal processing part. In biosensors, in which enzymes are immobilized on the surface of transducers, enzyme work as a bridge with analyte. These biosensors have many usages in different applications, such as monitoring of food safety. Good sensitivity and specificity and the least preparation of samples are some of their advantages.

One of the biosensor groups which is the greatest one and are produced commercially is electrochemical enzyme-based ones. The use of nanocomponents in biosensors would improve their sensitivity, stability, and detection limit.

Electrochemical enzyme-based biosensors have had successfully and commercially application as a bioanalytic system with a good selectivity and stability, much better than previous ones. These biosensors in nanoscale have more efficiency in being sensitive, selective, and stable as well as detecting limit and response time, comparing to macroscale ones, because nanomaterials increase sensitive surface of the transducer and effectiveness of enzyme immobilization by increasing the ratio of surface to volume. Nanomaterials have good electrical conductivity, magnetic properties, and catalytic activity, which could also improve biosensor properties. These materials have been classified into two groups: organic and inorganic; metals, quantum dots, and zeolites are the examples of the former group, and carbon nanotubes, graphene and graphene oxide, and calixarenes for the latter one.

Inorganic nanomaterials accelerate transfer of electron and catalyze electrochemical reactions, while the organic ones amplify electrochemical signals to increase the level of biocompatibility. These biosensors could have application in food industries, because of their good biocompatibility, which would make them stronger with more specific usage.

Biosensors can be used to recognize a variety of biomolecular complexes, such as antibody, antigen, living cells, and enzyme-substrate interactions. Their nanoscale can detect molecules like glucose because of being small, transportable, having linearity of response, as well as more selectivity, sensitivity, and stability.

In fact, a biosensor is composed of three important parts: a bioreceptor molecules (to classify target analyte), a biotransducer component (to convert a chemical or

biochemical signal into an electrical signal), and finally an electronic system including amplifier, processor, or recorders. The biosensors could be classified into electrochemical, potentiometric, calorimetric, amperometric, resonant, ion-sensitive, and optical. Nanobiosensors are included to optical, bio, chemical, physical, and sol-gel types. Nanosensors matrix would protect food from effects of enzyme or dye toxicity. Of course, their usage in food industries depends on the detection type. For example, chemical or bio detection for optical nanosensors, in which their color changes by reaction with chemical or biological reagents. In biological nanosensors, antibody/antigen; DNA; enzyme interactions would happen. While, chemical composition or molecular concentration may occur in chemical type, or pressure, force, mass, and displacement in physical ones.

Knowing the mechanism of nanobiosensors is very important, for example, by spraying a nano bioluminescent on a food, visual glow could indicate microbial contamination, or released nano particles, such as nano-silver used in active packaging will react with microorganisms. In amperometric nanosensors, organophosphorus pesticides could be detected by mechanism of dual enzyme electrode system.

Technology of biosensors effects on many sections, including pharmacology, medical care, and food industry, in which safety has an important role, as well as easy, quick, and precise control of nutrients and pollutants.

In fact, enzymatic biosensors are electrochemical ones for food analysis, in which substrates would be detected either from their transformed products obtaining by enzymatic catalysis or by the detection of enzyme inhibitors.

To detect xanthine in fish, immobilized xanthine oxidase (MWCNTs) on multiwalled carbon nanotubes and a copolymer to act as a biosensor were used. It was shown that MWCNTs could have increased sensitivity more than biosensor when was applied to immobilize alone. By immobilization of glutamate dehydrogenase in MWCNTs, detection limit of monosodium glutamate was improved.

Nanotechnology has an important role in the agri-food industries for very distinctive properties of nanomaterials. Recently, they are used to detect food adulterants, pathogens, and toxins. Enzymatic nanobiosensor is made of an enzyme immobilized on the surface of a transducer.

