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Recent Advances in Food Biotechnology

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Foreword

I serve as the President of International Bioprocess Association in Malaysia; Director of Sustainable Food Processing Research Centre and Co-Director of Future Food Malaysia, Beacon of Excellence, and a Full Professor at the Department of Chemical and Environmental Engineering, Faculty of Science and Engineering, University of Nottingham Malaysia. I am delighted to accept the invitation from Dr. Vijai Singh in order to give introductory statements to the *Recent Advancement in Food Biotechnology*, a timely volume on the rapidly growing field.

The book covers basic understanding of food biotechnology and state-of-the-art technology. It has covered a wide range of topics including functional foods, agronomics, fortifications, genetic improvements of crops, removal of anti-nutritional factors, use of CRISPR-Cas9 in food complex, development of transgenic animals, genetically modified food, activities of bio-surfactants and its recent food application, microbiomes, next-generation probiotics, encapsulation of probiotics, and many more. Chapters are written by eminent scientists from across the globe who have established expertise on advanced food biotechnological concepts such as microbe-based food hydrocolloids, plant-based meat analogue, field testing tools and fortification programme analysis, bacteriocins, gene editing, food safety, and regulation.

I am pleased to recognize the valuable efforts of Dr. Ajay Kumar, Dr. Kiran Patruni, and Dr. Vijai Singh who together brought out an excellent volume through the world's leading publisher in Science—Springer Nature. This comprehensive book contains various aspects of fundamentals of food biotechnology, microbial advancements, macromolecules applications, novel cutting-edge technologies, and food safety. I believe that this volume is a collection of outstanding and informative text with a simple and easy-to-understand format.

This book offers a valuable source of information not only for beginners in food biotechnology but also for students, researchers, scientists, practitioners,

policymakers, and stakeholders who are interested in harnessing the potential of food biotechnology in many areas.

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Pau-Loke Show

Preface

Food science and biotechnology are integrated fields. The scientific merit of biotechnological applications in food sciences aims at fulfilling the basic needs of human nutrition, enhances its quality with safety, perception, and cost-effectiveness for promoting healthy living standards. As ever, the increasing global population demands innovations in terms of production of cost-effective raw materials, process and product refinement, bioactive value addition, improved sensory methods, etc., which is challenging in the competitive market. There is also a great extent of research work done and documented in the area of food biotechnology which covers fundamental knowledge of plants, animals, and microbiological applications. Research progress in cutting-edge technologies for advanced multidisciplinary food applications has also been documented in the similar area. There remains a gap to be bridged between the fundamental knowledge and its recent advancements in diversified applications with scientific input. In view of this, the compilation of this volume amasses diverse topics from related experts in dedicated areas on recent advancements of biotechnological innovations and its application in food which can help to achieve quality cultivation to consumption. The central idea behind this book is to highlight several aspects of food biotechnology which is not only limited to plants, animals, and microbes but also applicable to advance biomolecules application, novel processing technologies, ensuring food quality and safety parameters which further fulfill the gap related to novel route for synthesis, processing, and quality-enriched product formulations.

The contents of the book are divided into five parts. Each part offers chapters in a particular field. Further, each chapter covers the fundamental concepts, recent advancements, challenges, and upcoming trends. Readers are urged to refresh their basics of molecular biology, microbiology, metabolic pathways, enzymology, biochemistry, and food chemistry to understand the concepts of molecular insight and modern cutting-edge technologies in foods.

Part I deals with *fundamental concepts on recent trends of food biotechnology*. This part covers the ongoing trends in plant and animal biotechnology, futuristic prospects and its applications in the food sector. The novel topics agronomics bio-fortification, removal of anti-nutritional factors, use of the CRISPR-Cas9 system in food, tools, and techniques for transgenic animals have been discussed.

Part II focuses on *microbial applications in the food industry*. This part covers the modern aspects, developments, and challenges of microbial applications pertaining to food industry such as the use of biosurfactants, probiotics for functional foods, microbial productions of natural flavours and fragrance, psychotropic bacteria in dairy industries, bacteriocins, microbial surfactants, algae, and cyanobacteria as food supplements and concepts of improving crop yield using microorganisms.

Part III serves as a bridge to more advanced concepts such as *the role of macromolecules and micromolecules in food*. This part helps the reader to link the fundamental food biotechnology concepts related with the present need in the food industry. The advanced concepts covered in this part are utilization of various enzymes for process and product development, concepts related to enzyme inactivation and kinetics for food processing, and development of gluten-free products. Furthermore, significant reviews pertaining to mixed biopolymer applications, implementation of food fortification with nutrients, green-antioxidants synthesis and its impact on designing novel value-added foods, plant-based meat analogues are covered in this part.

Part IV deals with the applications of *novel technology in the food industry*. In this, extensive compilation of recent literature on genetically modified crops has been provided, along with the latest technologies. This part covers advanced technologies used in food sector such as bio-encapsulation, micro-encapsulation, nano-encapsulation, micelles formation, and non-conventional methods for extraction of bioactive molecules.

Every food professional should recognize that the ethics and safety of food are very important. Lastly, Part V covers the safety aspects related to *food quality and management*. This part provides significant information on food quality and safety concepts such as control over bacteriocins of food-borne pathogens, identification of toxic components in food, and ethical, biosafety, and regulatory issues in food biotechnology. This would help in recognizing the role of microorganisms, enzymes, bioactive components, and advanced methods in food processing. The essence of this book is that it brings together the diversified areas of food biotechnological tools and concepts.

This volume is an indispensable treatise that offers a holistic view of all the aspects related to the subject and will be a useful source not only to academicians but also to people working in the industry. It is also hoped that both students and professionals will equally benefit from this volume.

Kanpur, Uttar Pradesh, India — Ajay Kumar
Mehsana, Gujarat, India — Kiran Patruni
Mehsana, Gujarat, India — Vijai Singh

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Ajay Kumar

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I am also thankful to Almighty God who has given me the wisdom to edit this book. Finally, I dedicate this book to my parents for their countless blessings to accomplish the target.

Kiran Patruni

I acknowledge my sincere thanks to all the authors for their scientific contributions and reviewers for their valuable comments and suggestions to improve the quality of the chapters. I would like to express my sincere gratitude to Dr. J.-S. Yadav, Director (Research), Indrashil University, India, who gave me outstanding support and motivation to complete this book.

At a personal level, my sincere thanks to my beloved parents, students, family members, and friends for their moral support and motivation. I would like to warmly thank faculties and staffs of Indrashil University for providing a great working environment.

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Vijai Singh

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I am delighted to thank all the authors for their excellent contributions to this book. I would like to thank Dr. Madhurima Kahali (Editor-Medicine and Life Sciences) and Mr. *Lenold Esithor* from Springer for their excellent management of this project.

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I am aware that even despite our best efforts, the first version always comes with some error that may have crept in the compilation. I would be delighted to receive feedback from readers to further improve the future book. Last but not least, my sincere thanks to GOD for his supreme POWER for endowing me to live with joy and victory in shaping of this book.

Contents

About the Editors

Ajay Kumar is a Professor and Head of the Department of Biotechnology at the Faculty of Engineering and Technology at Rama University Uttar Pradesh, Kanpur. Dr. Kumar has successfully served more than 20 years in research and teaching. He has a proven experience in Genomics and Proteomics, Bioprocess Engineering, Bioinformatics, Microbiology, Industrial Microbiology, Genetic Engineering, Fermentation Technology, and Food Biotechnology. He has held several key positions in well-renowned Universities and Engineering Institutes. Dr. Kumar received his M. Tech. (Biotechnology) from the Institute of Engineering and Technology, Lucknow, India, and Ph.D. from the ICAR-Central Institute for Research on Goats, Mathura, India. His expertise lies in research that includes computational vaccine and drug development, genomics and proteomics, and fermentation technology. He has published 90 articles, 5 chapters, 3 books, and 2 patents. He has also served as chief and associate editor, editorial board member, and reviewer of several peer-reviewed journals. He is also a member of the Board of Study and Academic Council of Rama University, Kanpur, and Regional Food Research Analysis Centre, Department of Horticulture and Food Processing, Lucknow, Uttar Pradesh. Being a member of a professional body such as the International Association of Engineers (IAENG) and INSA, he has rendered consultancy services in vaccine research.

Kiran Patruni is currently working as Assistant professor in the Department of Biosciences, Indrashil University, Rajpur, Mehsana. He has received his Ph.D. from the Indian Institute of Technology (IIT)—Kharagpur. During his Ph.D., he developed reconstituted aloe vera hydrogel protocols for health promoting plant-based gels and published five international articles with multidisciplinary research approach. He is well versed in correlating the mechanical behaviour with structural aspects of biopolymer mix gels, complex suspensions, hydrogels, sol-gel, fluids, and mix gels. He has extensive research experience in the field of process development and characterization of biopolymer food gel formation. His research interest in the area of mix food gels formation, food fortification, and 3D food printing. He serves a reviewer of many peer-reviewed journals.

Vijai Singh is an Associate Professor and Head of the Department of Biosciences, School of Science at Indrashil University, Rajpur, Mehsana, Gujarat, India. He was

an Assistant Professor in the Department of Biological Sciences and Biotechnology at the Institute of Advanced Research, Gandhinagar, India and also an Assistant Professor in the Department of Biotechnology at the Invertis University, Bareilly, India. Prior to that, he was a Postdoctoral Fellow in the Synthetic Biology Group at the Institute of Systems and Synthetic Biology, Paris, France, and the School of Energy and Chemical Engineering at the Ulsan National Institute of Science and Technology, Ulsan, South Korea. He received his Ph.D. in Biotechnology (2009) from the National Bureau of Fish Genetic Resources, Uttar Pradesh Technical University, Lucknow, India. During his Ph.D., he has cloned, expressed, and characterized haemolysin from *Aeromonas hydrophila* in *Escherichia coli*. He has extensive experience in Synthetic Biology including MAGE, small regulatory RNAs, pathway designing, CRISPR-Cas systems, and microfluidics. His research interests are focused on building novel biosynthetic pathways for the production of medically and industrially important biomolecules. Additionally, his laboratory is working on CRISPR-Cas9 tools for genome editing. He has more than 9 years of research and teaching experience in synthetic biology, metabolic engineering, microbiology, and industrial microbiology. He has published 76 articles, 40 chapters, 11 books, and 2 patents. He serves as an associate editor, editorial board member, and reviewer of a number of peer-reviewed journals. He is also a member of the Board of Study and Academic Council of Indrashil University and is the Member Secretary of the Institutional Biosafety Committee (IBSC) at the same university.

Part I

Fundamental Concepts on Recent Trends of Food Biotechnology

Recent Trends in Food Biotechnology Contributing in Food Production and Processing

1

Ajay Kumar and Ramneet Kaur

Abstract

Food biotechnology is the use of living systems and organisms to develop or make useful products or any technological applications that can either develop or make or alter products or processes in order to meet human needs by using biological systems or organisms. It includes food fermentation to enhance properties such as the aroma, shelf life, taste, nutritional value and texture of food. Food biotechnology includes the production of enzymes to bring about desirable changes in food, both in the manufacture of food additives and fragrances, and flavourings, food ingredients are synthesised as well as other high value-added goods., genetically modified foods, genetically modified starter cultures, the use of all these modern technologies in diagnostics for food testing, the role of food biotechnology in increasing food production, improved harvesting, nutritional value and storage, better raw materials, better flavour and the production of food containing vaccines, the safety of food produced with food biotechnology as well as the risks and benefits of food biotechnology in food production. In terms of research and development studies as well as legal legislation in terms of food intake and human health, the application of new food biotechnological methods in food ingredients is assessed. Safe food production, on the other hand, is critical for detecting, managing and controlling physical, chemical and biological hazards in food. For health, safety, financial, social and ethical reasons, as well as national, geographical and international protection,

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modern food biotechnological research on transgenic plants, cattle and microorganisms are conducted today.

Keywords

Food biotechnology · Fermentation · Food processing enzymes · Food microorganisms · Transgenic plants · Genetically modified foods

1.1 Introduction

The term "food biotechnology" can be interpreted as a broad term used to describe new technologies that have had an effect on health, environment and agriculture ever since the late twentieth century (Gültekin 2005). Food biotechnology research and education are extremely important in both developed and emerging nations, and several regional and global organisations support them. Some countries also take the lead in these fields by launching large-scale investment campaigns (Hall 2005).

Food biotechnology has various fields of implementation in the field of food industry and science. The growth and research in this field have the greatest significance in the modern era when it comes to the creation of new and unique foods as well as the enhancement of technological advancement, and it has a long tradition in the field of food industry. Biological processes are left untouched; there is no significant modification. For the production of bread, some types of fermented drinks and yoghurt, as well as vinegar, the traditional biotechnological method was used. On the other hand, modern food biotechnology, also known as genetic modification, is the mechanism by which biological systems are modified using biological methods in order to construct new biological systems and enhance the qualities of products developed through biotechnology, including genetically modified food, human hormones, human insulin, enzymes and biotech vaccines, valuable products for both human and animal health use. In addition to the new and conventional versions, the model is being employed today in various expansions (Pinstrup-Andersen and Cohen 2000; WHO 2005; Jauhar 2006). However, there is an increasing need for new legal guidelines in the use of biotechnology that are more appropriate for newly developing countries as the rate of biotechnology use has risen dramatically; it is aimed to discuss how food biotechnology addressed the potential effects on health, safety, culture, economics and ethics in both national and international platforms while at the same time to benefit from the opportunities offered by this biotechnology (Cömert 2011). The food industry and agriculture have recently made it easier for numerous companies to coordinate research and to work together to make progress, even though they have different goals and interests, as a phenomenon biotechnology increases (Başkaya et al. 2009).

1.2 Implications of Food Biotechnology in Food Processing

Due to the emergence of new food processing technologies, some people are becoming worried about the effects of biotechnology on food. Food biotechnology can also be employed to harness the potential of food available. Between the farmer and the supermarket, food is the lifeline. Except for fruits and vegetables, almost all agricultural products are processed after harvesting. The safety of the food supply and nutritional quality can be enhanced by food biotechnology (Haroon and Ghazanfar 2016). This method is applied to determine food safety and examine ways in which food biotechnology can be applied to food production.

1.2.1 Genetic Development of Food Fermentation Microorganisms Using Food Biotechnology

Advances in agricultural technology have provided humanity with a way to produce, process and serve food for centuries across the world, benefiting the people greatly. Biotechnology may be said to be part of the food production. Several selection and mutation techniques were employed to develop fermented products such as sausages, wines and bread. Preservatives, antioxidants, flavour enhancers and vitamins are made by bacteria. Fermented foods have been consumed since time immemorial (Harlander 1990; Fraiture et al. 2020). Traditionally, specimen based on the collection of bacteria and mutations is uncontrolled, although it's possible. Furthermore, screening from all potential mutations is extremely laborious and time-consuming (Ross et al. 2002). However, the advancement of modern biotechnology with genetic engineering offers a system for species selection and transition that is both controllable and predictable (Demain 2000; Mosier and Ladisch 2009). Food fermentation on production using food biotechnology that used enhanced microorganisms used in and genetic effect are presented in Table 1.1.

Table 1.1 Effects of genetically enhanced microorganisms used in food biotechnology on production (Harlander 1990)

Food product	Effects	Product improvement effects
Cheese	Bacteriophage (virus) resistance	To minimise economic losses of valuable bacteria
Sausage	Bacteriocin production	Inhibition of pathogens
Beer	α-Amylase production	Low-calorie beer
Bread	Higher levels of maltose	Improved leavening
Wine	Malolactic fermentation in commercial yeast	Reducing the acidity level of the wine

1.2.2 Food Microorganisms

There are several types of microorganisms that are crucial to food preservation, flavour-boosting and producing aromas and textures of fermented foods. One of the latest applications of food biotechnology, which has provided promising results in the industry (Koffas and Marienhagen 2014), is known as microbial beta-galactosidase regulation with Bulgarian and Mexican yeast through genetic engineering of *Streptococcus thermophilus* (Markakiou et al. 2020) bacteria and *Lb. delbrueckii subspecies*. An increased number of research studies that deal with various genetically modified lactic acid bacteria have been performed in order to develop high-yield commodities (Rothstein et al. 2020).

1.2.3 Probiotics

Bacteria which have the capability of surviving or settling in the animal and human gastrointestinal systems, and competing, may be able to expand and do so in the large intestine (Kerry et al. 2018). The species that are called "probiotics" for their positive influence on the health of the host organism are termed "pro-biotics" for their benefits on the organism (Gorbach 2002), and probiotic microorganisms are also shown in Table 1.2. Competitively inhibiting pathogenic intestinal organism's strains may be extremely useful in the field of the food industry and agriculture. In various environments, including in plants, poultry, animal food and food-animal

Table 1.2 Probiotic microorganisms used in food biotechnology (Holzapfel et al. 2001; Mercan 2020)

Lactobacillus species	*L. bulgaricus, L. cellebiosus, L. delbrueckii, L. lactis, L. acidophilus, L. reuteri, L. brevis, L. casei, L. curvatus, L. fermentum, L. plantarum, L. johnsonii, L. rhamnosus, L. helveticus L. salivarius, L. gasseri, L. amylovorus, L. crispatus, L. gallinarum*
Bacillus species	*B. subtilis, B. pumilus, B. lentus, B. licheniformis, B. coagulans*
Pediococcus species	*P. cerevisiae, P. acidilactici, P. pentosaceus*
Streptococcus species	*S. faecalis, S. cremoris, S. lactis, S. intermedius, S. thermophilus*
Bacteroides species	*B. capillus, B. suis B. ruminicola, B. amylophilus*
Yeasts	*S. cerevisiae, C. torulopsis, saccharomyces boulardii*
Bifidobacterium species	*B. adolescentis, B. bifidum B. breve, B. infantis B. longum, B. thermophilum, B. animalis, B. lactis*
Propionibacterium species	*P. shermanii* ssp. *freudenreichii*
Leuconostoc species	*L. mesenteroides*
New-generation probiotic (NGP) bacteria	*Bacteroides xylanisolvens* DSM 23694, *Bacteroides ovatus* D-6, *Bacteroides ovatus* V975, *Bacteroides ovatus* V975, *Bacteroides dorei* D8, *Bacteroides fragilis* ZY-312, *Bacteroides acidifaciens* JCM 10556(T), *Clostridium butyricum* MIYAIRI 588, *Faecalibacterium prausnitzii, Lactococcus lactis trefoil factor* 1 or IL-10

agriculture, lactic acid bacteria are present. Lactic acid bacteria in food and drug technology are crucial because of their usefulness in both areas, so they have a significant economic impact (Wuyts et al. 2020). There are several new methods and techniques being investigated to find even more efficient ways to recognise and describe microorganisms in which to culture which to use the benefit of creating an isolate, for example, RNA and DNA sequence analysis microfluidic methods and dot-plate scanning. Micro-droplets that can possibly affect the activity of the probiotic can also be used to describe isolates and are advanced products of short-chain fatty acids (Mekonnen et al. 2020). Molecular methods may include an in-depth examination of the effects of the probiotic in the intestinal microbiota. Soon these approaches will be widely used (Fuller 1989).

1.2.4 Healthy Microbes

It is estimated that approximately 70% of the world's population has lost the enzyme needed to hydrolyse lactose into galactose and lactose. According to many studies, lactase-deficient people absorb lactose faster than other dairy foods. Increasing the amount of beta-galactosidase and streptigosid released by the two bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* contained in yoghurt makes the beverage easier to digest for lactose-intolerant people (Savaiavo and Levitt 1987). Extracted from microbial sources, a vast number of metabolites currently produced by bacteria and other microorganisms are used in processed foods (Neidleman 1990). These include flavours (diacetyl, pyrazines, lactones and esters), acidulants (acetic, lactic, benzoic, propionic), flavour enhancers (MSG), pigments (monascin, astaxanthin), stabilisers and thickeners (xanthan gum, dextrans), sweeteners (aspartame), nutritive additives (vitamins, amino acids), enzymes (proteases, lipases, cellulases, pectinases) and preservatives (nisin). These items provide versatility, prolong shelf life and increase quality and protection. Many of the above ingredients have a long history of healthy use in food. However, many microorganisms may create compounds suitable for use in processed foods. Additionally, many bacteria can develop polymers outside the cell that can be used as stabilisers, surfactants, viscous gelling agents and soluble dietary fibres in the diet. This enzyme plays a vital role in the development of these biopolymers.

Advantages of the Use of Microbes in Food Biotechnology The benefits of using microbes as a food source are given below (Caplice and Fitzgerald 1999)

- The growth of microbes is very fast, and they do not need much space as the conventional ways require.
- Microbe's proteins consist of all the essential amino acids.
- Helpful in recycling the waste materials and cleaning up the waste products.
- Their growth can be obtained from a wide range of cheap, agricultural waste products and industrial by-products, that is, ethanol, methanol, sugar, other petroleum products, waste from paper mills, molasses etc.

- The protein content of microbe's cells is very high (40–50% in bacteria and 20–40% in algae).
- High yielding ability is another main feature.
- Some microorganisms (yeasts) have a high content of vitamins.
- Less affected by environmental factors (climate).