Enzymes in nanobiosensor adjust the sensitivity and stability of them; therefore, more substrate specificity makes more enzyme turnover number and thermostability, the main necessity for a biosensor. Bioconjugation is a biosensing method with cost-effectiveness, based on the nature of interaction between nanomaterials and the enzyme surface functional groups. In nanobioconjugation technique, physical (adsorption, entrapment), covalent (covalent binding, cross-linking), and bioaffinity types are used to plan a strong nanobiosensor. Weak bioconjugation could have a little activity of biosensor, following the change of the enzyme active site conformation. Therefore, by more understanding functionality of a biomolecule at interface, better choice to plan and improve a bioconjugation would happen.

Nanobiosensors have been used to detect the pathogens in foods, and the optical assays like fluorescence and surface plasmon resonance are the most usable techniques. It was demonstrated that by using covalently co-immobilized enzymes choline oxidase and peroxidase on zinc oxide nanorod films, detection limit would

be improved, as well as xanthine, when xanthine oxidase immobilized on nanocomposite.

Another example of developing nanobiosensor is detection of glucose in fruit juice by immobilizing pyranose oxidase on gold nanoparticles, which was considered as a quick and sensitive method. The other technique to detect this analyte is immobilization of glucose oxidase on nanoclusters. Fructose has been detected in honey by fructose dehydrogenase which was immobilized on carbon nanotubes. To detect Escherichia coli, immobilization of b-galactosidase on carbon nanotubes has an effect on the lowering boundary of detection. It is supposed that enzymatic nanobiosensors get a perfect and important part of food quality control.

17.4 Risk Assessment of Nanomaterials in Food Industries

17.4.1 Legal Aspects

It is true that universities, companies, and governments have become intensely interested in the application of nanotechnology and nanomaterials in the food industry. However deceiving this area might seem, it is of utmost importance to consider its regulatory issues if this is supposed to be successful. In other words, population acceptance is highly dependent on regulations that assure nanomaterials' safety. According to the latest guidance of EFSA (European Food Safety Authority) any new food which contains nanomaterials is considered a novel system that must be fully tested and authorized to be consumed by society. EFSA is also responsible for verifying the latest approaches which are useful for measuring their safety. It must not be forgotten that for all kinds of nanoengineered foods, it is still vital to follow all regulations regarding conventional, non-nanotechnological food components.

Additionally, it has been proven that size reduction process carried out in nanotechnology can induce specific changes in features and biokinetics of food matrices which consequently leads to toxicological effects compared with their conventional counterparts. So, their safety must not be assumed identical to their conventional non-nanomaterial peers, which means for a specific nanomaterial, specific considerations and data must be provided individually.

Detailed characterization, impurity recognition, and physicochemical knowledge also provide pivotal insight into safety assessment. Material composition and purity, empirical formula of a complete matrix, particle size, particle shape, structure, surface chemical composition, surface area, surface charge, appearance, melting point, boiling point, density, porosity, dustiness, pH, and the final form of the material (if it is powder, dispersion, etc.) are among physicochemical properties that must be taken into account while testing the nanomaterials' safety.

It is also probable that changes in production process may add new impurities or modify physicochemical and morphological properties of the food matrix, and hence alter its safety.

Direct and indirect exposure to nanomaterials is another influencing factor when analyzing a nanomaterial safety. Feed additives, inhalation, pesticides, dermal exposure, and migration or transfer of nanomaterials from the packaging agent into food are some areas which must be closely examined to assure a safe component being accepted in food chain.

17.4.2 Toxicity

As a result of the extremely wide application of nanomaterials in food industry, their safety has become a complicated matter to deal with as there has always been a gap in data provided for emerging nanoparticles' safety. These days, nanomaterials are being used from the farm up to the moment that the food is consumed by people at homes. Nanoscale pesticides, fertilizers, functional ingredients namely food additives, flavor enhancers, or packaging enzymes, and nano sensors are part of this hugely implemented technology. Therefore, precise investigation of its properties, potential threats, and daily safe intake dose is mandatory.