1.2.5 Microbial Food Ingredients

In various foods, microorganisms may generate a large number of various types of metabolic by-products, used as ingredients in products that have already been produced (Neidleman 1990). These regulators have been studied extensively and found to be benzoic, coryne, are lactic, and there are some others such as citric acid and succinic acid, for instance, both of which are acetic emmualwise abundant organisms in *Corynebacterium* organisms developed flavour stabilisers (Nakayama et al. 2019); *E. coli* ATF1 produces aromas such as diacetyl, pyrazines and lactone, for example, ethyl acetate (Lee and Trinh 2019, 2020); *Lactobacillus acidophilus*, for example, produces stabilisers and thickeners such as dextrans (Gibbons and Banghart 1967); *Monascus purpureus*, for example, produces the Monascus-nata colour pigment (Sheu et al. 2000). Sweeteners (aspartame) (Erbeldinger et al. 2000), vitamins, amino acids (Revuelta et al. 2016) and preservatives are examples of food additives (nisin) (Joshi et al. 2009).

When these ingredients are used in food processing, the product becomes more usable, has better consistency, has higher nutritional values, has a longer shelf life and is safer. With the exception of some relatively new ingredients, most of the ingredients are derived from well-known species that have been used in food for long periods of time. Additionally, certain microorganisms exist in nature and also appear in processed foods. Other ways, as an example, bacteria develop extracellular biopolymers such as stabilisers, surfactants, which can be used in non-caloric treatments, or soluble fibre. In the biotech world, genes coding for these biopolymers are of great interest to the food industry (Torino et al. 2015; Revuelta et al. 2016).

1.2.6 Enzymes

Enzyme preparations are often employed to maintain and improve food quality in the food industry for various purposes, including monitoring and maximising nutritional values, flavour and texture (Neidleman 1986). GMOs completely reshape how agriculture functions making the latter half of the twentieth century seems like the earlier half in terms of total nutritional breakthroughs (GMOs). There are two protease enzymes and two different carbohydrate-based polysaccharide-enzyme complexes isolated to give faster, higher yield of the enzyme production. When added to foods, enzymes have the ability to break down and transform ingredients like dairy into something as simple as "dairy"; they can then be converted into more

Table 1.3 Enzyme samples obtained from genetically modified microorganisms using food biotechnology (Zhang et al. 2019)

Enzyme	Application area	Microorganism	Improvement
α-Amylase	Breaking down maltose and dextrin. Stain remover, fortification of flour, glucose syrup	*Bacillus licheniformis*, *Rhizopus oryzae*	Sensitive to acid. Thermostable
Proteases	Bakery, detergent industry	*Aspergillus* ve *Bacillus* species	Enhanced glycine. Releasing activity
β-Glucosidase	Food, animal feed, textile, fuel and chemical industry	*Aspergillus aculeatus*, *Thermotoga maritima*	Hydrolytic has improved efficiency
Lipase	Breaking down animal and vegetable oils and paper, cosmetics, pharmaceutical and detergent industry and agricultural chemistry	*B. subtilis*	Thermostability

complex ingredients such as "sweeteners". Rennin and amylase are two examples of such enzymes (Haroon and Ghazanfar 2016). The first recombinant rennin has received "generally recognized as safe" status and is thought to be the first in food biotechnology. The quality of the recombinant product is better, because it contains more active enzymes per protein unit than conventional milk taken from calves (Flamm 1991).

Some techniques of genetic engineering (region-specific mutagenesis: specifically changing primary amino acid sequences of enzyme) so as to make food systems more efficient. Some genetically modified enzyme examples are listed in Table 1.3 (Zhang et al. 2019).

1.3 Safety of Products and Foods Produced with the Help of Food Biotechnology

Food safety is the term describing the procedures and techniques to be implemented for producing, preserving, transporting and distributing food that is considered safe and secured (Artik et al. 2021). Risk in food processing is often considered at each point, beginning with the conception of risk of getting food and through to the possible foodborne illness that may occur at the time of consumption (Koç and Uzmay 2015). Food safety can be described as a person's ability to acquire the nutrients as well as the possibility that the acquisition process will continue indefinitely in the entire society. The system that provides food safety needs to meet the basic conditions which are described below:

1. *Availability*: We have the capacity to produce, store and import enough food to satisfy the needs of all the different and numerous classes, if not the exacted needs of each individual.
2. *Sufficiency*: To allow an atmosphere of confidence, year-round food threats must be acknowledged and prepared for, and food security should be established as a goal that is sure to be met, along with environmental sustainability.
3. *Accessibility*: To ensure that those who have access to food can feed when they are in political and foreign situations and to ensure that everyone's basic food needs are met.
4. *Acceptability*: For that purpose, people's dietary habits, food supply is irrelevant. Also, food does not conflict with human rights and ethics and honour.
5. *Individual and institutional factors*: Policies, like that of the Food and Drug Administration, the Food Standards Agency and the Federal Trade, oversee and direct the entire food safety management and oversight processes for those who do not bear the responsibility (Çankaya and Sancar 2009; Koç and Uzmay 2015).

In a nutshell, food safety concerns both human health and societal issues. But because of this, only genetically modified (GM) foods are being proposed (Çankaya and Sancar 2009). In medicine and food industries, modern food biotechnology has a wide range of applications. The use of genetically modified foods in the food supply chain and the effectiveness of herbicides and disease vectors have improved as a result of the production of genetically modified (GM) products in the laboratory. The plants' overall nutrition and resistance have improved, and the perishability of the products has decreased. It is made more aesthetically pleasing in terms of shape and size, as well as being free of contaminants and dyes (Gültekin 2005). The Codex Commission's studies focused on the criteria and principles for evaluating the food safety of products of modern food biotechnology, and as a result of this study, food products of modern biotechnology are evaluated under three key topics (WHO 2009):

- Food safety evaluation and management of food products derived from recombinant DNA microorganisms.
- Food safety evaluation and management of food products derived from recombinant DNA plants.
- Food safety evaluation and management of food products derived from recombinant DNA animals.

The public's generally accepted food safety standard for new foods represents people's experience of healthy eating. Accepted fact is that to substantiate experience is the source of food risk management expertise in many cases is typically garnered over a long period of time. Foods on this list are usually thought to be safe. Predefined food production risk analysis is advantageous for using the latest biotechnological methods, providing a basic framework for concepts that identify general food production risks. The main goal of these concepts is to help with risk analysis, food nutrition and food protection in the context of food biotechnology.

The Codex Alimentarius study and these principles concern only the nutritional, legal, economic and agricultural processing of biotechnologically derived foods. In order to reduce the risk, a nutritional hazard assessment must decide whether or not there are any, as well as collect information on the nature of the problem. Since both new biotechnology and conventional agriculture techniques are addressing similarities and differences, safety should be considered when looking at their effects on food. An expansion to describe and measure should include finding out what kinds of changes or new developments have occurred that can increase or decrease the threat or the threat's severity, respectively, to human health. The safety assessment consists of the comparison of looking at the food or its constituents with any appropriate or similar food of the same kind.

- Considers both the desired and unintended consequences.
- Determines and identifies new dangers.
- It must make the assessment of improvements in nutritional standards to human health if any is involved.

As a result, all new food and animal-derived foodstuffs must be looked at with equal suspicion (WHO 2009; Amin et al. 2011; Kramkowska et al. 2013). As a priority, the primary objective is to detect any kind of additional or unique threat as well as existing threats to plant, animal or recombinant DNA sources, and quantitative factors (as opposed to testing only for hazard) should be done after some form of relative and quantifiable study has been done:

- Evaluation of toxicity
- Composition analysis of key components
- Food processing
- Nutritional modification
- Potential accumulation of substances important for human health
- Evaluation of metabolites
- Assessment of allergy status (proteins)
- Use of antibiotic resistance marker genes
  Food that has been genetically modified is tested in the same way non-genetically modified food is tested. A wide range of genetic characteristics are being investigated, not just for the organism's characteristics but also for its origin (WHO 2009).

1.4 Legal Regulations in Biosafety

GM foods (i.e. those produced using genetic modification) first became available two decades ago. The impact of food biotechnology products on human health and food safety remained a contentious issue during this period (Dinçoğlu 2016). As necessary, the people's health and welfare have to be taken care of; and other topics, if uncovered, should be demolished as scandals for the sake of the health of the

public. In Europe, EU regulations provided the foundation of the safe production of food biotechnology GMOs. Topics like science, measurements, trade rules and environments are typically covered with respect to foreign statutes; Europe prepares and implements its policies and laws. This food and GMOs themselves, or products derived from these foods, may be used, produced or sold in the European Union under the European Parliament and Council's Chap. 61 regulations. All goods that contain GMOs must also meet labelling and traceability regulations. In 2010, Turkey passed a biosafety law to create a biosafety scheme, protect biodiversity and avoid any potential threats from GMOs and related items. In the light of these protocols, auditing, assessments and principles, various measurements were developed with regard to the aid, as well as various activities performed inside of a facility on GMOs. It was discovered that the Biosafety Committee provided a great deal of valuable scientific input. To ensure that the organisation is totally separate from everyone, even organisations or corporations and an autonomous board were established. The aim of this Biosafety Committee is to look at possible uses of GMOs and GMOs as food, feed and processing additives and assist with evaluations.

According to the fifth Article of the Biosafety Law, the following actions are prohibited:

- Releasing GMO and related products to the market without obtaining approval.
- Production of genetically modified plants and animals.
- Using GMO and related products by the owner or third parties against the decisions of the Committee.
- Using GMO and related products in infant food, baby formulas, follow-on baby food, follow-on baby formulas and baby and children supplementary food products.
- Using GMOs and similar goods for applications other than those specified by the Committee prior to their release to the market.

This responsibility falls on the Ministry of Agriculture and Fisheries and Forestry, which is in charge of overseeing the handling of GMOs in the food supply chain. Analyses and procedures are assigned to food and feed labs by the province according to the potential risks they pose to the province under the circumstances that are prescribed by the Ministry of Health determine the presence of hazardous control conditions (Başkaya et al. 2009).

1.5 Conclusion

Bacteria and viruses can also be detected more rapidly in food as biotechnological science and development advances. By avoiding the inexpressibilities, this can help to decrease the risk of food poisoning and prolong the shelf life of the food. Proteins which cause the production of many allergic reactions have begun to be hunted down and destroyed, so people with allergies may 1 day be able to eat foods that do not cause allergic reactions. In addition to providing food that is safe, food

biotechnology may also be used to employ expansive applications that help people and animals. Any of the food products being introduced to today's supermarkets have more nutritious qualities than their conventional counterparts. Via biotechnology, or biotechnology, foods can also help prevent or treat long-term illnesses by the presence of healthful components, including additional vitamins and free radicals, and by lowering unhealthy fats. The upshot of food biotechnology is more beneficial today than when viewed against the potential side effects. As the supply of food has been the subject of concern of nearly all global political and economic discourse for some time, it is critical to enhance food production for future populations.

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Exploration of Modern Biotechnology Trends in Functional Foods

2

Bhanu Solanki, Rupesh Maurya, Archana Mankad, and Vijai Singh

Abstract

Modern biotechnology has played a significant role in human welfare in a more sustainable way. In the past two decades, biotechnology has improved agriculture, medicine, environment and food industries. Biotechnology has enhanced the quality, shelf life, nutrition, processing and production of food. Functional foods have a great potential to address hidden hunger, i.e. a lack of micronutrients. Functional food not only possesses nutrition but also shows disease curing properties. Hence, functional food contributes towards the problem of global hunger and human health. There is a requirement to scale in food and nutrition by using different biotechnological techniques. The present chapter investigates and explores modern biotechnological tools in functional food as well as contribute to future perspectives where modern biotechnological techniques can be utilized for improving functional food. This chapter also explores the interrelationship between food, nutrition and techniques in biotechnology.

Keywords

Functional foods · Biofortification · Fermentation · Biotechnology

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2.1 Introduction

Modern biotechnological techniques have played a significant role in human welfare and quality of life. Biotechnology has taken a successful part in treating diseases, agriculture, forensic and bioremediation. Recombinant DNA technology, DNA sequencing, genome editing and CRISPR technology are the major techniques that have affected human life very systematically. Technological aspects related to biotechnology have improved agricultural, medical, environmental and industrial processes in the last two decades.

Hunger is a major global issue; mostly children, women and people living in rural areas are adversely affected by hunger. India had targeted an end to the hunger issue till 2015 which was not met (Saxena 2018). Biotechnology can provide a significant help in solving the global hunger and malnutrition issue. Questionnaire-based survey reveals that there is a significant relationship between lifestyle, health behaviour and preference for functional food (Szakály et al. 2012) also helps to reduce healthcare costs (Shahidi 2009). Food biotechnology plays a valuable role in enhancement of quality and quantity of food to overcome malnutrition and diseases (Haroon and Ghazanfar 2016).

2.1.1 Functional Food

Functional food started from Japan with the concept of fortified nutritional products with advantageous effects on physiology. Later it became, 'Natural food that contains known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven or strong documented health benefit for the prevention, management or treatment of chronic disease' (Martirosyan and Singh 2015; Reynolds and Martirosyan 2016).

There are five major types of functional food (Lau et al. 2013) mentioned below:

1. *Fortified products*: These are food products that are fortified with important constituents like minerals and vitamins, thus improving the quality of food and possessing advantageous physiological effects and public health benefits (World Health Organization 2006; Dwyer et al. 2015; Sharanya Rani and Penchalaraju 2016). Microbial fortification (involves probiotic bacteria such as lactic acid), commercial/industrial fortification (addition of micronutrients during manufacturing) and home fortification (micronutrient-enriched food supplied to deficient population in the form of packages and tablets) are the three types of fortification (Williams et al. 2006). One of the best examples of fortified products is transgenic golden rice which possesses a significant amount of iron and β-carotene (Liyanage and Hettiarachchi 2011). Fortified juices with vitamin C and grains with folic acid are other examples of fortified products (Spence 2006; Siro et al. 2008).
2. *Enriched products*: These are food products with additional nutrients and components added which are normally not present or present in a very small

quantity. Margarines are example of enriched products, which possess stanol esters, sterols, probiotics and prebiotics as bioactive compounds, where its clinical trials prove that it reduces total and low-density lipoprotein (LDL) cholesterol (Hasler 2002; Siro et al. 2008). Prebiotics and probiotics enhance the environment of the gastrointestinal tract (Spence 2006).

3. *Altered products*: When the deleterious components are removed or replaced with some important components with health benefits, such products are considered as altered products (Siro et al. 2008). The best example of this is alteration of fat in grains with fibres, and thus reduced fat product is available with good fibre enthralling health benefits (Spence 2006; Guine et al. 2020).
4. *Non-altered products*: Products with presence of naturally occurring nutritional components are non-altered food products. The presence of β-glucan in oats is an example of non-altered products (Kotilainen et al. 2006). Broccoli is an example of non-altered products which contains two bioactive components named diindolylmethane and selenium which possess anticancer properties. Berries, tomatoes, oats, apples, strawberries, oranges, grapes, peanuts, green tea, almonds and dark chocolate are the other examples of products of natural origin (Das et al. 2010).
5. *Enhanced products*: These are products with enhancement in the food component which can be achieved by providing different conditions during the growth phase or with the support of genetic manipulation in living beings and by new feeding composition to animals. One of the examples is altered feeding composition to chicken leads to a good content of omega-3 in eggs (Siro et al. 2008). It is more popular amongst dairy products, beverages, bakery products and confectionery (Kotilainen et al. 2006). Phytonutrient production in fruits, enhanced content of vitamins in fruits and vegetables, eggs with increased omega-3 content and lysine-rich corn are also examples of enhanced products (Spence 2006; Guiné et al. 2020).

Several important bioactive compounds are helping in healthcare and fitness, such as beta-glucan in cereals for chronic health problems (Vasanthan and Temelli 2008) and neochlorogenic and chlorogenic acids in prunes as laxatives (Stacewicz et al. 2001), and in intestinal health (Cencic and Chingwaru 2010). Nutraceutical shows many beneficial functions such as antioxidant, detoxifying and apoptotic actions (Ferrari and Torres 2003).

Tables 2.1, 2.2 and 2.3 indicate the presence of bioactive compounds that have a significant role in health. A functional food arena is based on information in plants (Marriott 2000), animals (Prates and Mateus 2002) and microorganisms (Dewapriya and Kim 2014). Plants are used as food as well as medicine (Heinrich et al. 2016). Ethnobotanical and ethnopharmacological studies have already produced data about use of plants in diet (Shi et al. 2018). About 2300 species of plants and fungi are enlisted which are consumed in the Mediterranean (Rivera et al. 2005) and 600 species in India (Singh and Arora 1978). Still many wild crop relatives of crops need to be explored. It can be a possible solution to mineral deficiency and global hunger.

Table 2.1 Functional foods and their bioactive compounds are listed below (plants as functional food)

Sr. No.	Functional foods	Bioactive components	Health benefits	References
1.	*Smallanthus sonchifolius* (yacon)	Rich source of fructooligosaccharides and inulin	Enhances mineral absorption and gastrointestinal metabolism; regulation of serum cholesterol	Delgado et al. (2013)
2.	*Linum usitatissimum* (flaxseed)	Sources of phenolic compounds and high-quality protein	Helps in disease prevention	Oomah (2001)
3.	*Cynara scolymus* (globe artichoke)	Cynarin (1,3-O-dicaffeoylquinic acid)	Antioxidative activity	Lattanzio et al. (2009)
4.	*Olea europaea* (olive) oil	Oleuropein	Antioxidant activity	de la Torre (2008)
5.	*Cocos nucifera* (virgin coconut) oil	1,1-Diphenyl-2-picrylhydrazyl and phenolic compounds	Antioxidant activity	Marina et al. (2009)
6.	*Camellia sinensis* (green tea)	Epigallocatechin-3-gallate	Anti-obesity	Sergent et al. (2012), Chu et al. (2017)
7.	*Solanum lycopersicum* (tomatoes)	Lycopene	Reduces pancreatic cancer risk	Johnson and Krewski (2005)
			Cardiovascular diseases	Rao and Agarwal (2000)
8.	*Allium sativum* (garlic)	Allicin	Antimicrobial, ability to reduce cardiovascular disease	Rahman (2007)
9.	Cruciferous plants	Glucosinolates	Inhibit carcinogenesis	Hayes et al. (2008)
10.	Citrus fruits	Limonoids	Reduces risk from cancer	Tian et al. (2001)
11.	*Beta vulgaris* (beet) root	Betalains	Antilipidemic activity	Gengatharan et al. (2015)
12.	*Lathyrus aphaca* (yellow pea)	Fibre	Improve weight control	Lambert et al. (2014)
13.	Cereals	β-Glucan and arabinoxylan	Lowering cholesterol and rate of fat absorption, improving gastrointestinal health	Otles and Cagindi (2006)
14.	*Triticum aestivum* (wheat)	Phenolic acid	Antioxidant activity	Borneo and Leon (2012)
15.	*Adansonia digitata* (baobab tree)	Polyphenols	Reduces glycaemic response and reduces starch digestion	Coe et al. (2013)

(continued)

Table 2.1 (continued)

Sr. No.	Functional foods	Bioactive components	Health benefits	References
16.	*Citrus sinensis* (orange)	Polyphenols	Anti-allergic, antiviral, antioxidant, anti-inflammatory, antiproliferative and anticarcinogenic	Campone et al. (2020)
17.	*Zingiber officinale* (ginger)	Gingerols, shogaols, parasols and essential oils	Neuroprotective, respiroprotective, anti-inflammatory, antioxidant, antinausea, antidiabetic, cardiovascular protective, anti-obesity, antimicrobial and anticancer	Mao et al. (2019)
18.	*Asparagus racemosus* (shatavari)	Steroidal saponins, alkaloids, polysaccharides, polyphenols, flavonoids and vitamins	Anti-ulcer, anti-diarrhoeal, antidiabetic, immunomodulatory activities, adaptogenic, anti-dyspepsia, cardioprotective, galactagogue and shows improvement in neurological disorders	Veena et al. (2014)
19.	*Coffea* sp. (coffee), *Malus domestica* (apple), berry and *Vitis vinifera* (wines)	Caffeic acid and esters	Protects against allergic reactions, asthma, immunoregulation diseases. It also shows some activity against colon cancer	Guine et al. (2009)
20.	*Withania somnifera* (ashwagandha)	Withaferin A	Cytotoxic activity	Dutta et al. (2019)
21.	*Curcuma longa* (turmeric)	Curcumin	Anti-angiogenic, anti-inflammatory, antioxidant and antiproliferative	Prakash et al. (2017)
22.	Palm oil and wheat bran	Chromanols	Inhibits growth of breast cancer cells	Prakash et al. (2017)
23.	*Elaeagnus angustifolia* (Russian olive) root	Echinacoside	Antioxidant and free radical scavenging activity	Zhao et al. (2015)
24.	*Oryza sativa* (rice) bran	α-Lipoic acid	Lowers glycaemic index and controls body weight	Gul et al. (2016)
25.	*Cynara cardanculus* (cardoon)	Caffeoylquinic acid, flavonoids and cynarin	Antioxidant property	Tighe-Neira et al. (2017)