Silver, silica, iron, and titanium dioxide are considered as most consumed nanoparticles in food industry, especially in beverages. For instance, Ag NPs are used as an antimicrobial agent in food products. However, consumption of Ag NPs in edible products has caused some concerns as various experiments declared Ag NPs toxicity for cells as they can change the normal functionality of mitochondria, produce reactive oxygen particles, and boost membrane permeability.

Silicon dioxide (SiO2) nanoparticles, which are widely used in powdered products as an anticaking agent to improve their flow properties, have been proven to be risky when accumulated in liver to a certain extent. Silica nanoparticles which are used as anticaking agents can be cytotoxic in human lung cells when subjected to exposure (Athinarayanan et al., 2014). On the contrary, Yun et al. reported no toxicity of these nanoparticles when consumed orally by rats during 13 weeks. Therefore, more studies are needed to avoid any ambiguity concerning the nanoparticles safety.

Titanium dioxide NPs are widely consumed in cosmetics and medicine; in food products titanium dioxide nanoparticles act as white coloring agent. Higashisaka et al. (2015) claimed that titanium dioxide shows no toxicity in human and animal cells. Iron oxide nanoparticles, which are among interdisciplinary substances, showed to be noncytotoxic when used at concentrations below 100 mg/mL. However, massive consumption may end in accumulation in a targeted organ, possibly leading to iron storage, which can be hazardous toxic. Hence, their consumption is safe provided that they are used in a limited amount below 100 mg/mL (Buyukhatipoglu & Clyne, 2011).

17.5 Future Trends

Enzyme immobilization is a matter of concern in both academia and industrial sections. As a consequence, combination of nanotechnology and biotechnology boosts actions which result in immobilized enzymes' success. In order to increase the use of nanobiocatalysts, the following factors must be taken into account: (1) the ability to be separated and recycled easily and reusing high activity enzymes, (2) minimizing the structural changes of enzymes during stabilization and decreasing the inactive sites of nanobiocatalysts to reduce costs, (3) increasing the biocompatibility and stability of nanobiocatalysts in alimentation and pharmaceutical applications in various temperatures and pHs, and (4) simultaneous use of multiple enzymes with high compatibility in sequential reactions. Rapid advancement of nanotechnology can create opportunities to produce nanobiocatalysts to be used in food, medicine, pharmaceutical, and biological applications.

17.6 Conclusion

The latest attentions to nanomaterials and their wide applications have made it possible to immobilize enzymes, maintain their enzymatic activity, reuse, and finally recycle them. Various nanostructures such as nanofibers, nanoparticles, nanotubes, nanosheets, and others have been proven to act as efficient media for enzyme stabilization. If produced under good manufacturing practice and according to health regulations, these particles are preferred in comparison with their chemical counterparts. Enzymatic biocatalysts have become the center of attention as sensors in order to ensure food quality and safety. They are used as indicators of pesticides' residues, pathogenic microorganism, and their toxic metabolites. Nanotechnology and stabilization on nanostructures have been widely used in the production process of these biosensors.

References

Agustian, J., Kamaruddin, A. H., & Aboul-Enein, H. Y. (2016). Enantio-conversion and-selectivity of racemic atenolol kinetic resolution using free Pseudomonas fluorescens lipase (Amano) conducted via transesterification reaction. *RSC Advances, 6*(31), 26077–26085.

Alehosseini, A., Ghorani, B., Sarabi-Jamab, M., & Tucker, N. (2017). Principles of electrospraying: A new approach in protection of bioactive compounds in foods. *Critical Reviews in Food Science and Nutrition, 8398*, 1–18.

An, J., Zhang, M., Wang, S., & Tang, J. (2008). Physical, chemical and microbiological changes in stored green asparagus spears as affected by coating of silver nanoparticles-PVP. *LWT-Food Science and Technology, 41*(6), 1100–1107.

Athinarayanan, J., Periasamy, V. S., Alsaif, M. A., Al-Warthan, A. A., & Alshatwi, A. A. (2014). Presence of nanosilica (E551) in commercial food products: TNF-mediated oxidative stress and altered cell cycle progression in human lung fibroblast cells. *Cell Biology and Toxicology, 30*(2), 89–100.

Bajpai, V. K., Kamle, M., Shukla, S., Mahato, D. K., Chandra, P., Hwang, S. K., Kumar, P., Huh, Y. S., & Han, Y. K. (2018). Prospects of using nanotechnology for food preservation, safety, and security. *Journal of Food and Drug Analysis, 26*(4), 1201–1214.

Baskar, G., Banu, N. A., Leuca, G. H., Gayathri, V., & Jeyashree, N. (2015). Magnetic immobilization and characterization of α-amylase as nanobiocatalyst for hydrolysis of sweet potato starch. *Biochemical Engineering Journal, 102*, 18–23.

Bayramoglu, G., Doz, T., Ozalp, V. C., & Arica, M. Y. (2017). Improvement stability and performance of invertase via immobilization on to silanized and polymer brush grafted magnetic nanoparticles. *Food Chemistry, 221*, 1442–1450.

Beig Mohammadi, Z., Hamidi Esfahani, Z., Sahari, M., & Khosravi Darani, K. (2020). Enzymatic degumming of soybean oil by immobilized phospholipase A1 on plasma surface modified chitosan nanofibrous membrane. *Iranian Journal of Chemistry and Chemical Engineering (IJCCE), 40*, 1421–1430. https://doi.org/10.30492/ijcce.2020.40607

Beig Mohammadi, Z., Hamidi-Esfahani, Z., Sahari, M., & Khosravi-Darani, K. (2016). Optimization of phospholipase A1 immobilization on plasma surface modified chitosan nanofibrous mat. *Applied Food Biotechnology, 3*(1), 25–34.

Beverlya, R. L., Janes, M. E., Prinyawiwatkula, W., & No, H. K. (2008). Edible chitosan films on ready-to-eat roast beef for the control of Listeria monocytogenes. *Food Microbiology, 25*(3), 534–537.

Buyukhatipoglu, K., & Clyne, A. M. (2011). Superparamagnetic iron oxide nanoparticles change endothelial cell morphology and mechanics via reactive oxygen species formation. *Journal of Biomedical Materials Research Part A, 96*(1), 186–195.

Cao, Y., Wen, L., Svec, F., Tan, T., & Lv, Y. (2016). Magnetic AuNP@ Fe3O4 nanoparticles as reusable carriers for reversible enzyme immobilization. *Chemical Engineering Journal, 286*, 272–281.

Cushen, M., Kerry, J., Morris, M., Cruz-Romero, M., & Cummins, E. (2012). Nanotechnologies in the food industry—Recent developments, risks and regulation. *Trends in Food Science & Technology, 24*(1), 30–46.

Dwivedi, C., Pandey, I., Misra, V., Giulbudagian, M., Jungnickel, H., Laux, P., Luch, A., Ramteke, P. W., & Singh, A. V. (2018). The prospective role of nanobiotechnology in food and food packaging products. *Integrative Food, Nutrition and Metabolism (IFNM), 5*, 1–5.

Emamifar, A., Kadivar, M., Shahedi, M., & Soleimanian-Zad, S. (2010). Evaluation of nanocomposite packaging containing Ag and ZnO on shelf life of fresh orange juice. *Innovative Food Science & Emerging Technologies, 11*(4), 742–748.

Emamifar, A., Kadivar, M., Shahedi, M., & Soleimanian-Zad, S. (2012). Effect of nanocomposite packaging containing Ag and ZnO on reducing pasteurization temperature of orange juice. *Journal of Food Processing and Preservation, 36*(2), 104–112.