(continued)

Table 2.1 (continued)

Sr. No.	Functional foods	Bioactive components	Health benefits	References
26.	*Capsicum annum* L. (bell pepper)	Capsaicin	Neurotoxic and cytotoxic activity	Mercy and David (2018)
27.	*Coriandrum sativum* L. (coriander)	Phenolics	Antioxidant activity, hepatoprotective	Bhat et al. (2014), Ganesan et al. (2013)
28.	*Allium ampeloprasum* (wild leek)	Phenolics	Antioxidant activity	Arfa et al. (2016)
29.	*Daucus carota* (wild carrot)	α-Carotene, falcarinol and falcarindiol	Neutralizes free radicals Anti-inflammatory	Choudhary et al. (2009), Teodoro (2019)
30.	*Bacopa monnieri* (brahmi)	Saponins and their bacosides	Neuropharmacological effects	Devendra et al. (2018)
31.	*Cordyceps* (mushroom)	Adenosine, cordycepin, polysaccharides, mannitol and ergosterol	Treats inflammatory disorders and boosts immune system	Das et al. (2016)
32.	*Avena sativa* (oats)	β-Glucan	Reduces cholesterol and lowers density lipoprotein (LDL), also functions as antioxidant	Wani et al. (2014)
33.	*Glycine max* (soy)	Isoflavones, lecithin, saponins, fibres	Lowers cholesterol, LDL and triglycerides, anticancer properties	Basharat (2020)
34.	*Hordeum vulgare* (barley)	β-Glucan	Insulin resistance, involves in hypercholesterolaemia and hypoglycaemia and decreases the risk of colon cancer	Din et al. (2018)
35.	*Eleusine coracana* (millets)	Polyphenols	Antimicrobial, antioxidant and antidiabetic	Devi et al. (2014)
36.	*Sorghum bicolor* (black amber)	Phenolics	Anticancer	Das et al. (2016)
37.	*Trigonella foenum-graecum* (fenugreek)	Alkaloid, saponin, flavonoid and steroidal sapiogens	Antidiabetic, antioxidant, anticarcinogenic, hypoglycaemic and hypocholesterolaemic	Khorshidian et al. (2016)
38.	*Cucurbita pepo* (pumpkin)	Tocopherol	Antidiabetic effect	Dar et al. (2017)

(continued)

Table 2.1 (continued)

Sr. No.	Functional foods	Bioactive components	Health benefits	References
39.	*Vaccinium macrocarpon* (cranberry)	Proanthocyanidins	Treats urinary tract infections (UTIs)	Lee (2013)
40.	*Theobroma cacao* (cocoa)	Flavonoids, procyanidins and polyphenols	Treats neurodegenerative diseases, cardiovascular diseases, obesity, atherosclerosis, cancer, kidney stones, tuberculosis and fever	Scapagnini et al. (2014)
41.	*Arachis hypogaea* (peanut)	Flavonoids, phytosterol and phenolic acids	Blocks cholesterol absorption from diet	Arya et al. (2016)
42.	*Fragaria ananassa* (strawberry)	Tannins and ellagic acid	Anticancer, anti-inflammatory and treats hypertension	Basu et al. (2014)
43.	*Rosmarinus officinalis* (rosemary)	Caffeic acid, rosmarinic acid, chlorogenic acid, carnosic acid and carnosol	Antispasmodic, antimutagenic, antirheumatic, analgesic, diuretic, expectorant, chloretic and hepatoprotective Relieves respiratory disorders and promotes hair growth	Zhao et al. (2015)
44.	Umbelliferae and Rutaceae	Coumarins	Anticoagulant, antimicrobial, anticancer, anti-inflammatory and free radical scavenging activity	Zhao et al. (2015)
45.	Kale, spinach and yellow carrot	Lutein	Vision protection	Zhao et al. (2015)
46.	*Tanacetum parthenium* (feverfew)	Parthenolide	Reduces migraine headaches	Zhao et al. (2015)
47.	*Piper nigrum* (black pepper)	Chavicine	Memory enhancer	Mgbeahiruike et al. (2017)
48.	*Coptidis rhizoma* (Huanglian)	Berberine	Antiviral, antifungal, antibacterial, antidiabetic, anticancer and cardioprotective effects	Wang et al. (2019a, b)
49.	*Juglans* sp. (walnut)	Myricetin	Antidiabetic, antioxidant and anti-inflammatory	Unuofin and Lebelo (2020)
50.	Citrus and grapefruit	Naringenin	Antioxidant	Unuofin and Lebelo (2020)

Table 2.2 Animal source of functional food

Sr. No	Source	Components	Health benefits	References
1.	Milk protein	Bioactive peptides	Opioid-like activity, antimicrobial, immunomodulatory and antihypertensive	Shandilya and Sharma (2017)
2.	Fish oil	Fatty acids (eicosapentaenoic acid and docosahexaenoic acid)	Normal development of the brain, nervous system and healthy eyesight It also maintains blood lipid levels and integrity and mobility of joints	Das et al. (2010)
3.	Dairy product	Polydextrose	Prevents colon carcinogenesis, regulates bowel function, improves ease of defecation and calcium absorption in the bone and maintains blood sugar	Veena et al. (2016) Do Carmo et al. (2016)
4.	Milk	Galactooligosaccharides (GOS)	Promotes growth of bifidobacteria in thr GI tract which in turn prevents pathogenic diarrhoea, detoxifies liver, prevents inflammatory bowel disease (IBD), increases bone mineralization and reduces risk of bone fracture by stimulating calcium absorption	Sangwan et al. (2011) Oh et al. (2019)
5.	Yogurt	Xylooligosaccharides (XOS)	Reduces cholesterol level, lowers cariogenicity and maintains gastrointestinal health	Mumtaz et al. (2008)
6.	Milk	Lactulose	Increases the growth of bifidobacteria and reduces the intestinal transit time	Muehlhoff et al. (2013)
7.	Beef (buffalo meat)	Conjugated linoleic acid (CLA)	Anticarcinogenic and antioxidative	Fernandez et al. (2005)
8.	Mutton (goat meat)	Iron Vitamins	Antioxidant	Kausar et al. (2019)
9.	Pork meat	Polyunsaturated fatty acid (PUFA) Docosahexaenoic acid (DHA) Conjugated linoleic acid (CLA) Eicosapentaenoic acid (EPA) Vitamin E Selenium	Antioxidant	Bonos et al. (2014)

(continued)

Table 2.2 (continued)

Sr. No	Source	Components	Health benefits	References
10.	Fish meat	Docosahexaenoic acid (DHA) Eicosapentaenoic acid (EPA)	Contributes to the development of infant brain and liver	Sarojnalini and Hei (2019)
11.	Eggs	N3 fatty acids Sphingolipids	Reduce risk for cancer and cardiovascular diseases (CVD)	Prates et al. (2002)
12.	Chicken meat	Histidyl dipeptide (carnosine and anserine)	Antioxidant	Nisa et al. (2017)

Table 2.3 Microbes as source of functional food

Sr. No	Source	Component	Health benefits	References
1.	Lactobacilli and Bifidobacteria	Probiotics	Keeps gastrointestinal tract metabolically active	Shandilya and Sharma (2017)
2.	*Saccharomyces boulardii* (yeast)	Probiotics	Biotherapeutic agent (diarrhea, clostridium)	Zhang et al. (2010)
3.	*Spirulina* microalgae	Peptides, vitamins (ascorbic acid, B1, B2, B3, B12, biotin and folic acid), β-carotene	Anti-obesity, anticarcinogen, anti-inflammatory and antioxidant	Magalhães et al. (2017)
4.	*Lactobacillus helveticus*	Peptides	Antihypertensive, antimicrobial, antioxidative and antimutagenic	Gobbetti et al. (2010)
5.	Photosynthetic and non-photosynthetic bacteria	Carotenoids	Protective effects against heart disease, degenerative eye disease and cancer	Shandilya and Sharma (2017)
6..	*Lactobacillus acidophilus*	Probiotics	Functional in health concern like cancer, hypertension, stomach health, cholesterol lowering, urogenital tract health and immune function	Hasler (2002)

2.1.2 Agronomic Biofortification in Food Crops

There are two main approaches in biofortification, i.e. by adding nutrition to crops (fertilizer management) and genetic improvement (White and Broadley 2009). Foliar spray of zinc and iron in wheat (Aciksoz et al. 2011), iron and boron in rice (Jin et al.

2008) are useful for improving minerals in plants. Foliar application has a significantly higher micronutrient recovery percentage over soil application. Breeding approaches increase the level of nutrition and decrease the level of antinutrients by genetic modification (Goldman 2014).

There are some limitations to breeding approach, i.e. adverse soil factors. Use of cellular characteristic and molecular targets has also been used to develop future functional food for optimization nutrients and minimization of disease risk (Milner 2002). Mutations are induced in crops to improve the yield of food and to address the problems of global food security and nutrition (Jankowicz et al. 2017). Countries like China and India are practicing mutation breeding to meet the demand of food of ever-increasing population, and around 3000 mutant varieties are officially recorded over 60 countries which include sorghum, rice, legumes, barley, wheat, cotton, ornamental plants, fruits and edible oils (Mohan and Suprasanna 2011). Physical and chemical mutagen treatment induces mutation by breaking the nuclear DNA, and changes are made during DNA repair which are new and heritable (Mba 2013).

2.1.3 Genetic Improvement in Food Crop by Using Genetic Engineering

Genes in plants are quite diverse in terms of function and sequence (Backer et al. 2000). Microinjection, particle gun and electroporation techniques are used to deliver foreign DNA sequences into the target DNA which is utilized in crop plants to improve them genetically (Gasser et al. 1989). *Agrobacterium tumefaciens*-mediated gene transfer is used to make the transgenic plants and mostly applicable on dicotyledonous crops like tomatoes, potatoes and tobacco (Shetty et al. 2018). Transgenic plants are produced to improve yield, nutritional values and disease resistance crops. Expression of beta carotene in super bananas (Englberger et al. 2003) and golden rice development containing PSY bacterium genes (Beyer et al. 2002) improves vitamin A deficiency; lysine and methionine by using promoter:: gene phaseolin::BN* in canola, phaseolin::15 kD zein in soybean and glutelin:: amaritin in corn (Galili and Amir 2013); production of omega-3 fatty acid in canola by using *Mortierella* fungus' delta 6-desaturase enzyme (Lee et al. 2013); iron in rice by adding ferritin, iron storage protein from soybean to rice endosperm (Goto et al. 1999) and phytase in maize from *Aspergillus niger* (Kamthan et al. 2016); and folic acid fortification in tomatoes (Garza et al. 2007).

2.1.4 Removal of Antinutritional Factors

Many antinutritional compounds are present in smaller or larger quantities and are a major issue in developing countries such as food poisoning and renal and liver issues (Soetan and Oyewole 2009). A high concentration of phytates affects digestion (Nwokolo and Bragg 1977). Antinutritional factors such as phytoestrogens have oestrogen effects (Gatta et al. 2013). Oxalates and aflatoxins (Inuwa et al. 2011),

cyanogenic glycosides and trypsin inhibitors in *Manihot esculenta* (Sarkiyayi and Agar 2010) and phytic acid in grapes (Aina et al. 2012) are considered as antinutritional components. The biological importance of trypsin inhibitors, phytic acid, saponins and isoflavones was later realized and used for soybean processing (Anderson and Wolf 1995). Some popular methods are cooking, fermentation (Reddy and Pierson 1994), plant breeding (Clarke and Wiseman 2000), post-harvesting wilting, repeated water washing, autoclaving *Ricinus communis* seeds (Kumar 1992) and lowering down antinutritional components. Thermal processing also helps in lowering antinutritional factors (Sharma et al. 2018).

Several reports for stopping the production of antinutritional compounds came in the last decades such as silencing glycoalkaloid metabolism 4 for stopping steroidal glycoalkaloid production in potato and tomato fruits (Itkin et al. 2013) and down regulation of galactinol synthesis reduction of antinutritional oligosaccharides (Bock et al. 2009). RNA interference is a promising technique for the removal of allergens by silencing allergen proteins from peanuts, tomato, rice soybean and apple (Singh and Bhalla 2008; Gallo and Sayre 2009).

2.1.5 Protein Content Improvement by Genetic Engineering

Protein deficiency causes several diseases such as anaemia of malignant malnutrition (Altmann and Murray 1948), kwashiorkor (Vis 1969), marasmus (Khan et al. 2017) and cachexia (Morley et al. 2006). Breeding techniques were used by farmers for improving genome later. Technology like recombinant DNA and advanced selective breeding techniques were utilized in making genetically modified crops (Uzogara 2000). Cereals and legumes can provide the three most important amino acids: tryptophan (neurochemicals' precursor) (Richard et al. 2009), lysine (defence of mammalian cells towards viruses and prevents osteoporosis in human) (Tien et al. 2016) and methionine (deficiency leads to methylation-related disorders) (Martin et al. 2007). Genetic engineering is necessary in crop improvement as they lack important amino acids (Rommens 2007). The Ama1 gene of *Amaranthus* is incorporated into potatoes to enhance the essential amino acids like lysine, tyrosine, methionine and cysteine, and it increases about 60% of the total protein in potatoes (Chakraborty et al. 2010). Oxalate decarboxylase, enzyme from *Flammulina velutipes*, is introduced into soya bean and grass pea to degrade oxalate as it causes diseases like hypocalcaemia, neurolathyrism, coronary diseases and kidney stones (Kumar et al. 2019; Irfan and Datta 2017). Bacterial cysteine synthase is inserted into potatoes, and it improves the level of lysine by two- to threefold (Beauregard and Hefford 2006). The use of aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) for modification in biosynthetic pathways in canola and soybean increases the level of lysine by two- to fivefold (Wang et al. 2017). By modifying enzymes during biosynthesis in maize mutant opaque-2 results in the reduction of zein proteins which are lysine-poor and increase in the lysine-rich proteins (Sun 2008).

2.1.5.1 Breeding and Biotechnology

Breeding and biotechnology together have the potential to improve nutritional quality. In the process of developing new genotypes and commercial cultivars, the availability of new sources of quality attributes and nutrition attributes are required (Scalzo et al. 2005). Molecular markers have the capacity to enhance the quality of old plant breeding with marker-assisted selection (MAS). Breeding program targets set many traits classified in five broader areas, i.e. (1) marker-assisted evaluation of breeding material, (2) marker-assisted backcrossing, (3) pyramiding, (4) early generation selection and (5) combined MAS (Collard and Mackill 2008). Crops such as rice (Wang et al. 2005) and cereals (Collard and Mackill 2008) have been improved by using molecular breeding. Traits such as resistance against diseases (Chen and Sullivan 2003; Okada et al. 2004; Zhou et al. 2005) and insects (Jiang et al. 2004) and fragrance (Steele et al. 2004) have been improved. *Brassica napus* seeds NAPUS 2000 improved with lecithin and protein fractions and antioxidants like tocopherols and resveratrol (Leckband 2002).

Breeders transfer the genes of desirable traits and improve the crops (Brown and Thorpe 1995). Micropropagation includes callus, protoplast, organ, embryo, tissue and plantlet cultures (De Filippis 2014).

2.1.5.2 Biotechnology for Improving Bioactive Compounds in Plants and Others

Plants are known for their bioactive compounds and health benefits since ancient time. Plants are a major source of primary and secondary metabolites. Bioactive compounds are used in various industries such as cosmetics, pharmaceuticals, fragrance, dyes etc. Genetic transformation of plants with *Agrobacterium rhizogenes* has played a successful role in improving bioactive compounds (Chandra 2012). It enhances polysaccharide (Wang et al. 2006), growth and biomass (Tiwari et al. 2007), saponins (bacopasaponin D and bacopasaponin F) in *Bacopa* (Majumdar et al. 2011), glucosinolate biofortification (Park et al. 2011), tylophorine (Chaudhuri et al. 2005), tropane alkaloid content by use of Ri T-DNA (Jung and Tepfer 1987), glycoside (Piatczak et al. 2006), oil yield (Sujatha et al. 2013) and geraniol and aromatic compounds (Pellegrineschi et al. 1994).

2.1.5.3 Recombinant Somatotropin for the Improvement of Animal Products

Bovine somatotropin is a hormone produced in pituitary glands of animals and is easily degradable by natural digestive processes. Recombinant bovine somatotropin is synthesized and produced from animals by using recombinant DNA techniques (Beitz 2001). Both natural and recombinant bovine somatotropin increases the amount of production of milk by enhancing the production of insulin-like growth factor-1 (IGF-1) as per the amount of feed without sacrificing the quality of food (Ghimire et al. 2014). As somatotropin is species-specific, it remains inactive in humans; it is also recommended by the Food and Drug Administration (FDA) as safe, and the pasteurization process also inactivates it (Bauman 1992; Fesseha et al. 2019). The studies also proved that the quality of meat is improved when goats are

injected with recombinant somatotropin. There was a sharp rise in feed conversion ratio, average daily gain, body weight and dry matter intake in recombinant somatotropin-treated goats as compared to the control ones in earlier studies (Sanjrani et al. 2016).

2.1.5.4 Use of CRISPR/Cas9 Complex in Food

CRISPR (clustered regularly interspaced short palindromic repeats) is an adaptive immune system of type II bacteria and archaea (Wang et al. 2019b), discovered for the first time from dairy production of *Streptococcus thermophilus*, while Cas9 (CRISPR-associated protein-9) is a RNA-guided endonuclease (Hsu et al. 2014). CRISPR is a family of a deoxyribonucleic acid (DNA) present in the genomes of primitive cellular organisms (Deshpande et al. 2015). CRISPR/Cas9 functions as a vector to alter the genome of targeted plants by random or point mutation and by whole genome insertion. CRISPR/Cas technology also knocks out the undesired traits sequence from DNA (Song et al. 2016). Expression, interference and adaptation are the three stages of the CRISPR/Cas system (Ansari et al. 2020). The technology develops the yield and quality of crops like rice (disease resistant, stress tolerance and fragrance improvement) (Mishra et al. 2018), cassava (carotenoid biosynthesis) (Haque et al. 2018), potato (improved starch, herbicide resistant and decrease in accumulation of glycoalkaloids) (Nadakuduti et al. 2018), wheat (disease resistant) (Borelli et al. 2018), tomato (lycopene biosynthesis and fruit development) (Li et al. 2018), beet, bean, grape (disease resistant) (Toffazal 2019), apple (carotenoid biosynthesis) (Charrier et al. 2019), soybean (improves oleic acid content) (Bao et al. 2020), maize (anthocyanin biosynthesis and stress tolerant) (Singh et al. 2020), *Cannabis sativa*, *Brassica napus*, *Arabidopsis thaliana* (oleic acid improvement) (Mishra and Zhao 2018), pennycress (improved oil content) (McGinn et al. 2019), cotton (stress tolerant and fibre improvement) (Khan et al. 2018), barley (crop development) (Han et al. 2020), flaxseed (herbicide resistant) (Adhikari and Poudel 2020), watermelon, sweet orange (carotenoid biosynthesis) (Tian et al. 2017), *Salvia miltiorrhiza* (inhibits tanshinone biosynthesis) (Li et al. 2017), tobacco, cucumber (virus resistant) (Borrelli et al. 2018; Zhao et al. 2020) and citrus (citrus canker resistant) (Sun et al. 2019). The technique improves crop by deletion and addition of DNA sequences with the help of CRISPR/Cas9, and later it is repaired with the help of HDR (high-fidelity homology directed repair) or NHEJ (error-prone non-homologous end joining) pathway (Bhatta and Malla 2020).

2.1.5.5 Fermentation and Bioprocessing

Fermentation is a process in which compound molecules are converted to simpler ones with the help of selected microorganisms, as they mediate oxidation and reduction of molecules. During fermentation, many bioactive components such as conjugated linoleic acid (CLA), vitamins and folate are produced which are the basic components of functional foods. Probiotic organisms like *Lactobacillus* and *Streptococcus* species are the best example of fermentation microbes used in dairy products (Yadav et al. 2011). *Lactobacillus*, *Leuconostoc*, *Bacillus* and *Pediococcus* species are used in the fermentation of vegetables like kimchi (fermented cabbage

and radish), natto (fermented soybean) and sauerkraut (fermented cabbage). Lactic acid bacteria are used in the fermentation of tomatoes increasing the availability of lycopene and also in the preservation of tomatoes (Admassie 2018). They are also used in the fermentation of fish, meat, bread and beverages. *Yeast*, *Aspergillus* and *Penicillium* strains are also useful in fermenting beverages. These fermentations and bioprocessing not only produce bioactive components and make food functional but also increase the shelf life of the product. These bioactive components help in keeping cardiovascular, digestive and bone health good and also function as antioxidant, antimicrobial and anti-ageing and improve immunity (Liang et al. 2016; Marshall and Megia 2011). It reduces and depletes antinutritional and toxic components like tannins, haemagglutinin and cyanogenic glycosides. The controlled fermentation also enhances proteins, carbohydrates and other nutritional factors in food and also modifies starches (Niba 2003). The major purposes of fermentation are enrichment of food and bioactive components, food preservation, detoxification and reducing cooking time and fuel requirements (Mani 2018).

2.1.5.6 Types of Fermentation

Following are the types of fermentation processes used in food processing depending upon their end product:

1. *Lactic acid bacteria (LAB) fermentation*: Lactic acid bacteria fermentation is done by the lactic acid bacteria or microflora to preserve and to maintain the nutrients in food longer. LAB status is generally recognized as safe (GRAS) microorganisms due to their potential in a wide range of applications (Widyastuti and Febrisiantosa 2014). It is used for the fermentation of dairy products like milk, cheese, yogurt and non-dairy products like meat, vegetables, fruits and cereals with the help of salt and acid used during fermentation (Soro-Yao et al. 2014). Addition of salt inhibits the growth of gram-negative (spoilage) bacteria and allows the growth of LAB which are gram-positive bacteria as it shows high tolerance to salt. LAB produce peptides like bacteriocins (nisin and pediocin) which functions as antimicrobial components and inhibits the growth of spoilage microbes (Mora et al. 2020). LAB also produce hydrolytic enzymes like peptidases and lipases which break down macromolecules like proteins and lipids into simpler forms which further form the specific precursors of aroma (Antara et al. 2019). Acid produced by LAB lowers the pH level and thus prevents the spoilage of products by inhibiting the growth of spoilage bacteria. LAB produce bacteriocin and reduce the pH level in meat which increases the shelf life and prevents spoilage (Sanlieret al. 2019). Probiotics are the bioactive components, which keep the digestive tract and cardiovascular healthy and prevent colon cancer, and are produced by fermentation with the help of bacteria like Bifidobacterium, Lactobacillus, Saccharomyces and Pediococcus species in dairy products. Fruits and vegetables are also good sources of probiotics (Wedajo 2015; Liptakova et al. 2017). Kimchi, pickles, sauerkraut and yoghurt are the products of lactic acid bacteria fermentation (Mani 2018).

2. *Alcoholic fermentation*: The process in which sugar (glucose, sucrose or fructose) is converted to ethanol, energy (ATP) and carbon dioxide is known as alcoholic fermentation. Yeast performs this process in the absence of oxygen and is considered as anaerobic fermentation. It is applied in the bread industry, cheese industry and wine industry (Walker and Stewart 2016; Mani 2018). Koumiss is a beverage prepared from unpasteurized mare's milk with the help of alcoholic and lactic acid fermentation. Yeasts convert sugar to ethyl alcohol and carbon dioxide, whereas LAB convert lactose to lactic acid. Koumiss is reported to treat avitaminosis, anaemia and intestinal and gastric diseases. It also shows positive effects on cardiovascular, nervous and immune systems. Kefir (fermented milk drink) produced from kefir grains is formed with the help of acid-alcoholic fermentation. Kefir is proved best as anti-inflammatory, anticarcinogenesis, antibacterial, antidiabetic, hypocholesterolemic, anti-hypertensive, antimutagenic, probiotic effects and antioxidant (Sanlier et al. 2019).
3. *Acetic acid fermentation*: In this process, the acetic acid bacteria (AAB), gram-negative bacteria, convert ethanol to acetic acid and glucose to gluconic acid. The process occurs in the presence of oxygen and is considered as aerobic fermentation. Vinegar, kombucha and water kefir are the products of acetic acid fermentation. Gluconacetobacter, Gluconobacter, Acetobacter and Komagataeibacter are the main genera involved in the vinegar production. Acetobacter, Komagataeibacter and many genera of yeasts also function in the production of kombucha (Gomes et al. 2018). Acetobacter species are used in water kefir production (Lynch et al. 2019). Vinegar prevents cardiovascular diseases and diabetes, while kombucha treats gastrointestinal tract disorders (Gomes et al. 2018).
4. *Alkaline fermentation*: In alkaline fermentation, the proteins are converted into amino acids, peptides and ammonia generally with help of Bacillus species of bacteria. This bacterium species increases the pH level and thus prevents the spoilage from several microorganisms. Alkaline fermentation of legumes, fish and protein-rich oil seeds leads to the production of condiments (Ugwuanyi and Okpara 2019). Natto, Thua-nao, Ogiri and Daradawa are also the products of alkaline fermentation process. All of these products are used as meat substitutes as they match the meat protein and amino acid profile (Shiferaw and Augustin 2019).

Sr. no.	Enzymes	Sources	Application	References
1.	α-*Amylases*	Bacteria (*bacillus*, *pseudomonas* and *clostridium*) and fungi	Baking industry (converts bread dough starch into small dextrins which allows dough fermentation and increases the shelf life of products)	Souza (2010) John (2017)
2.	*Lactases*	White rot fungi (*Pleurotus pulmonarius*, *P. ostreatus*, *Agaricus bisporus*, *Trametes versicolor*), few bacteria, plants and insects	Baking; removal of polyphenol from wine	Mayolo et al. (2020) Brijwani et al. (2010)
3.	*Catalases*	*Aspergillus Niger*, *Penicillium variable*, *Saccharomyces cerevisiae*, *staphylococcus*, *micrococcus lysodeikticus*, *Bacillus subtilis*, *Thermoascus aurantiacus* and *rhizobium radiobacter*	Hydrogen peroxide is used during cold pasteurization for cheese production, but it hinders the bacterial cultures which are responsible for the actual cheese production, so catalase enzyme removes hydrogen peroxide by breaking it into water and oxygen	Sharma and Ahmad (2014) Kaushal et al. 2018
4.	*Rennet*	*Rhizomucor pusillus*, *R. miehei*, *aspergillus oryzae*, *Endothia parasitica* and *Irpex lactis*	Clots milk for quality cheese production	Shinde et al. (2015)
5.	*Lipases*	Microbial, plants and animals	Egg processing (enhances the emulsification properties of yolk lipids), baking and dairy product processing for aromatic notes	Guerrand (2017)
6.	*Proteases*	Plants, animals and microorganisms	Cheese production, fruit juice fortification, maintains gluten in bread and recovers meat proteins	Singh et al. (2016)
7.	*Glucoamylases*	Animals, plants and microorganisms	Light beer production (converts dextrin to fermentable sugars with low calorie value), sake and soya sauce production	Raveendran et al. (2018)
8.	*Phospholipases*	*Bacillus*, *fusarium* and *Streptomyces*	Egg yolk industry, refinement of vegetable oil and bread-making	De Maria et al. (2007)
9.	*Esterases*	*Rhizomucor miehei*, *aspergillus*, *bacillus*, *Candida*, *Rhizopus* and *Penicillium*	Synthesis of flavour esters, cheese flavour enhancement and fat function modification	Chaudhary et al. (2015)
10.	*Cellulase*	Fungi (*Trichoderma reesei*, *Humicola*, *Penicillium* and *aspergillus*) and bacteria (*bacillus*, *Acetobacter xylinum*, *clostridium*, *Caldocellum*, *Flavobacterium*, *pseudomonas*, *Ruminococcus* and *Thermomonospora*)	Baking industry (break up roughage of dough), olive oil extraction and increases the shelf life of fruit juice	Kumar et al. (2019)

(continued)

Sr. no.	Enzymes	Sources	Application	References
11.	*Xylanase*	Bacteria, fungi, actinomycetes and protozoans	Beer quality improvement, bread-making and fruit juice clarification	Raveendran et al. (2018) Harris and Ramalingam (2010)
12.	*Pectinase*	Plants, bacteria and fungi	Extraction, clarification and stabilization of fruit juices	Kubra et al. (2018)
13.	*Glucose oxidase*	*Aspergillus Niger*	Removes small amount of oxygen from food and glucose from diabetic drinks and increases shelf life of food	Salim et al. (2017)
14.	*Laccase*	Fungi, bacteria and higher plants	Juice processing, wine stabilization, bioremediation of waste water and baking	Brijwani et al. (2010)
15.	*Peroxidase*	Bacteria, fungi, algae, plants and animals	Determination of the extent of lipid peroxidation in meat products	Pandey et al. (2017)
16.	*α-Acetolactate dehydrogenase*	Yeast	Beer maturation	Dulieu et al. (2000)
17.	*Asparaginase*	Mammals, birds, plants, yeast and bacteria	Acrylamide formation in baked and fried food	Cachumba et al. (2016)
18.	*Naringinase*	Grapefruit leaves, celery seeds, fungi and bacteria	Debittering of fruit juices and aroma enhancement in wine	Ribeiro (2011)
19.	*Lipoxygenase*	Plants, animals, bacteria and fungi	Aroma production, baking conditioner and flour treatment agent	Shi et al. (2020)
20.	*Cyclodextrin*	Starch	Encapsulation of flavour compounds; removes bitterness from juices, coffee tea and milk; and reduction of cholesterol in food	Matrina et al. (2013)
21.	*Glucosyltrans ferase*	Plants	Aroma and fragrance	Schwab et al. (2015)
22.	*Fructosyltrans ferase*	Plants and microorganisms (*Aureobasidium* and *aspergillus*)	Produces prebiotic compound (fructooligosaccharide)	Antosova and Polakovic (2001)
23.		*Transglutaminase*	Microorganisms (*Streptoverticillium*, *Streptomyces*, *Providencia*, *Enterobacter*, *Actinomadura*, *Corynebacterium* and *bacillus*	Improves texture and quality of meat, fish, milk and bakery products
	Kieliszek and Misiewicz (2014)			
24.	*β-Galactosidase*	Fungi, bacteria, yeast and plants *Aspergillus* and *Kluyveromyces* (commonly used)	Hydrolyses whey and produces sweet syrups; production of galactooligosaccharides (GOS) which is the key component for prebiotic food; lactose hydrolysed milk production	Saqib et al. (2017)

(continued)

Sr. no.	Enzymes	Sources	Application	References
25.	*β-Glucanase*	Archaea, algae, fungi, bacteria, molluscs and higher plants	Used in botrytis wine production (for removal of β-glucans to reduce their viscosity)	Martin et al. (2007)
26.	*Invertase/β-fructo furanosidase*	Plants and microorganisms. *Saccharomyces cerevisiae* (chief strain)	Non-crystallizable sugar syrup production from sucrose; lactic acid, glycerol and alcoholic beverage production by sucrose fermentation	Kulshrestha et al. (2013)
27.	*Pullulanase*	Hyperthermophilic archaea	Starch processing, baking and cyclodextrin production	Ramdas and Malviya (2010)
28.	*Phytases*	Microorganisms and plants	Improves mineral bioavailability; used in cereal bran fractionation, bread-making, corn wet milling and production of plant protein isolates	Greiner and Konietzny (2006)

2.2 Conclusion

Although there is improvement in crop varieties and cultivation patterns in the last few decades, the milestone of solving hunger and malnutrition is far away. Biotechnology can be boon in the field of food supply. Some agricultural systems are depending on harmful chemicals. Only biotechnology can fulfil the future expectations for human as well as ecological health. Functional food not only fulfils nutritional needs but also prevents various diseases.

Human population is increasing rapidly; fulfilling its requirement for safe and nutritious food is an important task for humanity. The low production of food is considered as the main cause of hidden hunger and poverty. Use of modern biotechnological techniques for improving crops and use of functional food can solve chronic as well as transitory food insecurity. Gene editing techniques such as insertion or deletion of genes carrying specific traits from an individual for obtaining improved animals or crops are significant tools for solving food insecurity. Functional food ingredients come from significant genetic resources. Biotechnology consists of many modern techniques for management of such important genetic resources for improvement of health and prevention from diseases. Techniques belonging to genetic engineering, DNA recombinant technology, DNA interference, CRISPR/Cas9 are the most promising techniques which can fulfil consumer requirements.

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The Current and Future Prospects of Animal Biotechnology Applications in Food

3

Prameela Kandra

Abstract

Advances in animal biotechnology catered to make several applications in the field of food industry to improve living standards. Various food processing techniques can be applied for the betterment of food products. The biotechnological tools help to produce nutritionally enriched, long shelf life, more productive food products. The quality attributes in the production of livestock products envisaged for high yield. However, in the current scenario there is a demand to improve the production of foods to meet the people's requirements. Hence, this book chapter unfolds with the introduction to animal biotechnology followed by applications in food. This chapter also highlights the various methods of production of transgenic animals; further, it reviews the safety with future prospects.

Keywords

Biotechnological tools · Food applications · Transgenic animals · Quality · Safety

3.1 Introduction

Development of science and technology advances in the product based scientific developments throughout the world. Biotechnology is an interdisciplinary field that has many applications in agriculture, animal sciences, medicine, etc. The production of genetically modified foods through genetic engineering tools is one of the approaches to see the improvement in animal biotechnology. The quality in terms

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of taste, shelf life, more yield, and rich nutrients are increased progressively through animal biotechnology. Several molecular-based techniques in modern biotechnology will help change the living organisms to improve the production of commercial food products like high-protein meat, beverages, and vitamin-enriched foods. Genetically modified foods will help to eradicate malnutrition and hunger by improving the productivity even in low-income developing countries like in Africa, Asia, and Latin America where 24,000 lives are claimed per day due to hunger and malnutrition (James 2003). This situation has a great impact on humanity. Due to human activities, the natural resources are depleting, and it is believed that by 2050, nearly ten billion people may not have resources (UNFPA 1995). To improve nourishment for all kinds of people, exploitation of genetic engineering is necessary to achieve the goal with existing resources. Hence, increased livestock production for sustainable lives is expected with improvement of nutrition, health, and animal products.

For the past 25–30 years, animal biotechnology is rapidly growing. Initially genetically modified animals were used for basic research and as disease models. The main idea is to check the overexpression or no expression of genes, to identify mutated genes, and to find disease-causing genes. In all these conditions, how the organism will react and give responses to various situations can be understood. Any change at the molecular level may affect the animal phenotype (Olsson and Sandøe 2004). The intended genetic variations in welfare problems are very hard to avoid as it may affect the animal at any point. In human beings Huntington's disease which causes premature death due to loss of neural control (Naver et al. 2003). In intended genetic modifications, we can find effects of similar type of mutations in species. Therefore, to evaluate welfare consequences, some studies will help before the production of genetically modified animal (Dahl et al. 2003). Different methods of approaches are helpful to produce genetically identical individuals, but still we need to understand some of the concepts of epigenetic factors to produce genotypic and phenotypically 100% similar donor animal (CeBRA 2005a, b). Hence, modification in the production is necessary. Genetic modification is advantageous in producing more food from animals to meet the food requirements of mankind. However, there is a low success rate in the cloning of animals, and most of the individuals born may have complications in health and welfare. The produced animal may have large offspring syndrome (LOS). The problems included in LOS are immune dysfunction, fetal overgrowth, hypoxia, placental abnormalities, malfunctioning of the urinogenital tract and organs like the brain and liver, respiratory failure, microbial infections, etc. (van Reenen et al. 2001). Transgenic animals are genetically modified animals produced by transgenesis. Several animals like rats, rabbits, mouse, sheep, and pigs have been produced through this technology. In transgenesis the genes can be manipulated to improve livestock milk and meat-producing species.

In the 1980s, Steen Willadsen introduced cloning by embryo cell transfer method for the production of cloned sheep (Willadsen 1986). From 1970 to 1972, transformation of bacterial plasmid DNA into *E. coli* was performed. Later in 1975, monoclonal antibody was developed, and in 1978, the first recombinant DNA product called somatostatin was produced. The first human recombinant insulin named Humulin was produced in 1982. In 1985, Protropin was developed by

Cohen and Boyer, and a patent was filed. Similarly several recombinant products like vaccines, interferons, and tissue plasminogens were developed from 1986 to 1989. The work of Ian Wilmut produced the first cloned sheep named as Dolly in 1996 (Wilmut et al. 1997). Similarly several genetically modified crops were in the market by the end of 2004. Most of the work was carried out on mice, cattle and sheep, goats, pigs, cats, and horses. Several recombinant products were produced such as recombinant vitamin C, recombinant rennet, baker's yeast, and several enzymes like lipase, amylases, and some of lactic acid starter cultures with bacteriophage resistance. Many industries like Calgene, Novomil, Novo Maturex, Genecor, and Unilever come forward for the production of such genetically modified products. Hence, animal biotechnology in one way is helpful in producing animals for research purposes and as model systems to study several diseases like cancer, Parkinson's diseases, diabetes, stroke, and cystic fibrosis. In another way it will help to develop special animal for food applications in order to improve better health with special traits.

In 2006, scientists were able to produce human protein-rich animals (CeBRA 2005a). However, many of them opposed the genetically modified animals for agricultural production system. Due to various reasons and debate on GM-based animals, the practices are extended from 2006. The ultimate goal of the scientists and researchers is to establish possible methodologies for the efficient production of GM animals (Khanna and Hunter 2005; Emborg 2004; Swanson et al. 2004; Olsson and Sandøe 2004). For example, introduction of human gene into the animal genome to produce milk protein especially a sheep was developed with alpha-1-antitrypsin which is used for the treatment of lung-associated disorders (NAS 2002). Other way to produce leaner meat and functional foods in the animals is with increase growth rate, disease resistance (Kues and Niemann 2004). The first luminescent fish was developed for aquarium in 2003 named as Glofish and other pet animals (Caplan 2004; Holt et al. 2004; CGS 2005). Nowadays, researchers are working towards the successful production of wild ox, tigers, and mammoths.

3.2 Applications

Genetically modified plants and animals have many applications. Most recombinant proteins are produced in large quantities in plants than in animals as plants have the advantage of freedom from human pathogens. Even though the genetically engineering methodologies started in the eighteenth century, for the past two decades it has improved a lot, hence having many products in the market. All biopharmaceuticals produced through genetic engineering are from animals, but none of the products are from plants, because the yield is less and the steps for purification and application of methodologies are costly compared to that from animal. Hence, in the future plant-based DNA vaccines may have a potential role in animal biotechnology. However, transgenic plants express new traits with additional nutritional values or resistance to pests. Some of genetically engineered products are produced through fermentation process. Different pharmaceutical

products like erythropoietin, human insulin, GMSF (granulocyte-monocyte colony-stimulating factors), streptokinase, hepatitis, human growth hormone, and interferon are all produced by animal, plant, and microbial domains. These three domains are involved not only in biomedical components but also in the production of food products. Many enzymes in food industries are produced by genetic engineering, crop breeding, transgenic plants, animals, animal breeding, and production of functional foods and nutraceuticals. Some of the complications in traditional animal breeding (several generations) can be solved by genetic engineering (single generation).

3.3 Development of Transgenic Cattle for Milk Production

Bovine milk which is produced by transgenesis maintains the dairy milk enriched with essential amino acids, vitamins, and calcium. As milk is rich in many nutrients like calcium, carbohydrates (lactose), proteins (casein), and vitamins like A, D, E, and B complex, it can be considered as a balanced diet which will supplement daily calcium requirements by two servings of milk (Rinzler et al. 1999). The protein present in the milk casein represents 80% of total milk (Brophy et al. 2003). Casein is a phosphor protein and could bind to calcium, zinc, and magnesium ions as it is negatively charged (Dalgleish et al. 1989; Jimenez Flores and Richardson 1988; Brophy et al. 2003). The amount of casein in the milk is very important for making several dairy products like cheese, butter, cheddar, etc. If there is an increase in casein concentration by 20%, by using genetic engineering the production rate of dairy products is also increased simultaneously in the industrial economy (Wall et al. 1997). All these characteristics were initiated in mice as these animals are best models and have shown increase protein expression in the mammary glands (Colman 1996). Two types of caseins (β and k) were produced by inserting CSN2 and CSN3 genes in transgenic cows. Nuclear transfer technology was used for transferring of genes. The experiments conducted with CSN3 in mice have shown a low expression; to improve the CSN3 levels, a fusion gene CSN2/3 was constructed and transferred into BFF (bovine fetal fibroblast) cells. This concept was successful in nine functional healthy cows, resulting in the increase of β-casein to 8–20% and k-casein to 100% (Brophy et al. 2003). In a nuclear transfer methodology, insertion of genetic material from donor nucleus is transferred to mature unfertilized egg. Now this may be transferred to a foster mother to develop an animal that is genetically identical to the donor (Wolf et al. 2001). In other gene transfer technology called microinjection, the foreign gene is transferred into the male pronucleus of fertilized egg where the egg is a single-cell stage, ensuring all somatic cells in the animal should contain transgene. Now this embryo is transferred to a surrogate mother (Wall et al. 1997). However, the major idea of genetic engineering programs in livestock is to improve production rate in healthier animal food products. Hence, the production of transgenic animals through genetic engineering is advantageous as the expression of certain genes to improve the production and

quality of food based products and has significance in nutritional and commercialization of these products.

3.4 Production of Transgenic Cattle

Transgenic cattle were produced to increase the muscle growth by inserting growth differentiation factor called myostatin. This growth factor is involved in muscle mass regulation. Initially it was produced as a polypeptide chain having 375 amino acids. By removing the N-terminal amino acids enzymatically, the biologically active segment was produced which is made up of 109 amino acids (Gleizes et al. 1997). Similarly this gene is conserved in other vertebrates like human, baboon, murine, bovine, chicken, rat, ovine, porcine, turkey, and zebra fish. Due to the role of myostatin, scientists found an increase in the muscle mass of animals like Belgian Blue and cattle. To understand the role of myostatin in increasing the muscle mass, myostatin gene was knocked out, and the muscle weight was measured and compared with the wild variety. There was an increase of 30% muscle mass in transgenic animal compared to wild type. This is due to the increase in muscle fibers (McPherron and Lee 1997). The gene was constructed with rat myosin light chain1, MLC enhancer, and SV40 polyadenylation. The whole construct was inserted by pronuclear microinjection into the mouse genome. These results have shown without any health-associated problems, and with enhanced growth rate in muscle mass, it helps to compensate for the healthier meat production for consumer requirements.