Faramarzi, S., Anzabi, Y., & Jafarizadeh-Malmiri, H. (2020). Nanobiotechnology approach in intracellular selenium nanoparticle synthesis using Saccharomyces cerevisiae—Fabrication and characterization. *Archives of Microbiology*, 1–7.

Fathi, M., Karim, M., Khoigani, S. R., & Mosayebi, V. (2018). Use of nanotechnology for immobilization and entrapment of food applicable enzymes. In *Bioactive molecules in food* (pp. 1–25). Springer.

Fatima Mustafa, F., & Andreescu, S. (2018). Chemical and biological sensors for food-quality monitoring and smart packaging. *Food, 7*, 168. https://doi.org/10.3390/foods7100168

Fernández, A., Picouet, P., & Lloret, E. (2010). Cellulose-silver nanoparticle hybrid materials to control spoilage-related microflora in absorbent pads located in trays of fresh-cut melon. *International Journal of Food Microbiology, 142*(1-2), 222–228.

Fernandez, A., Picouet, P., & Lloret, E. (2010). Reduction of the spoilage-related microflora in absorbent pads by silver nanotechnology during modified atmosphere packaging of beef meat. *Journal of Food Protection, 73*(12), 2263–2269.

Fernández, A., Soriano, E., López-Carballo, G., Picouet, P., Lloret, E., Gavara, R., & HernándezMuñoz, P. (2009). Preservation of aseptic conditions in absorbent pads by using silver nanotechnology. *Food Research International, 42*(8), 1105–1112.

Fuertes, G., Soto, I., Carrasco, R., Vargas, M., Sabattin, J., & Lagos, C. (2016). Intelligent packaging systems: Sensors and nanosensors to monitor food quality and safety. *Journal of Sensors, 2016*, 4046061.

Gabrielczyk, J., Duensing, T., Buchholz, S., Schwinges, A., & Jordening, H. J. (2018). A comparative study on immobilization of fructosyltransferase in biodegradable polymers by electrospinning. *Applied Biochemistry and Biotechnology, 185*(3), 847–862.

Ghasemi, S., Jafari, S. M., Assadpour, E., & Khomeiri, M. (2017). Production of pectin-whey protein nano-complexes as carriers of orange peel oil. *Carbohydrate Polymers, 177*, 369–377.

Ghasemi, S., Jafari, S. M., Assadpour, E., & Khomeiri, M. (2018). Nanoencapsulation of d-limonene within nanocarriers produced by pectin-whey protein complexes. *Food Hydrocolloids, 77*, 152–162.

Ghorani, B., Alehosseini, A., & Tucker, N. (2017). 8. Nanocapsule formation by electrospinning. In S. M. Jafari (Ed.), *Nanoencapsulation technologies for the food and nutraceutical industries* (pp. 264–319). Academic Press.

Ghosal, K., Chandra, A., Praveen, G., Snigdha, S., Roy, S., Agatemor, C., et al. (2018). Electrospinning over solvent casting: Tuning of mechanical properties of membranes. *Scientific Reports, 8*(1), 5058.

Ghouri, M. Z., Khan, Z., Khan, S. H., Ismail, M., Aftab, S. O., Sultan, Q., & Ahmad, A. (2020). Nanotechnology: Transformation of agriculture and food security. *Bioscience, 3*, 19.

Handford, C. E., Dean, M., Spence, M., Henchion, M., Elliott, C. T., & Campbell, K. (2014). *Nanotechnology in the agri-food industry on the island of Ireland: Applications, opportunities and challenges*. Institute for Global Food Security at Queen's University, Belfast, and the Teagasc Ashtown Food Research Centre.

Hansen, S. F., Maynard, A., Baun, A., Tickner, J. A., & Bowman, D. M. (2013). 22 Nanotechnologyearly lessons from early warnings. In *Late lessons from early warnings: Science, precaution, innovation* (Vol. 32).

Haroon, F., & Ghazanfar, M. (2016). Applications of food biotechnology. *Journal of Ecosystem and Ecography, 6*(215), 2.