3.5 Production of Transgenic Swine

Consumption of high-fat diet especially exogenous fats may increase the body fat, phospholipid contents and cholesterol. Fat produces high calories of energy when compared to other nutrients like carbohydrates and proteins. The excess energy in the body is stored in the form of fat. Fats are composed of fatty acids with glycerol. Most of the fatty acids are palmitic (C16), stearic (C20), and oleic (C18) acids. Some of the fatty acids are essential which are not synthesized by the human body and hence are to be supplied in the diet, whereas some other fatty acids are synthesized by the human body (Campbell and Reece 2002). However, most of the unsaturated fatty acids like oleic acid, linoleic acid, linolenic acid, and arachidonic acid are essential fatty acids. Saturated fatty acids are considered as bad fats because they increase LDL (low-density lipoproteins) (NRC 2002). According to the American Diabetes Association, Dietetics Association, and Heart Association, intake of fat should be restricted to 30% only to meet daily calories needs; intake beyond that may cause several health issues. USDA conducted a study on the insertion of recombinant bovine growth hormone (rBGH) into pigs to check the amount of fatty acids expressed in animals. Many studies were conducted to understand the relationship between the hormone and fatty acids and increase in fatty acid content and

cholesterol compared with wild varieties (Persuy et al. 1995; Solomon et al. 1994; Etherton 1991).

3.6 Production of Transgenic Fish and Poultry

Advances in animal biotechnology have directed the use of poultry eggs as bioreactors for many exogenous proteins (Tranter and Board 1982; Harvey et al. 2002). Researchers succeeded in bacterial gene expression in transgenic chickens (Ivarie 2003; Harvey et al. 2002). A transgene named β-lactamase was secreted in the blood and egg white and was maintained constantly for four generations. These eggs are good enough to be used as bioreactors (Harvey et al. 2002). Transgenic fish is more suitable for human consumption, and it got the approval to be used as food (Niiler 2000). Few companies got FDA approval to use growth hormone gene from Chinook salmon (Zbikowska 2003; Du et al. 1992; Devlin et al. 1994). Transgenic *Oreochromis niloticus* fish was developed by the scientists of the University of Southampton in the UK using several gene constructs (Rahman et al. 1998). However, they got better results with Chinook salmon GH gene with antifreeze promoter using cytoplasmic microinjection into the fertilized fish egg and succeeded in the integration of two G1 and G2 generations. The transgenic fish has shown 33% less food conservation than wild tilapia. This may help to reduce the production cost of farmers. Similarly antifreeze properties are also increased in ocean pout and winter flounder fish species. Specific proteins like antifreeze proteins and antifreeze glycoproteins may protect the fish from ice freezing by inhibiting crystal formation since the wild varieties of Atlantic salmon and tilapia lack these proteins. There are four types of antifreeze proteins represented as AFP I, AFP II, AFP III, and AFP IV (Davies and Hew 1990; Davies and Sykes 1997). This development has increased and is applied to several species of fishes to increase the productivity. There are two types of AFP which are a part of AFP-I type. They have two different isoforms like intracellular skin type and immature protein liver type (Hew et al. 1986; Gong et al. 1996). *Salmo salar* has shown stability to freezing temperature. Expression of antifreeze proteins was successfully observed in salmon for three generations. Another application is the production of Glofish. A green fluorescent protein was isolated from jellyfish, and the gene was inserted into a zebra fish embryo (Gong et al. 1996). Researchers also developed engineered zebra fish to detect environmental pollutants. Most of the pollutants are detected like halogenated hydrocarbons, mercury, copper, and quinines (Carvan et al. 2000). A gene from sea coral was used to develop red fluorescent zebra fish. Green fluorescent rice fish, a medaka, was developed. Many Glofishes were developed with red, green, orange-yellow, blue, and purple colors as ornamental fishes. Genetically modified fishes are reachable to public nowadays. Recently few fishes were created to act as sensors by incorporating light-emitting reporter gene (luciferase) (Legler et al. 2000; Chen et al. 2010). De Coster and Larebeke (2012) developed fish sensitive to retinoic acid and estrogen.

3.7 Modified Foods

Genetically modified plants and animals have shown benefits in terms of crop yield, pesticide resistance, increased nutritionally rich crops, and reduced costs with greater food quality. To resolve some issues like mineral-tolerant varieties, drought- and salinity-resistant crops can be developed through the advances in genetic engineering. Figure 3.1 shows the few examples of GMO commercial products. However, there are some foods developed in view of health promotion which are considered as designer foods (Bhat et al. 2017). Designer food was first introduced by Japan in 1980, and in 1934 designer egg was developed by modification in fatty acid content (Cruickshank 1934; Arai 1996). These eggs are developed by few scientists by enriching them with polyunsaturated fatty acids, vitamin E, selenium, and carotenoids which improves the antioxidant activity and immunity (Surai 2001; Raes et al. 2002; Jiang et al. 1994). Similarly, milk quality was improved by changing casein concentration, fatty acids, and amino acids and lowering carbohydrates (Rajasekaran et al. 2013). Removing the lactoglobulin gene (β-LG) from dairy animals like cow and bovine helps to reduce milk allergies in children (Sabikhi 2007). Selenium-enriched poultry foods have gain attention to improve the health of individuals (Fisinin et al. 2009).

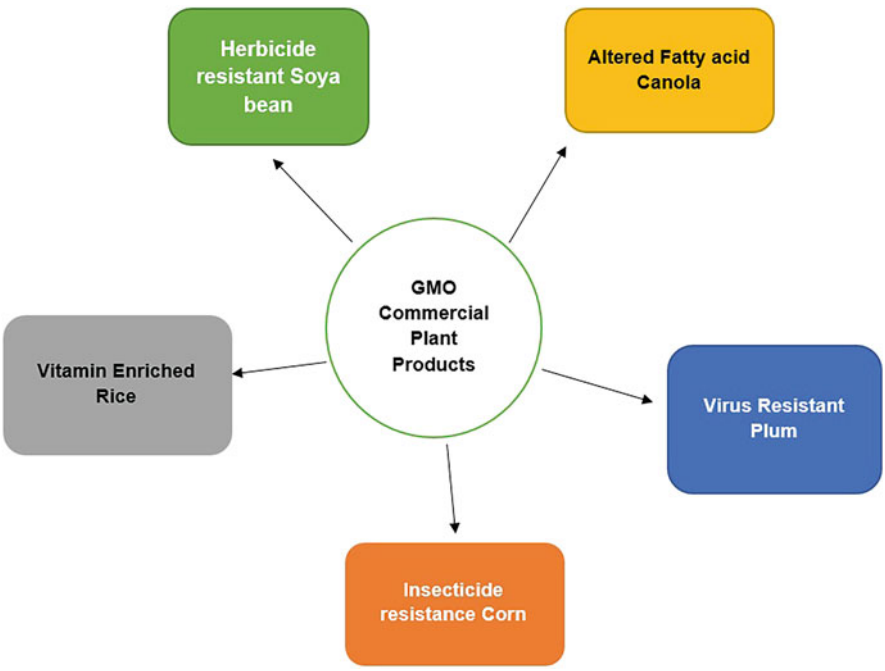

Fig. 3.1 Genetically modified commercial plant products

3.8 Current Technologies

Several techniques are available to produce advanced animals having food applications. Artificial insemination has an effect on dairy. In the 1980s, GM animals developed were rats, mice, sheep, rabbit, and pigs. Techniques that are used for the production of transgenic animals are in vitro fertilization, embryo cloning, and nuclear transplantation. With the help of recombinant technologies, the genetically superior breeds can be developed. Nearly 70% of cows (Holstein) in the USA were used for dairy applications and are artificially inseminated. The first patent on recombinant DNA techniques was initiated in 1980. Many steps will assist the reproductive technology. Artificial insemination and estrus synchronization are the initial steps in the production of superior breeds. Eggs are fertilized in the donor, and embryo transfer is the next step where a donor animal is induced for superovulation.

3.8.1 Methods for the Production of Transgenic Animals

In the transgenic animal production, the foreign gene is introduced into the germ line of the recipient (Vijg et al. 1996). One of the methods for insertion of foreign DNA is by pronuclear micro injection. However, the transgene DNA constructed by using short segment of DNA using PCR (Polymerase chain reaction) or using plasmid or viral vectors and corresponding DNA fragments are ligated by ligases and transgene contain promoter helps to determine the transgene where and when is active, exon, intron and poly (A) and enhancer sequences (Clark et al. 2004). Hence, cloning strategy may help to understand the functional and expression of a transgene. From the upstream regions a promoter sequences are isolated and a protein coding sequence contains start codon and stop codon. In a transgene intron may have effect on mRNA stabilization. Once transgenes were constructed the transferring of transgene is one of the key steps in production of transgenic animals. The foreign gene can be inserted into gametes or embryos by different methods. The methods are microinjection, embryonic stem cell-mediated, retrovirus-mediated, sperm-mediated, transposon-mediated, and nuclear transfer (Fig. 3.2).

3.8.1.1 DNA Microinjection

In superovulation process the female eggs can be produced by injecting gonadotropin which results in the release of many eggs at a time. These eggs are fertilized in vivo or in vitro. If fertilization occurs in vivo, the fertilized eggs can be inserted into the pronuclei by microinjection in mammals, but pronuclei is not visualized in non-mammalian species; hence, foreign DNA must be injected into the cytoplasm. A pseudopregnacy was developed in female by injecting hormones and mating with vasectomized males. Fusion occurs with embryos that are implanted into pseudopregnant foster mother, and this process helps to prepare the female reproductive system for transplantation. Even though DNA insertion by microinjection method was a first technique and the insertion is in a random, the success rate for this method is low in chicken, fish medaka, and *Xenopus* due to the inability to integrate

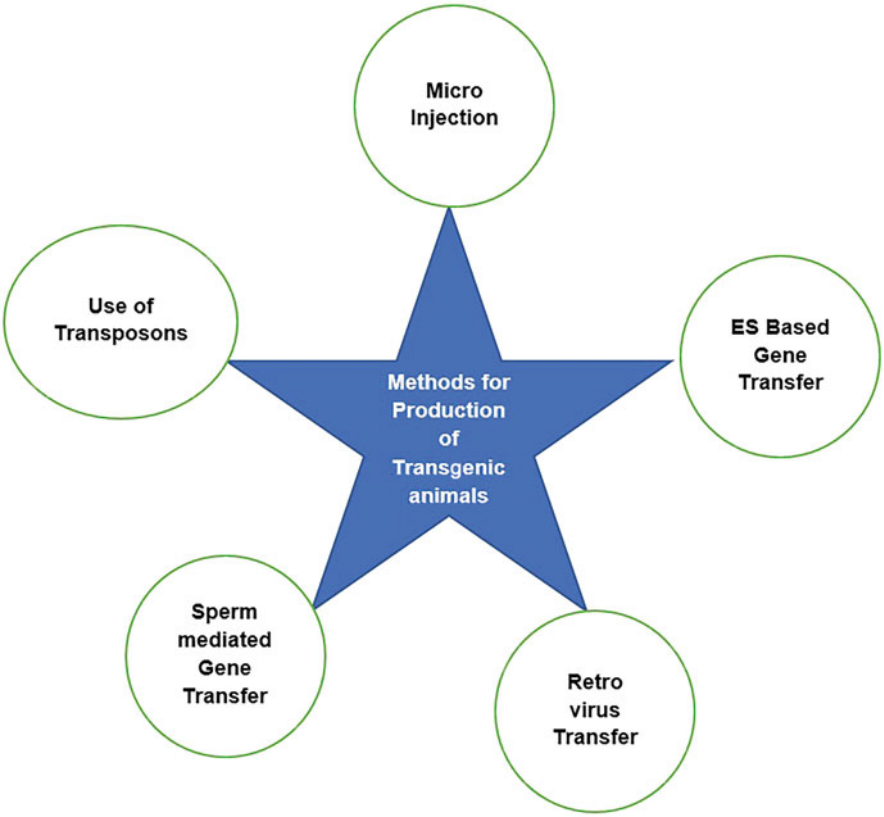

Fig. 3.2 Different Methods for production of Transgenic animals

into the genome of these species. In insects (*Drosophila*) and worms, foreign DNA is injected into the gonad syncytium.

3.8.1.2 ESC (Embryonic Stem Cell)-Mediated Gene Transfer

In general embryonic stem cells are derived from blastocyst (inner cell mass). These cell lines are having self-renewal, pluripotency, and clonogenicity characteristics derived from mouse. However, worldwide nearly 120 human embryonic stem cells have been derived (ISSCR). When hESCs were injected into the host which is immune-deficient, it will form teratomas composed of ectoderm, endoderm, and mesoderm. To enrich clinical trials, the well-defined protocols provide knowledge to study the ESCs' role in gene transfer in transgenic animal production. The studies revealed that ESCs towards particular lineage like endoderm, neuron cardiomyocytes, hematopoietic cells, hepatocytes, osteogenic cells, and germ cells (Perrier et al. 2004; D'Amour et al. 2005; Passier et al. 2005; Ng et al. 2005; Zambidis et al. 2005; Schwartz et al. 2005; Bielby et al. 2004, Clark et al. 2004). However, other factors like free radicals, stimuli, and environment conditions which

mimic the early embryo also help to influence the differentiation of ES cells (Heng et al. 2004). From preimplanted embryo, the totipotent stem cells were isolated, and they can be cultured in the laboratory. Now these cells are transfected with transgene. The transfected embryonic stem cells can be screened with the help of specific marker gene. Now these transformed embryonic stem cells are injected into the blastocyst through microinjection. Although the transfected ES cells are transferred into the surrogate mother, to check the establishment of transgene in somatic and germ line, few successive generations were considered for observation. This method has given better success with mice (Sasidhara 2006).

3.8.1.3 Retrovirus-Mediated Gene Transfer

In this method retroviruses which carry RNA as genetic material are used as vector for transferring genetic material into the host, as retroviruses do not have the capacity to replicate them. The property of retrovirus where they can make DNA from RNA through reverse transcriptase will help to integrate foreign genes. Now these viruses are injected into the embryo through microinjection and allow the embryo to grow to blastocyst stage. Now these cells can be transferred to the surrogate mother. This is one of the best techniques proven even for birds and mammals (Sasidhara 2006; Pfeifer 2006; Lillico et al. 2007).

3.8.1.4 Use of Transposons

This is another method for insertion of foreign DNA into the host. Transposons are DNA sequences containing at least one gene coding for transposons that trigger integration. This method is successful in case of chicken, silkworm, *Drosophila*, and medaka. Transposons help to enhance the integration frequency. In vitro foreign genes can be introduced into transposons to produce recombinant transposons. Now these can be inserted into the embryo (1 day old). This method is more efficient in non-mammalians and mammals (Ding et al. 2005).

3.8.1.5 Sperm-Mediated Gene Transfer

Other strategy to transform exogenous DNA is through mature spermatozoa which act as a vector in developing transgenic animals. The spermatozoa act as a vector for their own genome. But this method is very difficult due to the frequent degradation of DNA (Smith and Spadafora 2005). This method is improved by intracytoplasmic sperm injection for in vitro fertilization (Shen et al. 2006). However, the plasma membrane have damaged storage, thawing during this process. This method has been efficient in pigs and mice (Yong et al. 2006; Moreira et al. 2007). To produce transgenic mouse, lentiviral vectors can be used and are injected to infect spermatogonia in seminal tubules (Readhead et al. 2003). The produced sperm cells are introduced into the oocytes through artificial insemination, and this procedure has shown better success in cattle (Sperandio et al. 1996). Dolly, the transgenic sheep, was developed through single-cell nuclear transfer methodology (Wilmut et al. 1997).

3.9 Safety in Usage of Transgenics

The safety, risk, and usage of genetically modified animals and their products can be evaluated through the guidelines reported by the NRC (National Research Council) in 2002. This committee measures the criteria for assessment and risk factors and effect on severity. Mainly the committee focuses on ethical and social issues in the production of genetically modified organisms, misapplication, technologies, and methodologies adopted for the production. Environmental concern, food safety, and institutional and animal welfare are the major important areas of concern of the committee, and they design rules and regulations accordingly (NRC 2002). However, federal guidance for agricultural products was published by the OSTP (Office of Science and Technology) White House in 1986. The NRC ensured the GE products and framed new application regulations to address the unique problems in animal biotechnology when federal agency responsibilities are not clear. In 2009, the FDA released guidelines for the industrial usage of genetically engineered animals (FDA Guidance for Industry). The products whichever are used will not affect the structure and function in the animal body or man, and according to the FFDCA, if any new animal drug is intended to be used in animals that is not recognized as safe and that has not been prescribed or recommended. The FDA states that usage of new GE products is not safe unless they get approval from the FDA. Hence, it is necessary to get approval before they market. The FDA will examine toxicity, marketability, and potentiality of the food and edibility. Mostly the guidelines will address the allergies of some seafood, dairy products, meat products, etc.

However, genome manipulation may have greater efficiency in the production of several products, but Rollin has suggested two principles: a more palatable one and a highly contentious one of welfare conservation to help mitigate inhumane procedures related to genetic engineering (Rollin 1995). There are many arguments in GE animal production (Rutgers et al. 1999; Bovenkerk 2002). The production of GE-based products is also a public concern since unhealthy transgenic animals will be euthanized. For example, in 1985 Beltsville pigs were produced by USDA Agricultural Research Service. Nineteen animals have experienced painful conditions like ulcers, physical deformities, arthritis, and reduced immunity, and all these animals were euthanized (Rollin 1995). Similarly, in 2003 Dolly produced by Scotland experienced premature aging and cancer several (300) times to fuse nuclei with blastocyst (Wilmut 1997; Varner 1999). A number of hoofed animals were produced by gene transfer technologies. In 2001 industry guidelines were implemented by the FDA to resolve some issues in marketing of cloned animals. In 2008 continuous moratoriums were initiated by stakeholders on the safety aspects of animal cloning. In 2003 a draft was made on risk assessment on consumption of clones of sheep, cattle, pigs, and goats (FDA). In 2006, the guidelines were released for safety of animal clone and offsprings. In 2006 a draft of the safety guidelines for meat and dairy products was released. On public comments ethically and expert suggestions, the FDA has designed guidelines for the risk and safety of consumption and marketing of genetically engineered animals.

3.10 Future Prospects and Conclusion

Production of transgenic animals is an advantage to fulfill the requirements of the people as this technology will enhance yield. Few products are commercialized. For example, emerging in animal biotechnology environmental concern product is the development of Enviropig. This waste of this modified animal will produce less phosphorous content which is advantageous to the environment. However, other genetically modified animals like salmon, insects, fish, and shellfish are disrupting ecosystems and introduce new genes in the natural population which changes the biodiversity. The FDA will act in accordance with agency requirements to the National Environmental Policy Act. The production of environmental concern transgenic animals is one of the future challenges. Unexpected changes in animals through gene modification have negative results on human health. Hence, genetically modified foods should be produced according to food safety concerns to avoid many health-associated issues. Sometimes the genetically modified foods may cause allergy. So the development of non-allergic-oriented genetically modified foods is the second future prospective. Change in strategies in the insertion of modified DNA is another future challenge; this may reduce the risk of clinical applications. Enhanced bioavailability and reduction of toxicity of proteins developed through transgenesis are another thrust area. The production of people-demanded products with economical viability, limited market price, and consumer acceptance is one of the near-future prospects.

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Part II

Microbiological Applications in Food Industry

Utilization of Bio Surfactants in Food Technology

4

Monisa Anwer and Ajay Kumar

Abstract

Several microorganisms are capable of producing a wide range of amphipathic compounds that carry both hydrophilic and hydrophobic moieties in the same molecule, and this allows them to exhibit surface activities at the interfaces; such molecules are termed as bio surfactants. Bio surfactants are the surface active compounds that are classified mainly on the basis of their mode of action, physiochemical properties and also their molecular weight. Bio surfactants include the low molecular weight compounds that reduce interfacial tension and surface at the gas-liquid-solid interface. Even though a large population of microorganisms is responsible for the production of bio surfactants, their significant role is not clear, but due to their diverse chemical structure and properties, these surface active agents might have different natural roles in the growth of microorganism producing them and also positively affect the ecological niche. Due to their diverse characteristic features, bio surfactants are compounds of keen interest and are being studied and used widely for different purposes. Such detailed studies have revealed their potential to be used as various agents and additives in different industries including medical, agriculture, fermentation and food industries. One of their major and important contributions is in the food processing industries where they find their application as food formulation ingredient, anti-adhesive agent and antimicrobial agent. These compounds are responsible for promoting the formation and stabilization of emulsion. They are also used as agents to control the agglomerations of fat globules and effectively enhance the food texture and shelf life of starch-containing products. As anti-

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adhesive and antimicrobial agents, they potentially inhibit the colonization of pathogenic food spoilage microorganism and also inhibit the formation of biofilm by these pathogens on the surface of food processing materials.

Keywords

Bio surfactants · Bioemulsifiers · Antimicrobial agents · Antiviral agents

4.1 Introduction to Bio Surfactants

Bio surfactants by nature are secondary metabolites developed from living or static cells of bacteria, fungi and yeast which have huge promising applications. Surfactants usually are compounds that confer the presence of both hydrophobic and hydrophilic moieties which possess the ability to accumulate in between the molecules of oil in combination with water as well as air and water and thus possess prodigious applications. Bio surfactants can be regarded to contain both moieties which means having hydrophobic and hydrophilic natures. They are amphiphilic in nature and are excavated from unicellular organisms, namely, bacteria, fungi and yeast. Because of the above-mentioned properties, these are also known to possess emulsifying activities (Ahimou et al. 2001). Chemical nature possess by Biosurfactants are glycolipids, lipopeptides, protein complexes (Polysaccharide in nature), Phospholipids and fatty acids.

Biosurfactants have several industrial applications, one of which well used is removal of petrochemical wastes. Biosurfactants have a market size of around 1.6 billion CGAR from 2020 to 2026. It is gauged that its demand will increase by 5.5% in the pursuit of removing petrochemicals, but it has been estimated that demands in the fields of food processing and application will emerge at a very faster pace (Fig. 4.1).

The intercontinental market of bio surfactants has evolved to nine million tonnes. Bio surfactants are extricated out from non-replenishing energy sources which possess immediate feedback mechanism towards oil sources.

The very common products which have been formed are sophorolipids, methyl ethyl sulphonates [MES], sorbitan esters and lipopeptides as well as rhamnolipids.

The industry supporting usage of biosurfactants possess variety of it because of majority of customers have emerged a knowledge that the products employed to their personal care or food supplements should be derived from organic sources and should completely lack synthetics.

As a matter of fact, the lavish quantity of bio surfactants requires a huge focus on down-streaming of products; the process of better endowment requires optimization of certain conditions which include flexible ranges of pH, temperature, speed of agitation and suitable duration provision of air; rates of dissolution are also implemented for better mixing and amalgamation. These criteria employ huge-scale machinery setup and manual handling, thus providing employment to several people.

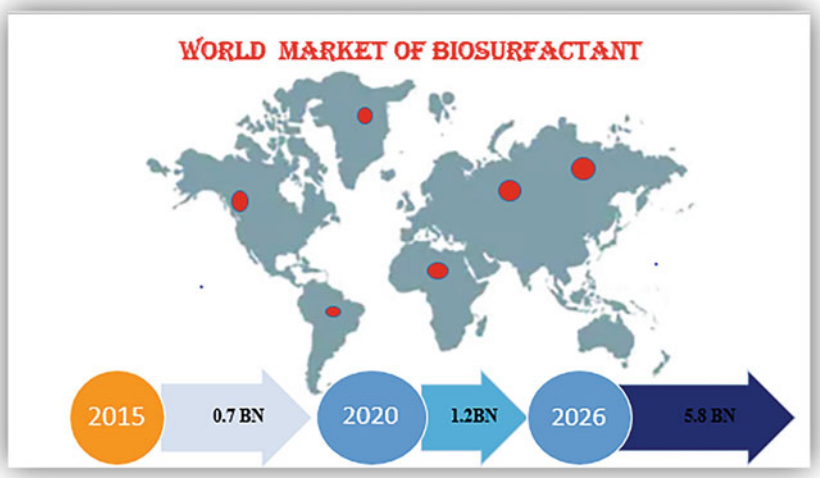

Fig. 4.1 Bio surfactant market in the span of 2020–2026

This chapter will deal with certain properties of bio surfactants which will demonstrate its helpfulness in the food industry and related co-operations.

4.2 Antibacterial, Antifungal and Antiviral Actions as Supreme Actions of Bio Surfactants

4.2.1 Antimicrobial/Antifungal and the Low Toxic Nature of Bio Surfactants

A range of antimicrobial activities of bio surfactants has been described in several literatures against bacteria, fungi and algae as well as viruses (Nitschke and Costa 2007). Most of the literature report that lipopeptides as well as glycolipids are the most widely studied bio surfactants which portray a huge spectrum of antimicrobial effects (Hayes et al. 2019). Sophorolipids, rhamnolipids and trehalolipids are the major forms of glycolipid-based bio surfactants; also, a category of mannosylerythritol lipids is included in lipopeptide bio surfactant (Adcox et al. 2005).

Poisoning of food and development of food-borne pathogens have been a major hitch in public health worldwide. In a detail provided by the WHO, for the people of about 500, 1 among each of 10 dies from eating contaminated food. Remediation of food-borne pathogenicity by utilization of bio surfactants as natural antimicrobial agents has become a revolution. Our environment comprises several pollutants which when take their way into the body cause several problems including the

toxicity caused by microorganisms, namely, the species of *Vibrio parahaemolyticus, Clostridium perfringens, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa* and so on.

Among bio surfactants, lipopeptides form the widely reported class having antimicrobial action. The genus *Bacillus* is responsible for producing the most well-known lipopeptide bio surfactants. *B. subtilis* produces the first bio surfactant that presents antimicrobial property, that is, surfactin (Cawoy et al. 2015). It also produces other lipopeptides with the same property: fengycin, iturin, bacillomycin and mycosubtilin (Cawoy et al. 2015).

There has been wide range of bio surfactants potentially existing to combat against several pathogenic eukaryotes as reported by Nitschke and Costa (2007). The lipopeptides which are usually derived from bacterial sources are known to carry antibacterial properties.

The antibacterial efficacy, antifungal potential and antiviral activity of bio surfactants make them eligible to be prospective treatment of several diseases; they are not only therapeutic, but they could also be incorporated into food products which somehow manages the prevention of any further intoxication (Cameotra et al. 2004). The lipopeptide category of surfactants contains hydrophobic aptitude related to hydrophilic stream which together becomes capable of exhibiting various biological activities which confers the actions on surfaces or internal mechanism of enzymatic activities (Roongsawang et al. 2011).

In a study carried out by Yuliani et al. (2018), they mentioned microorganisms, namely, *Staphylococcus aureus, Escherichia coli, Salmonella typhi and Listeria monocytogenes* as well as fungi *Candida albicans* are causdal of several food borne infections and found out that bio surfactants produced by gram-positive *Bacillus subtilis* C19 is hazardous for above mentioned microbes (Table 4.1).

4.2.2 Bio Surfactants as Antiviral Agent

The antiviral efficacy of the bio surfactant has been reported in the literature; as reported by Muthusamy et al. (2008), the number of incidence of HIV in women is increasing, and this major issue has led to the need to develop agents with higher efficacy that could act as a safe vaginal topical microbicide agent. The sophorolipid surfactants obtained form *C. bombicola* as well as the analogues of this compound for example the sophorolipid diacetate ethyl ester carry a potential spermicidal and veridical agent, and the reports have also shown that this substance carry the veridical activity similar to the potential of nonxynol-9 against the human semen. The surfactant and its analogues have also been reported to have a good antiviral activity (Naruse et al. 1990) against enveloped viruses than the non-enveloped viruses, and this fact suggests that the physiochemical interaction between the surfactants and the virus envelope protein might be the reason behind the inhibitory action (Vollenbroich et al. 1997). The studies suggest that the formation of ion

Table 4.1 Microorganisms derived from various sources having susceptibility against bio surfactants

Food material	Microorganism	Antimicrobial effect	Reference
Milk, milk-derived products	*E. coli, B. subtilis, P. aeruginosa*	Bio surfactant derived from *B. subtilis* C19	Yuliani et al. (2018)
Packaged food material	*S. cerevisiae, Cladosporium herbarum, Aspergillus niger, A. cinnamomeus*	Glycolipids and other bio surfactants usually derived from *Pseudomonas* species	Sobrinho et al. (2008)
Probiotics, health drinks	*Cladosporium, Aspergillus, Penicillium, Rhizopus, Lactobacillus paracasei*, all LAB microorganisms, *L. brevis, L. buchneri, L. plantarum, L. perolens* and yeast *Zygosaccharomyces bailii*	Bio surfactants derived from varied microorganisms, bio surfactants made from lactic acid bacteria	Schuller et al. (2000), Rodriguez et al. (2013)
Food sale by street vendors	*Shigella flexneri, Salmonella typhimurium, Vibrio cholerae*	Bio surfactant derived from *Bacillus cereus, Staphylococcus aureus*	Fakruddin et al. (2017)
Food served by family restaurants	*Salmonella* spp., *Shigella* spp., *Bacillus cereus*	*Sophorolipids, rhamnolipids*	Anibijuwon et al. (2012)
Raw materials Cereals pulses, wheat, rice	*Clostridium botulinum, E. coli, Clostridium perfringens, Staphylococcus aureus*	Sophorolipids, rhamnolipids, bio surfactants extracted from the materials of lactobacillus bacteria	Diaz et al. (2016)

channel that causes viral proteins to leak out and disturb the normal metabolism process of the virus could be the mechanism of antiviral potential of some of the lipopeptides (Seydlova and Svobodova 2008). Two major productions from *Bacillus subtilis* fmbj, that is, surfactin and fengycin, under in vitro studies have shown the potential activity to inactivate the cell-free virus stocks of different viruses like Newcastle disease virus and porcine parvovirus. Both of these compounds could also potentially resist the infections as well as replication mechanisms of these viruses (Huang et al. 2006). A study reported that the trehalose lipids, namely, TDM and trehalose dimycolate, are able to confer resistance against intranasal infection-causing influenza virus in mice models; they induced the proliferation of T-lymphocytes that carry the gamma/delta T-cell receptors (Franzetti et al. 2010). The complex of rhamnolipid with alginate has shown a good antiviral activity against type 1 and 2 herpes virus by inhibiting the cytopathic effects in the Madin-Darby bovine kidney cell line (Remichkova et al. 2008).

4.3 Physical Applications of Bio Surfactants

4.3.1 Bio Surfactants as Bioemulsifiers

There are numerous food contents which comprise a combination of water and oil; it is a matter of fact whenever liquids with two different densities get mixed with each other. In a solution of two different densities, the liquid of lighter density gets separated, and there is formation of air bubbles. To resist this formation of air bubbles, emulsifiers are added in the food product to bring stability to it. The food materials that usually contain emulsifiers are margarine, ice creams and usually milk and other products that constitute them. Surfactants as clear from the name are the agents that act on the surfaces of two different compounds (Hasenhuettl and Hartel 2008).

Bio polysaccharides are heavy molecular weight compounds consisting hetero- and lipo-polysaccharides as well as proteins and lipoprotein in nature. They are heavy in molecular containment than bio surfactants. Emulsion of oil and water is generally of two different categories; one among them comprises oil in water which forms bubbles in water, while the emulsion of oil in water creates suspension (Calvo et al. 2004). The application of adding emulsifier is that it is responsible in reducing surface tension between the molecules of different densities, thereby causing their miscibility (Haften 1979). In the sector of food industry, the addition of emulsifier is the major cause to deploy solubilization of fat, vitamin stabilization amino acid equalization.

There are a huge number of applications of an emulsifier in the industry of food and packaged food materials:

1. The first and foremost application of emulsifier in food industry requires the formation of pellucid solution. The pellucid or clear solution can only be observed if the two solutions of liquid phases get contacted to each other; the colours and their type require dissolution (Rosenberg et al. 1979).
2. Mitigation of tissue bonds in the case of starch to break them into simpler ones in order to obtain good entities of Pazzta, spaghetti and noodles (Sharma and Sharma 2018).
3. Starch response: Several emulsifiers possess an angulate category of fatty acid layer in their entity which amalgamates mixture of amylose. The attribute is very significant to delay or rupture the stalling as well as they are also responsible to reduce any sort of binding and adjoining between such products such as tomato puree and white sauce pasta (Lukondesh et al. 2003; Cirigliano et al. 1985).
4. Developing interconnectivity with proteins and peptides: The emulsifiers contain a specific interaction with protein molecules and thus are responsible to generate an interchangeable structure which provides flexibility and instructiveness amongst them (Ericson and Le 1975).
5. The creation and subtleness of foam are also very important in the food industry: In food products there are several served materials which are better with frothing; this is done by incorporating emulsifiers in the bottom of the material so that there is no hindrances left by the presence of saturated fatty acids. This type of action of emulsifier is required for the improvisation in the volume of the material (Haferburg et al. 1986).

6. Removal of oiliness from the surface of cookies and other related stuffs: Emulsifiers are added to the products having sugar crystals and oils which when coagulated together join together and give oiliness-free appearances; this makes the food look good and shows sincerity in servings (Mnif and Ghribi 2016).

It has been a well-known fact that chemical emulsifiers create problems like acidity indigestions or other stuff. Due to its toxicity and expensiveness in the market, researchers have stated that availability of any material will depend upon the mixing. According to several researchers, there are several microorganisms which produce byproducts that represent emulsifying properties (Torabizadeh et al. 1996). A varied number of bioemulsifiers have been processed, and they have been approved by several international health organizations including the World Health Organization, but several studies have been taken out to transfer. A varied number of metabolites extracted from the microorganisms have nutritional values as well. A huge number of biomolecules are employed in the production of materials used as supplementary in the food industry (Campus et al. 2015).

4.3.1.1 Extraction and Employment of Bio Surfactant-Derived Bioemulsifiers

With the help of pasteurization, yeast and fungi could be brought down to dormant stage, and the bioemulsifiers could be extracted out from them. Mannoproteins are common glycoproteins that are extracted out from fungi or yeast. It is a form of glycoprotein. Mannoprotein molecules are developed at pH 3–11 in which the yeast and fungi are suitably placed. Beta 1 and 3 glucanases are the enzymes which can be suitably employed; further, mannoproteins could be removed. When the protein segment gets subtracted from the main chain, the emulsifier group of mannoproteins expresses itself (Luna et al. 2015; Pinto et al. 2018). *Candida tropicalis* have been reported to produce mannan fatty acids (Siporin et al. 1975). *Candida lipolytica* have been known to produce sophorolipid (Shekhar et al. 2015); *Candida tropicalis* are known to produce liposan (Ruffino et al. 2008; Dilarri et al. 2016). *Penicillium chrysogenum* is known to develop polyketide derivative (Luna et al. 2015).

4.3.2 Bio Surfactants as Enzyme Inhibitors and Activators

Microorganisms and the extracellular material provided by them have been employed in fermentations and food processing from time immemorial (Tunga and Tunga 2003). Microbial enzymes contained in bio surfactants are more stable than enzymes of flora and fauna. Microbial enzyme alpha-amylases are responsible for varied actions that include clarification of fruit juices which means rupturing of any fibre present in the liquid (Sharma et al. 2014), formation of rice cakes (Sivaramakrishnan et al. 2006) and bread quality improvement (Gupta et al. 2003). Glucoamylase enzyme is used in the beer production (Mohan et al. 2014), and brewing is also one of its applications (Monfort et al. 1996). Beta galactosidase is

added in food materials to suppress the action of lactose intolerance (Gibson and Wang 1994). Although these enzymes are not directly considered as bio surfactants, attached entities with them show considerate effects and are responsible to perform these actions.

4.3.3 Employment of Bio Surfactants in Flotation or Froth Formation

In the last 80–90 years, several different structural contents of bio surfactants have been investigated. Several strains which are capable of developing bio surfactants have been investigated and studied in several researches. The property of bio surfactants depicts the low toxicity suppression in wastes (biodegradability) and their counterresponses towards chemical surfactants. The varied number of bio surfactant characteristics makes them suitable for further processes in industries where produced materials are of importance (Li et al. 2014).

4.3.4 Anti-Adhesive Agent

The materials' surfaces used for the food processing environment are pre-treated with bio surfactants. The pathogenic microorganisms that are responsible for food-borne illness outbreak easily cause the biofilm formation on the food contact surfaces, and for these kinds of pathogenic microbes, the sanitation process is ineffective, affecting the free-living cells (Kim et al. 2006; Stepanovic et al. 2004). The preconditioning of the surface with surface active agents like bio surfactants can be the most interesting and effective strategy for the prevention of food-borne pathogens that adhere to the solid surfaces. As demonstrated by Meylheu et al. (2006b), when the surface of stainless steel is preconditioned with the bio surfactant produced by the bacterium *Pseudomonas fluorescens*, it carried the reduction in the number of *Listeria monocytogenes* LO28 adhering cells; along with this it also promoted disinfectants like hydrogen peroxide and sodium hypochlorite for their bactericidal activities. They reported the similar findings with the bio surfactant obtained from *Lactobacillus* helveticus which inhibited the adhesion potential of four strains of *Listeria* on the stainless steel surface (Meylheu et al. 2006a). A study reports that the bio surfactant fraction obtained from the bacterium *Lactobacillus plantarum* shows a potential, more than 50%, anti-adhesive activity in the range of 4–25 mg/mL against food-borne pathogens like *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Yersinia enterocolitica* and completely inhibits their growth at the maximum concentration (Madhu and Prapulla 2014). Another work was reported to assess the anti-adhesive effects of the rhamnolipids and surfactant bio surfactants on stainless steel and polypropylene surface against food pathogens like *L. monocytogenes, S. enteritidis* and *E. sakazakii* (Nitschke et al. 2009). The findings from their work showed that the preconditioning with surfactin bio surfactant leads to the reduced number of the adhering cells and that mostly of

L. monocytogenes. The surfactin bio surfactant was capable of delaying the adhesion of the bacterial cells within a short period for the non-growing cells and a long period for the growing cells. Bio surfactants obtained from the bacterium *Lactobacillus paracasei* ssp. *Paracasei* A20 obtained from the Portuguese dairy plant carry a good antimicrobial property as well as a promising anti-adhesive potential against several pathogenic microbes (Gudina et al. 2010). The attributes depicted by the bio surfactants clearly suggest that these bioactive surface agents can be considered as an alternate or a new tool for the development of strategies that could help in the prevention and delaying of the microbiological colonization on the surface of industrial equipment that are used for the preparation and processing of food stuff.

4.3.5 Probiotics Bio Surfactants Activity

The mechanisms of action used by probiotics vary widely, but some of them include the production of certain agents that could impart antimicrobial activity like organic acids, hydrogen peroxides, bacteriocin and diacetyl (Merck et al. 2005). Probiotics are also known to create interference with the adherence of cells for the biofilm formations by pathogenic microbes on the epithelial cells lining the urogenital and intestinal tracts (Reid et al. 2001). This interference is caused by the release of some surface active molecules like bio surfactants (Gudina et al. 2010). As reported by Hong et al. (2005), the antimicrobial lipopeptides produced by the bacterium *Bacillus* probiotics carry an antimicrobial potential, and this product inhibits pathogenic microorganisms that are present in the gastrointestinal tract from growing. The *Lactobacillus* probiotics are very well known for interfering with vaginal pathogens by producing bio surfactants or by competing with them for adherence (Barrons and Tassone 2008). Investigations have reported that the nosocomial pathogens can be antagonized by the bio surfactants from the probiotic-type microorganisms (Walencka et al. 2008). Silicone rubber adsorbed with the bio surfactant layer obtained from the probiotic bacterium *Lactococcus lactis* potentially inhibits the adhesion of pathogenic bacteria and yeast. Similar authors also demonstrated that the probiotic bio surfactants also reduce the microbial load on the voice prostheses (Rodrigues et al. 2004). Several discoveries overall conclude that both the probiotics and their bio surfactants are potentially capable of antagonizing the growth and development of the major pathogenic microbes like *Streptococcus spp. S. aureus, E. faecalis* and *C. albicans* (Van Hoogmoed et al. 2000).

4.4 Limitations of Bio Surfactants

One of the major limitations for the use of bio surfactants is the high cost of their production which can be somewhat decreased by the use of agro-industrial waste as the raw material for its production on large scale. The major restriction of bio surfactant use in the food industry could be because of safety reasons as these compounds are derived from microbes and some of the bio surfactants are produced

by opportunistic bacteria, and hence there is a need to look for non-pathogenic source of bio surfactants like yeast and probiotic bacteria, or the strategies could be implemented to modify them structurally to reduce the toxicity strength. As the microbial diversity is considerably vast, there is a great opportunity for the exploration of new surfactants especially from non-pathogenic microorganisms and also from extremophiles. The emerging resistance to bio surfactants by food-borne pathogenic microorganisms is also an unexplored field that needs to be considered prominently to devise them as antimicrobial agents in food and feed industries.