He, X., & Hwang, H. M. (2016). Nanotechnology in food science: Functionality, applicability, and safety assessment. *Journal of Food and Drug Analysis, 24*(4), 671–681.

Higashisaka, K., Yoshioka, Y., & Tsutsumi, Y. (2015). Applications and safety of nanomaterials used in the food industry. *Food Safety, 3*(2), 39–47.

Homaei, A., & Samari, F. (2017). Investigation of activity and stability of papain by adsorption on multi-wall carbon nanotubes. *International Journal of Biological Macromolecules, 105*, 1630–1635.

Husain, Q. (2010). β Galactosidases and their potential applications: A review. *Critical Reviews in Biotechnology, 30*(1), 41–62.

Jafari, S. M., Fathi, M., & Mandala, I. (2015). Chapter 13-Emerging product formation. In C. M. Galanakis (Ed.), *Food waste recovery* (pp. 293–317). Academic Press.

Jafari, S. M. (2019). *Nanoencapsulation of food ingredients by specialized equipment: Volume 3 in the Nanoencapsulation in the Food Industry series* (Vol. 3). Academic Press.

Jafarizadeh-Malmiri, H., Sayyar, Z., Anarjan, N., & Berenjian, A. (2019). *Nanobiotechnology in food: Concepts, applications and perspectives*. Springer.

James, J., Simpson, B. K., & Marshall, M. R. (1996). Application of enzymes in food processing. *Critical Reviews in Food Science & Nutrition, 36*(5), 437–463.

Jia, F., Narasimhan, B., & Mallapragada, S. (2014). Materialsbased strategies for multienzyme immobilization and colocalization: A review. *Biotechnology and Bioengineering, 111*(2), 209–222.

Jin, T., & Gurtler, J. B. (2011). Inactivation of Salmonella in liquid egg albumen by antimicrobial bottle coatings infused with allyl isothiocyanate, nisin and zinc oxide nanoparticles. *Journal of Applied Microbiology, 110*(3), 704–712.

Kharazmi, S., Taheri-Kafrani, A., & Soozanipour, A. (2020). Efficient immobilization of pectinase on trichlorotriazine-functionalized polyethylene glycol-grafted magnetic nanoparticles: A stable and robust nanobiocatalyst for fruit juice clarification. *Food Chemistry, 325*, 126890.

Kumar, H., & Neelam, R. (2016). Enzyme-based electrochemical biosensors for food safety: A review. *Nanobiosensors in Disease Diagnosis, 5*, 29–39.

Li, H., Li, F., Wang, L., Sheng, J., Xin, Z., Zhao, L., Xiao, H., Zheng, Y., & Hu, Q. (2009). Effect of nano-packing on preservation quality of Chinese jujube (Ziziphus jujuba Mill. var. inermis (Bunge) Rehd). *Food Chemistry, 114*(2), 547–552.

McClements, D. J., & Xiao, H. (2017). Is nano safe in foods? Establishing the factors impacting the gastrointestinal fate and toxicity of organic and inorganic food-grade nanoparticles. *NPJ Science of Food, 1*(1), 1–13.

McClements, D. J., Decker, E. A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition, 49*(6), 577–606.

Morais, M. G. D., Martins, V. G., Steffens, D., Pranke, P., & da Costa, J. A. V. (2014). Biological applications of nanobiotechnology. *Journal of Nanoscience and Nanotechnology, 14*(1), 1007–1017.

Mosafa, L., Moghadam, M., & Shahedi, M. (2013). Papain enzyme supported on magnetic nanoparticles: Preparation, characterization and application in the fruit juice clarification. *Chinese Journal of Catalysis, 34*(10), 1897–1904.

Mukhopadhyay, A., Bhattacharyya, T., Dasgupta, A. K., & Chakrabarti, K. (2015). Nanotechnology based activation-immobilization of psychrophilic pectate lyase: A novel approach towards enzyme stabilization and enhanced activity. *Journal of Molecular Catalysis B: Enzymatic, 119*, 54–63.