4.5 Conclusion and Future Prospects

The diverse functional properties of bio surfactants have made them to be one of the most important compounds to be considered in the current era for various strategies and applications. These compounds of microorganism origin carry an excellent detergency property and emulsifying, foaming and dispersive traits and are therefore considered as versatile chemical process. The different properties of bio surfactants can be used in food industries some of which are being highly exploited for the betterment of food processing strategies. The recent anti-adhesive potential of these compounds has driven the attention towards and are now used as alternate tools for the inhibition and disruption of biofilm formation in the food and food processing surfaces made by major pathogenic food spoilage microorganisms like *Listeria monocytogenes*. These compounds are also good bioemulsifiers, stabilize the emulsion formation and carry potential antimicrobial attribute and hence inhibit the colonization of pathogenic and spoilage-causing bacteria and fungi. These combined attributes of bio surfactants have made them as potential and effective multipurpose food additive. As their different biological and functional potentials are known, they are being addressed as the active component for food formulations; hence, the efforts could be made to discover some of their more effective attributes to broaden and diversify their application in these industries. These natural compounds are tolerant to common processing methods and can also effectively compete with synthetic surfactants for their ability to reduce interfacial tensions and surface and due to their low toxicity and biodegradability. The studies have shown that the bio surfactant-producing microbes like some yeast impart no toxicity or pathogenicity for the consumer, and this makes them as the ideal agent for food formulations. The research approaches made to determine the potential and biological properties of these compounds have depicted that along with its attributes suitable for the food industry, these compounds also carry valuable biological properties including antioxidant, anti-tumour, anti-inflammatory and antimicrobial, and these attributes enable them to be considered in the products for the avoidance of contaminations both during and after processing. All the characteristic features and attributes of bio surfactants allow them to be used as additives and versatile ingredients for the processing of food products.

Bio surfactants are one of the potential metabolites of microbial origin to have an extended spectrum of functional and biological properties and applications in various industries including food manufacturing and processing industries. The accurate use of these compounds can be implemented in the food and feed industries only when the exact knowledge of their toxicity is known. The ongoing potential applications of these compounds in the food processing industries encourage researches to consider and determine their better efficacy, toxicity, stability and more diversification. Even though the current knowledge on the application of bio surfactants and their biological potential is vivid, its application and usage in the food industry are limited, and hence research needs to focus on extending this for the betterment of food processing strategies.

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Probiotics: Promising Opportunity for Future Functional Foods

5

Mahima Verma and Pramod Kumar Rout

Abstract

The global market value of probiotics is increasing over the years. Probiotics are widely used by human beings and animals to enhance immunity and reduce stress levels. The strain characteristics, safety evaluation and the key health benefits linked to probiotics are discussed. The present paper also describes the various steps for the development of probiotic-based foods and their nutritional content. The major mechanisms of action for probiotic stimulating beneficial effects on human health are described. The increasing knowledge of the human microbiome and its functions are providing an opportunity to develop next-generation probiotics. Probiotic uses in daily food consumption have a great potential in personalised nutrition for various chronic disorders in human being and livestock. The microbial population of the gut in different environments modulates both physiological and psychological disorders. Therefore, it is necessary to evaluate the efficacy of probiotics in different physiological conditions. The issue of maintaining the safety and efficacy of probiotics is a major concern, and there is a need to harmonize guidelines for the production, marketing and use of probiotics globally.

Keywords

Probiotic characteristics · Microbiome · Epigenetics · Next-generation probiotics · Safety evaluation

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5.1 Introduction

The mammalian gastrointestinal tract is in a symbiotic relationship with a large number of microbes over millions of years. The diet has a major role in modelling the gut microbiota composition and determining the association between the gut microbiome and animal health (Shanahan et al. 2017). The gut microbiota has enormous potential to modulate immune mechanism for better human health. The microbial composition and their functions and interactions contribute significantly to improve human metabolism and also to regulate the balance between health and disease. The diversity in microbiome has been found to be associated with various metabolic pathways, thereby improving intestinal permeability and enhancing immunomodulation. Gut microbiota diversity is also the vital factor that affects the responsiveness in different nutrition status. Probiotics are potential functional foods and provide health benefits and well-being beyond the basic nutrition. Probiotics are gaining popularity due to continuously increasing clinical trial database expanding the scientific substantiation towards their beneficial health effects. Probiotics impart several health benefits such as improving immunity, maintenance of intestinal homeostasis, changes in bile salt conjugation, digestive and respiratory health, alleviating infectious disease symptoms or allergies, preventing colon cancer, prevention of diarrhoea or constipation, alleviating chronic gastrointestinal inflammatory disorders and anti-inflammatory activity. Moreover, probiotics also contribute to the synthesis of vitamins and bioactives and improve the bioavailability of nutrients. The foods and beverages containing probiotics are future foods with wider acceptability among consumers (Shi et al. 2016). The global market value of probiotics by 2024 is expected to reach about 94.48 billion USD (Fortune Business Insights 2020). The expanding novel information is setting grounds for the rationalisation of next-generation probiotics and standardisation of integrated dietary strategies for modulating the human gut microbiome. This will further lead to innovative formulations consisting of non-conventional indigenous bacteria from the gut to employ effective personalised nutrition strategies. Therefore, the present paper analyses the probiotic use, mechanism of action, the food safety evaluation, product development and the nutritional content.

5.2 Probiotics

5.2.1 Probiotic Definition and Classification

The concept of probiotics originated in the twentieth century, when Russian scientist Elie Metchnikoff proposed that fermented milk and milk products contribute to a healthy life. In 1908, Metchnikoff was awarded Nobel Prize in Medicine for representing that helpful microbes can replace destructive microbes to recover from intestinal illnesses. Pliny the Elder, a Roman naturalist, suggested the consumption of fermented milk as remedy to intestinal problems. Henry Tissler from

Pasteur Institute reported that the presence of bifidobacteria in infant gut reduced incidences of diarrhoea.

The term 'probiotic' is a combination of two Greek words 'pro' and 'bios' meaning 'for life'. The term probiotic was first used by Lilly and Stillwell in 1965 (Lilly and Stillwell 1965) as an anonym to antibiotic stating, 'substances secreted by one micro-organism which stimulates the growth of another'. Parker (1974) used the term probiotics for the first time in the sense that it is used today as 'organisms and substances which contribute to intestinal microbial balance'. Fuller (1989) revised it as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. Thereafter, it was referred to as viable, non-pathogenic micro-organisms (bacteria or yeast) that, when ingested, are able to reach the intestines in sufficient numbers to confer health benefits to the host (Schrezenmeir and de Vrese 2001). The FAO and WHO in 2002 revised it as 'Probiotics are live micro-organisms that when administered in adequate amounts confer a health benefit to the host' (FAO/WHO 2002). The ISAPP (International Scientific Association for Probiotics and Prebiotics) maintained the definition by the FAO/WHO in 2013. The WHO considered using probiotics as the next significant immune defense system when antibiotics become ineffective due to antimicrobial resistance; this is called as 'microbial interference therapy' (Fukao et al. 2009; Botes et al. 2008). In the past few years, more than 500 probiotic-based food items are introduced in the global market, and this number continues to grow. The probiotic foods produced by the fermentation of fruits and vegetables, cereals and meat products provide a variety for the different strata of consumers. The probiotics-rich food produced from different sources are described in Table 5.1. Probiotics are widely used in livestock, dog and poultry production for maintaining better health. The use of probiotics in animal nutrition is broadly accepted. Probiotics are increasingly used in commercial animal feed for poultry and cattle to modify gut flora. Probiotics are used in animals to increase immunity, reduce stress levels and improve overall growth. Probiotics are also used for enhancing the quality of eggs and meat and reducing the load of *Salmonella*. Probiotics are also gaining popularity among pet owners for maintaining the health of their animals, thus opening more opportunities for manufacturers.

5.2.1.1 Classification

The probiotic micro-organisms are categorised based on the following criteria:

1. Bacterial and non-bacterial probiotics: *Lactobacillus* and *Bifidobacterium* form the major genera of bacterial probiotics, and certain yeast and fungal probiotics are also used such as *Saccharomyces*.
2. Non-sporulating and sporulating probiotics: Non-spore-forming probiotics are *Lactobacillus* and *Bifidobacterium*, and *Bacillus subtilis* and *Bacillus amyloliquefaciens* are spore-forming probiotics.
3. Multi-strain and single-strain probiotics: Probiotic formulation for a particular health benefit may contain a single strain or may be additive effect of multiple strains, each in a specific dosage.

Table 5.1 Probiotic sources

Probiotics source
1. Dairy sources
Yogurt
Kefir
Buttermilk
Cream
Aged cheeses
2. Nondairy beverages
Kombucha
3. Vegetable and fruit
Sauerkraut
Kimchi
Sauerruben
Pickles
Chutney
Brined olives
4. Soy sources
Tempeh
Miso
Natto
Tamari
Soy sauce
5. Grain sources
Traditional sourdough bread
6. Meat source
Ham
Salami
Loin
Sausages

4. Allochthonous and autochthonous probiotics: Allochthonous probiotics refer to species of micro-organisms normally not present in the gastrointestinal tract (e.g. yeasts), whereas autochthonous probiotics are the indigenous strains of gut microflora (e.g. *Lactobacillus* and *Bifidobacterium*).

5.2.2 Probiotic Strains

Lactobacillus and *Bifidobacterium* are the two commonly available genera in the market. The most popularly used probiotic micro-organisms are *Bifidobacteria*, *Bacillus coagulans*, *Lactobacillus acidophilus group*, enterococci especially *Enterococcus faecium* SF68, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Escherichia coli* strain Nissle 1917, certain strains of *Lactobacillus casei* and yeasts *Saccharomyces cerevisiae* and *S. boulardii*. The genus *Bacillus* dominates the

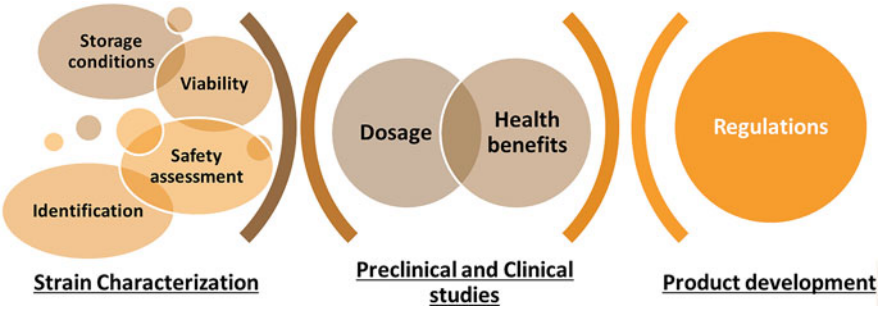

Fig. 5.1 Steps involved in product development using probiotics

bacterial spore-forming probiotics. These probiotics are ingested either as single strain or as combination of multiple strains. The development of novel probiotic genera and strains is being achieved through focused research efforts. There are two main forms in which probiotic organisms can be ingested, as live microbial cultures in fermented foods and as supplements (European Food Information Centre (EUFIC) 2003). Fermented foods can be pre-packaged foods or traditional local preparations of dairy and vegetable origin. Most common being yogurt, kefir, sauerkraut and pickles (Table 5.1). Probiotic supplements are used in the form of powder, tablet or capsule consisting of certain dose of freeze-dried (lyophilised) bacteria.

Probiotics function optimally only when the food carries the necessary minimum viable count at the time of consumption. This is referred to as dosage. Clinical trials are carried out at 10^6–10^{11} CFU (colony-forming unit). The food industry commonly utilises 10^9 CFU, with 10^6 CFU as the least recommended level depending on the strain.

Figure 5.1 describes the various steps for the development of probiotic-based foods. Developing effective probiotic supplements and foods largely depends on selecting the right strain and optimising the culture conditions. The strains of interest must possess certain qualities such as being culturable in industrial conditions and maintain stability and viability during the shelf life of the product. The microorganisms should be safe for human consumption and meet the regulatory criteria set by the regional or local authorities. It is required to follow scientific, ethical and regulatory guidelines and incorporate it into a product (a dietary supplement or functional food) to bring it to the consumer (Fig. 5.2).

5.2.3 Technological and Safety Profile

The probiotic strain must possess technological and safety profile. Viability of the strain is required for optimal function of product. The viability of probiotic microbes in food products is influenced by several factors during processing, manufacturing and storage. The viability of strains is affected by several factors such as the presence

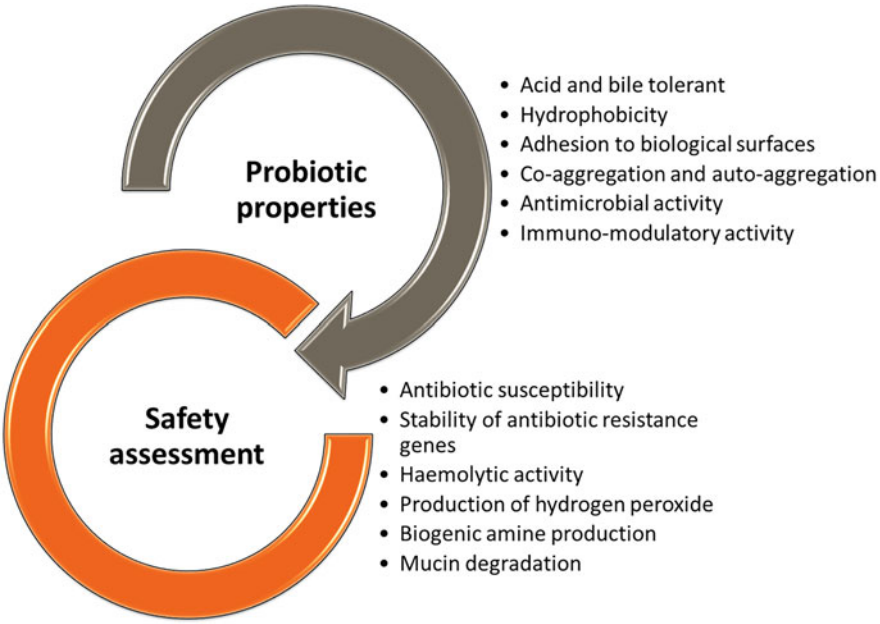

Fig. 5.2 Technological and safety assessment requirements for a probiotic microorganism

of certain chemicals, food parameters, processing factors and microbiological parameters. These factors include:

Microbiological factors: Inoculum load and probiotic strain
Processing factors: Incubation temperature, cooling rate, heat treatment, storage methods and packaging mode and materials
Chemical factors: Bacteriocins, food colouring agents, hydrogen peroxide and added artificial flavours
Food parameters: Water activity, molecular oxygen, titratable acidity and sugar and salt content

The characteristics of a probiotic strain are:

- Resistant to gastric acidity
- Adaptable to various digestive tract environments
- Able to maintain good viability
- Resistant to bile acid and has bile salt hydrolase activity
- Non-pathogenic and non-toxic
- Decreases pathogen adhesion to intestinal mucosa and efficient in attaching to intestinal epithelia and colonise
- Bacteriocin and acid production for antimicrobial activity against bacterial pathogens

- Better utilisation of nutrients and substrates in normal diet
- Stability in chosen characteristics during storage, processing and transportation
- Anti-inflammatory, immune-stimulatory and anti-mutagenic

In order to obtain the desired health outcomes from a probiotic, it is imperative to use the exact strain for which the benefit is established through research. It is necessary to use specific probiotic strain for reproducible clinical and health outcome. It has been observed that a different strain of the same species or close related strain may not produce the desired benefit and cannot be assumed to act in a similar manner. Similarly the inoculum load has to be optimised for processing and storage so as to maintain the adequate viable count at the time of consumption. The production process itself exposes the probiotic strain to different stresses affecting their viability and functionality (Fiocco et al. 2020). Fermentation temperature is the primary factor responsible for viability and qualitative properties of probiotics. Generally, the favourable growth temperature for most of the probiotic species ranges 37–43 °C (Lee and Salminen 2009). Other factors such as selection of culture medium ingredients, cryoprotectants and lyoprotectants have direct impact on the growth and survival of micro-organisms. The future of food industry, i.e. personalised nutrition, considers nutritional interventions tailored to individual needs to deliver optimum health and wellness. This is based on several factors such as intrinsic (microbiome, metabolism, phenotype, genotype), environmental (social context) and lifestyle (dietary habits and exercise). Personalised nutrition includes interventions which seek to improve stratification of diet by utilising the biological information and biomarkers. Science-led personalised nutrition is gradually being established, particularly in areas like gut health. With a huge potential for future foods, probiotics stand as a powerful candidate for personalised nutrition. In this transit from 'one size fits all' to 'personalised nutrition', our horizons are expanding in multiple dimensions seeking novel opportunities, but safety is inevitable. Food safety is a global health priority as unsafe food creates a vicious cycle of illness and affects nutritive value of food. A very significant feature associated with probiotics is to prove them safe for consumption and capable of encumbering with microbial infection as they are ingested as live micro-organisms (Fig. 5.2). Moreover, probiotics will play an effective role in fighting malnutrition by utilising traditional knowledge and will fulfil the SDG goal.

5.3 Safety Evaluation of Probiotics

Normally a probiotic should possess characteristics such as acid and bile tolerance, resistance to gastric juices, effective adhesion to the gut lining, genetically stable, lactic acid producer, anti-genotoxic property, short generation time, antimicrobial and bile salt hydrolase activity. Briefly, assessment of a probiotic includes:

1. Identification of strain by molecular techniques for DNA sequencing and submission to international repository

2. Strain characterisation/functional analysis by in vitro and animal test
3. Safety evaluation by in vitro studies and animal and human trials
4. Efficacy and dosage standardisation by randomised controlled human trial
5. Labelling and giving information to the consumers enabling them to make a decision and also claims communication

Probiotics are ingested as live micro-organisms; thus, they should not pose any risk to the host. Their safety has to be assured via various scientific studies before approval for use in food or as a supplement. The first step to evaluation is the identification of micro-organism, carried out by 16S rRNA sequencing, and the strain is submitted to an international microbial repository. Identification of the strain is the first indication, but to assure the safety, properties like lack of transferable antibiotic resistance genes or any undesirable genetic properties need to be established.

The next step is the characterisation of the identified strains at genomic and biochemical levels and their safety assessment based on antibiotic resistance, virulome factors, pathogenicity and toxicity. This comprises analysis of any virulence or toxic properties as invasion and translocation in epithelial cells, haemolysis, platelet aggregation, mucin degradation and production of gelatinase, enterotoxins and cytotoxins. The safety assessment of probiotics is carried out for genetic stability over time: anti-mutagenic, harmful metabolic activities, potential pathogenicity or toxicogenicity, presence of any undesirable transferable resistance genes and clinical studies proving no adverse effects of the strain.

The working group of the FAO/WHO has recommended safety assessment for probiotics in food application. The safety assessment covers all the environmental and health aspects such as assessing antimicrobial resistance factors and their transferability, evaluation of various undesirable metabolites, different adverse effects during clinical trials, epidemiological assessment of adverse effects after marketing and evaluation of toxicity, haemolytic activity and toxins. The probiotics used in food and supplements should possess the status of GRAS (generally recognised as safe). The antimicrobial resistance and horizontal transmission of antibiotic resistance genes in microbes are global health safety concerns. Therefore, antibiotic resistance genes or transfer of such genes should be absent in a probiotic micro-organism. Lactobacilli show high incidences of intrinsic resistance to vancomycin (Hamilton-miller and Shah 1998; Gueimonde et al. 2013) and transmissible antibiotic resistances (tetracycline and macrolides). These genes could be located at chromosome, transposon or plasmid. Another major technological and safety concern is mutagenicity. In order to reduce genetic drift, it is important that the number of generations remains as low as possible during probiotic production (Sanders et al. 2014). Confirmation and characterisation of a probiotic is followed by in vivo studies to demonstrate the efficacy and determine the dosage. The in vitro screening and potential in vivo tests and controlled human clinical trials establish the (1) safety, (2) efficacy, (3) effectiveness and (4) surveillance of probiotic claimed foods and beverages.

5.4 Effect of Probiotics on Human Health

Probiotics are popular for their associated health benefits such as improving digestion, reducing allergic effects, maintaining body pH balance, improving blood circulation, enhancing nutrient absorption and assimilation of food, restoring gut microbiota, stimulating immune system and preventing colon cancer (Gilliland 1990). The health benefits of probiotics are presented in Fig. 5.3. There is a lack of adequate scientific investigation to evaluate all the health claims. Limited data and few placebo-controlled studies uphold the scepticism about their use.

Most of the commercialised 'classical' probiotics are often isolated from biological samples and fermented foods due to their upright safety status and their technological advantages. The preclinical evidences have proposed applications for the prevention and treatment of various illnesses and disorders, such as infectious diarrhoea, irritable bowel syndrome, antibiotic-associated diarrhoea, chronic inflammatory bowel diseases (IBS), necrotising enterocolitis, allergies and obesity and its comorbidities. Probiotics produce lactic acid which lowers the intestinal pH, decreases toxic or carcinogenic metabolite production and inhibits bacterial pathogens such as *E. coli*, *Salmonella*, *Clostridium*, *Shigella*, etc. Furthermore, they synthesise nutrients, vitamins (B and K) and the enzyme β-D-galactosidase and enhance the bioavailability of copper, magnesium, calcium, manganese and other minerals. The key health benefits linked to probiotics are:

1. Maintain microbial homeostasis in gut
2. Prevent diarrhoea

Fig. 5.3 Health benefits associated with probiotics

3. Regulate mental health conditions
4. Help in reducing cardiovascular disorder conditions
5. Reduce the severity of certain allergies and eczema
6. Reduce symptoms of digestive disorders
7. Boost the immune system
8. Regulation of body condition

Probiotics restore the natural balance of gut microbiota for overall well-being by eliminating undesired microbes. This prevents certain infections and allergies. Candida is a commonly occurring problem due to the overgrowth of naturally present yeast, *Candida albicans*, in the human digestive tract and causes health problems. In healthy humans, probiotics keep a check on candida proliferation.