Mustafa, F., & Andreescu, S. (2020). Nanotechnology-based approaches for food sensing and packaging applications. *RSC (Royal Society of Chemistry) Advances, 10*, 19309–19336. https://doi.org/10.1039/d0ra01084g

Neethirajan, S., & Jayas, D. S. (2011). Nanotechnology for the food and bioprocessing industries. *Food and Bioprocess Technology, 4*(1), 39–47.

Neo, Y. P., Ray, S., & Perera, C. O. (2018). Fabrication of functional electrospun nanostructures for food applications. In *Role of materials science in food bioengineering* (pp. 109–146). Academic Press.

Netto, C. G., Toma, H. E., & Andrade, L. H. (2013). Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes. *Journal of Molecular Catalysis B: Enzymatic, 85*, 71–92.

Nile, S. H., Baskar, V., Selvaraj, D., Nile, A., Xiao, J., & Kai, G. (2020). Nanotechnologies in food science: Applications, recent trends, and future perspectives. *Nano-Micro Letters, 12*(1), 45.

Park, B., Fu, J., Zhao, Y., Siragusa, G. R., Cho, Y. J., Lawrence, K. C., & Windham, W. R. (2007). Bio-functional Au/Si nanorods for pathogen detection. In *Nanosensing: Materials, devices, and systems III* (Vol. 6769, p. 67690). International Society for Optics and Photonics.

Park, Y. W., Kim, S. M., Lee, J. Y., & Jang, W. (2015). Application of biosensors in smart packaging. *Molecular & Cellular Toxicology, 11*(3), 277–285.

Patel, S. N., Singh, V., Sharma, M., Sangwan, R. S., Singhal, N. K., & Singh, S. P. (2018). Development of a thermo-stable and recyclable magnetic nanobiocatalyst for bioprocessing of fruit processing residues and D-allulose synthesis. *Bioresource Technology, 247*, 633–639.

Pradhan, N., Singh, S., Ojha, N., Shrivastava, A., Barla, A., Rai, V., & Bose, S. (2015). Facets of nanotechnology as seen in food processing, packaging, and preservation industry. *BioMed Research International, 2015*, 365672.

Raghu, P., Reddy, T. M., Reddaiah, K., Swamy, B. K., & Sreedhar, M. (2014). Acetylcholinesterase based biosensor for monitoring of malathion and acephate in food samples: A voltammetric study. *Food Chemistry, 142*, 188–196.

Rashidi, L., & Khosravi-Darani, K. (2011). The applications of nanotechnology in food industry. *Critical Reviews in Food Science and Nutrition, 51*(8), 723–730.

Ravichandran, R. (2010). Nanotechnology applications in food and food processing: Innovative green approaches, opportunities and uncertainties for global market. *International Journal of Green Nanotechnology: Physics and Chemistry, 1*(2), P72–P96.

Rojas-Mercado, A. S., Moreno-Cortez, I. E., Lucio-Porto, R., & Pavo'n, L. L. (2018). Encapsulation and immobilization of ficin extract in electrospun polymeric nanofibers. *International Journal of Biological Macromolecules, 118*(Pt B), 2287–2295.

Ruane, J., & Sonnino, A. (2011). Agricultural biotechnologies in developing countries and their possible contribution to food security. *Journal of Biotechnology, 156*(4), 356–363.

Saallah, S., Naim, M. N., Lenggoro, I. W., Mokhtar, M. N., Bakar, N. F. A., & Gen, M. (2016). Immobilisation of cyclodextrin glucanotransferase into polyvinyl alcohol (PVA) nanofibres via electrospinning. *Biotechnology Reports, 10*, 44–48.

Shahiri Tabarestani, H., & Jafari, S. M. (2019). Production of food bioactive-loaded nanofibers by electrospinning. In *Nanoencapsulation of food ingredients by specialized equipment* (pp. 31–105). Academic Press.

Shahrestani, H., Taheri-Kafrani, A., Soozanipour, A., & Tavakoli, O. (2016). Enzymatic clarification of fruit juices using xylanase immobilized on 1,3,5-triazine-functionalized silicaencapsulated magnetic nanoparticles. *Biochemical Engineering Journal, 109*, 51–58.

Sharma, C., Dhiman, R., Rokana, N., & Panwar, H. (2017). Nanotechnology: An untapped resource for food packaging. *Frontiers in Microbiology, 8*, 1735.

Sheldon, R. A. (2007). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis, 349*(8-9), 1289–1307.

Singh, N., Srivastava, G., Talat, M., Raghubanshi, H., Srivastava, O. N., & Kayastha, A. M. (2014). Cicer α-galactosidase immobilization onto functionalized graphene nanosheets using response surface method and its applications. *Food Chemistry, 142*, 430–438.

Skoronski, E., Souza, D. H., Ely, C., Broilo, F., Fernandes, M., Junior, A. F., & Ghislandi, M. G. (2017). Immobilization of laccase from Aspergillus oryzae on graphene nanosheets. *International Journal of Biological Macromolecules, 99*, 121–127.

Sojitra, U. V., Nadar, S. S., & Rathod, V. K. (2016). A magnetic tri-enzyme nanobiocatalyst for fruit juice clarification. *Food Chemistry, 213*, 296–305.

Sundarraj, A. A. (2019). Nano-agriculture in the food industry. In *Plant nanobionics* (pp. 183–200). Springer.

Tavares, A. P., Silva, C. G., Dražić, G., Silva, A. M., Loureiro, J. M., & Faria, J. L. (2015). Laccase immobilization over multi-walled carbon nanotubes: Kinetic, thermodynamic and stability studies. *Journal of Colloid and Interface Science, 454*, 52–60.

Thakur, M. S., & Ragavan, K. V. (2013). Biosensors in food processing. *Journal of Food Science and Technology, 50*(4), 625–641.

Vinayaka, A. C., Basheer, S., & Thakur, M. S. (2009). Bioconjugation of CdTe quantum dot for the detection of 2,4-dichlorophenoxyacetic acid by competitive fluoroimmunoassay based biosensor. *Biosensors and Bioelectronics, 24*(6), 1615–1620.

Wang, X. Y., Jiang, X. P., Li, Y., Zeng, S., & Zhang, Y. W. (2015). Preparation Fe3O4@ chitosan magnetic particles for covalent immobilization of lipase from Thermomyces lanuginosus. *International Journal of Biological Macromolecules, 75*, 44–50.

Wen, P., Zong, M. H., Linhardt, R. J., Feng, K., & Wu, H. (2017). Electrospinning: A novel nanoencapsulation approach for bioactive compounds. *Trends in Food Science & Technology, 70*, 56–68.

Wong, D. E. (2016). *Enzyme stabilization in hierarchical biocatalytic food packaging and processing materials*. Doctoral dissertations, p. 672.

Xie, W., & Ma, N. (2010). Enzymatic transesterification of soybean oil by using immobilized lipase on magnetic nano-particles. *Biomass and Bioenergy, 34*(6), 890–896.

Yu, Z., Li, B., Chu, J., & Zhang, P. (2018a). Silica in situ enhanced PVA/chitosan biodegradable films for food packages. *Carbohydrate Polymers, 184*, 214–220.

Yu, H., Park, J. Y., Kwon, C. W., Hong, S. C., Park, K. M., & Chang, P. S. (2018b). An overview of nanotechnology in food science: Preparative methods, practical applications, and safety. *Journal of Chemistry, 2018*.

Zhou, L., Lv, S., He, G., He, Q., & Shi, B. I. (2011). Effect of PE/AG2O nano packaging on the quality of apple slices. *Journal of Food Quality, 34*(3), 171–176.