Probiotics can prevent and reduce the severity of diarrhoea and related gastric tract symptoms. These microbes help in conditions like peptic ulcers, acute diarrhoea, mild ulcerative colitis, antibiotic-associated diarrhoea (AAD), traveller's diarrhoea, irritable bowel syndrome (IBS), necrotising enterocolitis, vomiting, regurgitation by reducing inflammation and preventing recurrences of inflammatory bowel disease. The strain used and the viable count in the dosage together determine the efficacy of probiotic.

As they contribute in the improvement of the digestive system, the cholesterol level and blood pressure are regulated (Jackson et al. 2002; Hill et al. 2009; Ebel et al. 2014). Probiotics lower cholesterol by breaking down bile salts and preventing reabsorption. Probiotics also help in lowering high blood pressure by producing ACE inhibitory peptides during fermentation. Thereby, probiotics maintain heart health, serum cholesterol level and modestly lowers blood pressure.

Another health benefit of probiotics is that they are capable of reducing, controlling or even treating allergies. Few probiotics reduce the prevalence of eczema and other allergies and also reduce its severity in case of occurrence. Probiotics prevent food allergy by alleviating intestinal inflammation (anti-inflammatory properties) and promoting barrier mechanisms. Probiotics improve milk allergies, alleviate lactose intolerance (De Vrese et al. 2001) and lower the incidence of atopic eczema in infants (Isolauri et al. 2000; Kalliomaki et al. 2001; Wang and Wang 2015). Probiotics reduce serum IgE levels and Th2 cytokines.

Probiotics boost IgA-producing plasma cells, natural killer cells and T lymphocytes and protect against infections. Probiotics stimulate the immune system by inducing mucosal immune response and systematic immune response (Perdigon et al. 2001; Herich and Levkut 2002; Fuller 2000; Isolauri et al. 2001). They boost the number of IgA-producing cells, enhance the phagocytotic activity and also increase the number of T lymphocytes and natural killer cells (Reid et al. 2003).

Probiotics are also known to help in reducing dental caries (Nase et al. 2001). Some probiotics help in losing weight by preventing the intestinal absorption of dietary fat, burning more calories and storing less fat. However, few strains support in gaining weight.

Probiotics are also linked to improve mental health, mood, cognition, depression, anxiety, stress and memory, autism and obsessive-compulsive disorder (OCD). Gut

bacteria directly stimulate afferent neurons via the vagus nerve and improve NREM (non-rapid eye movement) sleep (Galland 2014). Probiotics are also therapeutically important in controlling disorders such as alcoholism, chronic fatigue syndrome, fibromyalgia and restless legs syndrome. Lactic acid bacteria (LAB) can produce neurotransmitters such as acetylcholine and gamma amino butyrate (GABA) and respond to hormones (Cryan and Dinan 2012). Probiotics provide vitamins and trace elements to the host by fermenting the digestive fibres in the large intestine (LeBlanc et al. 2013).

Probiotics show anti-mutagenic properties, detoxify carcinogenic substances and prevent colon cancer (Nase et al. 2001). Micro-organisms such as *Lactobacillus bulgaricus* help in the prevention of colon cancer by preventing the breakdown of β-glucuronidase that contributes to the growth of cancer-causing agents. The main focus of studies on the anti-carcinogenic effects of probiotics is on colorectal cancer and few reports on breast and bladder cancers as well (Boyd and McGuire 1990). Human trials reveal the association of lactobacilli (*L. acidophilus*) and bifidobacteria with low risk of colon cancer (Horie et al. 1999; Moore and Moore 1995). Probiotics are able to interfere with carcinogen-producing micro-organisms (Guarner and Malagelada 2003). It is also reported that consuming fermented milk products such as yogurt lowers the risk of breast cancer in women (Le et al. 1986; Van't Veer et al. 1989) and also reduces the prevalence of both gastric ulceration and bladder cancer (Burns and Rowland 2000).

The availability of data in clinical trials remains limited, and only a few are placebo-controlled; thus, the results are invariant, and this gap needs to be bridged with generation of statistics and identification of gut biomarkers. The use of probiotics, because of their health benefits, lays on the borderline of pharma and food industry. However, the food industry is able to utilise them for their technological properties and reach masses with health claims. In food industry, probiotics are implemented widely for fermentation to make yogurt, cheese, cultured butter, sour cream, sausages, pickles, sauerkraut etc. They contribute to the taste and aroma and impart texture of finished food product. Few strains producing exopolysaccharides (EPS) are employed in the manufacture of fermented milk to improve its viscosity and texture and those producing mannitol claimed to have several health benefits (Wood and Holzapfel 1995; Wisselink et al. 2002). Probiotics change the pH of food, which inhibits the growth of spoilage agents, thus contributing to enhanced shelf life of food. The antimicrobial activity of lactic acid bacteria attributes to metabolite production such as diacetyl, hydrogen peroxide, organic acids like lactic and acetic acid, ethanol, reuterin, acetoin, acetaldehyde, reutericyclin, carbon dioxide and bacteriocins. They are categorised as biopreservatives as they increase the shelf life of food by inhibiting food spoilage bacteria. The antioxidant activity of probiotics in fermented dairy products improves the nutritional value of these products, and the enzymes produced regulate gut-serotonin production.

5.5 Action Mechanism of Probiotics

The mechanisms of action by which probiotics articulate beneficial health effects need to be explored. Probiotics inhibit pathogenic growth by synthesising antimicrobial compounds, modifying gut pH, competing with pathogenic bacteria for binding to the epithelial layer and stimulating immunomodulatory cells (Amara and Shibl 2015). The major mechanisms of action for probiotic stimulating beneficial effects on human health are described:

5.5.1 Epithelial Barrier Function

The maintenance of intestinal barrier function is done by a series of related mechanisms such as secretion of mucus, chloride and water and apical junction joining the epithelial cells by tight junction proteins (Ng et al. 2009). Mucus secreted by goblet cells forms the integral part of the gut barrier defence system. Polymerisation of mucin forms the structural basis of mucus, which contributes to protection from pathogens, toxins, enzymes, abrasion and dehydration. *Lactobacillus rhamnosus* GG and *L. plantarum* 299v upregulate the production of mucins (MUC 2 and MUC 3) in the intestines to challenge the adherence of an enteropathogen such as *Escherichia coli* O157:H7 to the intestinal epithelia (Hardy et al. 2013). Further, *Streptococcus thermophilus* and *Lactobacillus acidophilus* reverse the secretion of chloride induced by *E. coli* (Brown 2011).

5.5.2 Competitive Exclusion of Pathogens

Probiotics create a hostile microenvironment for reducing the pathogenic bacteria by lowering gut pH to inhibit survival of pathogens and also by producing organic acids such as lactic acid and acetic acid (Brown 2011; Bermudez-Brito et al. 2012; Goudarzi et al. 2014). The alternative mechanisms by which the probiotics work are by blocking the available bacterial receptor sites (Goudarzi et al. 2014); by secreting antimicrobial substances such as bacteriocins; by competing for essential nutrients and energy sources (Brown 2011); and by releasing gut protective bioactive metabolites like short-chain fatty acids (SCFAs), glutamine, arginine, hydrogen peroxide, conjugated linoleic acids and diacetyl (Bermudez-Brito et al. 2012; Hemaiswarya et al. 2013).

5.5.3 Adherence to Intestinal Lining

Probiotics improve the host immune system by adhesion and colonisation in the gut. They work through stimulation of systemic and mucosal host immunity (Hemaiswarya et al. 2013). The mucosal immunity is imparted due to the release of cytokines and chemokines after the probiotic adheres to the gut lining.

5.5.4 Antimicrobial Peptide Production

Probiotics also work through the production of antimicrobial peptides such as bacteriocins, plantaricin, lactacin and reuterin. These inhibitory peptides possess broad-spectrum activity against pathogens such as bacteria, protozoa, fungi and viruses. Bacteriocins are antimicrobial peptides with potential applications in food preservation. Bacteriocins from LAB are largely utilised as biopreservatives, signifying potential alternative antimicrobial strategy in the scenario of antibiotic resistance issue (Suskovic et al. 2010). Bacteriocins are categorised into four distinct classes: (1) lantibiotics, membrane-active peptides which are small in size (<5 kDa); (2) heat-stable, small (<10 kDa), non-lanthionine peptides; (3) heat-labile, large (>10 kDa) proteins; and (4) complex (Saulnier et al. 2009). Commercial bacteriocins such as plantaricin from *L. plantarum*, nisin from *L. lactis* and lactacin B from *L. acidophilus* are narrow-spectrum bacteriocins, with activity against closely related bacteria. Reuterin produced from *Lactobacillus reuteri* are broad-spectrum bacteriocins, able to inhibit a range of pathogenic micro-organisms, and are expressed differentially by various *L. reuteri* strains (Saulnier et al. 2009).

5.5.5 Immune-Modulation

Probiotics exhibit immunomodulatory properties and stimulate immune response by increasing the phagocytic activity of macrophages, enhancing natural killer cell activity, stimulating IgA production and modulating cytokine production. *L. acidophilus* La1 and *Bifidobacterium lactis* Bb12 are found to increase the phagocytosis substantially (Delcenserie et al. 2008). Increased IgA secretion may decrease the numbers of pathogenic micro-organisms in the gut, thus restoring the microflora (Hawrelak 2013; Fuller and Gibson 1997). Due to these immunomodulating properties of probiotics, they are advocated to inhibit intestinal and urogenital pathogens and improve conditions, such as IBS, food allergies and pouchitis, and are also used as an adjuvant to vaccination (Schultz and Sartor 2000; Miele et al. 2009).

5.5.6 Interference with Quorum Sensing Signalling Molecules

The phenomenon of communication of bacteria with each other and their surroundings is called quorum sensing. This is driven through chemical signalling of molecules known as auto-inducers. Quorum sensing can control community-level gene expression in response to altering cell numbers. Probiotic strains of bacteria such as *Lactobacillus*, *Bifidobacterium* and *B. cereus* control virulence gene expression in pathogens via degrading the auto-inducers of pathogens by production of enzymes or antagonists. Probiotics *L. acidophilus*, *B. cereus* and *B. toyoi* prevent bacterial toxicity by using this mechanism (Brown 2011; Goudarzi et al. 2014).

5.6 Next-Generation Probiotic Development

The increasing knowledge of the human microbiome and functionality are expanding the opportunities of probiotic applications. Improved culture methods and advanced sequencing technology have allowed discovering the new range of micro-organisms from microbiota. These may be assessed for potential health benefits, expanding the health areas and, thus, providing an opportunity to develop next-generation probiotics (O'Toole et al. 2017). A number of bacteria, namely, *Eubacterium* spp., *Roseburia intestinalis*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* and *Bacteroides* spp., are isolated from the human gut for their probable probiotic potential (O'Toole et al. 2017; Brodmann et al. 2017). These candidates offer physiological functions as production of bioactives which are not conferred by conventional probiotics (Blaak et al. 2020). Commercialisation of these probiotics imposes multiple challenges. The preliminary challenge is optimisation of rich complex growth media. This demands huge investments. The other aspects concern the optimum growth conditions in industrial facilities for bulk production and optimising the processing, storage and transit conditions.

The locations for discovering new strains and species and the targets for probiotic intervention include the oral cavity, skin, female urogenital tract and nasopharyngeal tract (Maguire and Maguire 2017; George et al. 2016; Cribby et al. 2008). *A. muciniphila*, isolated in 2004, is a promising candidate (Derrien et al. 2004). It prevents obesity as shown in preclinical animal model tests and is proved safe for human use in initial proof-of-concept studies (Depommier et al. 2019). The new species such as *Staphylococcus hominis* (skin commensal isolate) reduces atopic dermatitis and eczema (Nakatsuji et al. 2017), and *L. crispatus* restores vaginal dysbiosis (Reid 2012). The major source of probiotics in the gut is most likely to be the fermented foods (Pasolli et al. 2020); these have immense potential for future food development. The commonly exploited probiotic strains are of generally recognised as safe (GRAS) status in the USA, or they have qualified presumption of safety (QPS) status with the European Food Safety Authority (EFSA). However novel prebiotic species cannot be assigned as safe due to no history of use. A comprehensive characterisation of these newly identified strains is obligatory. This should comprise of full genome sequence, virulence factors, toxin genes, retrospective association analysis of human disease linked with taxa, transferrable genetic elements, antibiotic resistance, proven safety in animal models, pharmacodynamics, pharmacokinetics and phase I–III trials.

A probiotic must maintain viability during processing and shelf life to give the desired health benefits. Considerable challenges are faced related to stability, deliverables, excipients, prebiotics, viability, processing and depreciation of the sensory acceptability of foods through the production of off-flavours. It is required to make research efforts for further developments in these areas and overcoming various challenges. Microencapsulation is an alternative for probiotics to provide stability and maintain viability and controlled release in the intestine and prevent proliferation in food. This includes the development of cost-effective polymer

microcapsules, symbiotic microcapsules and heat-labile microcapsules and determining their release mechanism and controlling factors in the gut.

5.7 Eubiosis and Dysbiosis

The aggregate of all microbiota that reside on and within human tissues and biofluids is known as human microbiome. As 70–80% of immune cells are located in the gut, the development of immune system depends on a balanced and diverse gut microbiota since early life for a well-established gut microbiota associated with improved health in later life. This reduces risk of allergies and persistence of allergic diseases. It corresponds to all anatomical sites including the skin, placenta, mammary glands, uterus, ovarian follicles, seminal fluid, oral mucosa, saliva, lungs, conjunctiva, biliary tract and gastrointestinal tract. These include all bacteria, viruses, archaea, fungi and protists. The Human Microbiome Project sequenced the genome of the human microbiota, identifying microbes from focus areas such as the skin, nose, mouth, digestive tract and vagina. There are three different types of bacteria: beneficial, neutral and harmful. The majority of bacteria in a healthy digestive tract is the beneficial or 'good' bacteria (also known as probiotics), along with neutral bacteria.

The state of homeostasis of gut microbiota, maintaining a healthy and balanced ecosystem, is described as eubiosis. Dysbiosis is characterised by reduced diversity of gut microbiota leading to challenged immunity causing allergies, disorders and diseases. It has been observed that dysregulation of the microbiome predisposes the host to conditions from chronic inflammation and obesity to metabolic syndrome and irritable bowel syndrome (IBS) and weakens the immune system. Dysbiosis is caused by aging, stress, hormonal imbalance, poor lifestyle, antibiotic treatment, excessive use of medications and poor quality of sleep. This may be reversed by simple changes in diet and lifestyle, avoiding stress, keeping the body hydrated, exercises, proper sleep, avoiding unnecessary medications and intake of probiotic-rich food. The consumption of probiotics alters the gut microbiome composition and provides protection against pathogens by forming barrier in the intestinal tract.

5.7.1 Epigenetics of Gut Microbiota

Stress results in inflammation, affecting different body parts and resulting in increased gut-barrier permeability. This perturbs the intestinal microbiome homeostasis and often leads to the condition of dysbiosis, which exaggerates pathogenic stimulation and is often accompanied by anxiety and depression. The gut microbiome is largely affected by a decrease in gut-barrier function. The human microbiome is comprised of 100 trillion microbes, residing in body parts interacting with the external environment; this prompts response to environmental stimuli causing epigenetic modification. The dysbiosis condition develops due to chronic stress, and the microbes may undergo mutation as adaptation (Household et al.

2017). These newly formed genes are transferred to the next generation within 20 minutes (Powell 1956) and trigger a signal to the body where inflammatory micro-organisms rapidly displace beneficial microbes (Reid et al. 2011). On the contrary, during positive mental condition, the microbiota improves barrier functions and activates anti-inflammatory and antiproliferative mechanism of host through a multitude of epigenetic effects (Househam et al. 2017). Gut microbiota produce HDAC inhibitor such as short-chain fatty acids, butyrate, lactate, pyruvate and propionate. Butyrate is the most effective and promotes gene expression by acetylation and other mechanisms. HDAC inhibitors hyperacetylate histones by exerting beneficial effects on health. Butyrate inhibits HDAC classes I and IIa which are responsible for suppressing proliferating regulatory T cell (Treg). This mechanism regulates T cell functions by Treg proliferation (Furusawa et al. 2013).

Similarly, it has been shown that meditation practice can improve gastrointestinal tract function and reduce the symptoms of functional gastrointestinal disorders (Aucoin et al. 2014; Schoultz et al. 2013, 2015, 2016). The change in microbial composition of the gut modulates both the psychological and physiological symptoms of GI disorders (Cryan and Dinan 2012). Beneficial microbes promote homeostasis and maintain a robust immune system (Kamada et al. 2013). It is necessary to evaluate the effects of meditation and yogic practices on microbiome and in improving physiological and psychological symptoms of various chronic disorders.

5.8 Prebiotics, Synbiotics and Postbiotics

Prebiotics are employed as natural feed additives in human food as well as in animal nutrition. The prebiotic concept was introduced in 1995. Prebiotics stimulate the growth activity of some micro-organisms in the colon due to its non-digestible fibrous food ingredients (De Vrese and Schrezenmeir 2008). A range of fermentable carbohydrates such as several dietary fibre types, phenolics, phytochemicals, human milk oligosaccharides (HMOs), long-chain fructooligosaccharides (lcFOS), short-chain galactooligosaccharides (scGOS), conjugated linoleic acid and polyunsaturated fatty acids (PFA) transformed into corresponding conjugated fatty acids acts as effective prebiotics. Prebiotic is usually resistant to acids, salts and hydrolysing enzymes in the GI tract and remains unabsorbed in the upper gastrointestinal tract and fermented by intestinal microbiota (Kuo 2013). The sources of prebiotics can be breast milk, unrefined wheat, soybeans, raw oats, yacon, inulin-rich food, unrefined barley, non-digestible carbohydrates and in particular non-digestible oligosaccharides. Generally prebiotics such as bifidogenic and non-digestible oligosaccharides (inulin) satisfy all the requirements for prebiotic classification (Pokusaeva et al. 2011). Prebiotics also exhibit several health gains like reducing inflammation, prevalence and time duration of diarrhoea and other IBS-associated GI symptoms and protect against colon cancer (Peña 2007). Prebiotics enhance the mineral uptake by increasing bioavailability, reduce the risk of cardiovascular disease and prevent obesity (Pokusaeva et al. 2011).

Gibson and Roberfroid introduced the term 'synbiotic' in 1995, which is 'a mixture of probiotics and prebiotics'. Synbiotics function by enhancing the viability and intestinal adhesion of live microbial dietary supplements. They stimulate selective growth and activate selective metabolism to promote specific strains of health beneficial bacteria (Gibson and Roberfroid 1995). Synbiotics have both probiotic and prebiotic properties and provide synergy between them and were formulated to accelerate the survival of probiotics in the gastrointestinal tract (Cencic and Chingwaru 2010; Rioux et al. 2005). Synbiotics are generally used as they impact the survival of probiotic bacteria in the upper intestinal tract.

Probiotics supply energy and nutrients, maintain the intestinal equilibrium and provide a protective barrier for the alimentary tract (Blay et al. 1999; Gibson 2003). Therefore, a single product should contain a combination of both components for better efficacy as compared to the activity of the probiotic or prebiotic alone (Bengmark 2005; Konstantinov et al. 2013).

Food fermentation and its health benefits and market demand gave rise to the postbiotics concept. Postbiotics are bioactive compounds, which are produced in a matrix in fermentation and impart beneficial health effects. Postbiotics are microbial fragments and metabolites (Konstantinov et al. 2013) and are capable to prompt many mechanisms of their live probiotic counterparts. Postbiotics can include many different constituents including short-chain fatty acids (SCFAs), teichoic acid, functional proteins, microbial cell fractions, metabolites, peptidoglycan-derived muropeptides, extracellular polysaccharides (EPS), pili-type structures and cell lysates. Postbiotics are commonly differentiated into two types: paraprobiotics and FIFs (fermented infant formulas). Paraprobiotics, or ghost probiotics, are defined as 'non-viable or inactivated microbial cells, which, when administered in sufficient amounts confer benefits to the host' (Aguilar-Toalá et al. 2018). Postbiotics provide the opportunity to utilise the functional active ingredients produced from a potential micro-organism. Postbiotics are safer and elegant method and eliminate probiotic challenges of storage and maintaining viability in shelf life. The postbiotics concept opens new horizons of applied microbiology in food and personalised nutrition.

The composition of the gut microbiota is associated with its functional and metabolic phenotype. Hence, the microbial metabolism of prebiotics varies from individual to individual (Panesar et al. 2009). This further links to the differences in the degree of health effects of pre-, pro-, post- and synbiotics among individuals (Umu et al. 2017). However, most of the health effects of prebiotics, probiotics, synbiotics or postbiotics depend eventually on the production of short-chai fatty acids (SCFAs) and other components like functional proteins, extracellular polysaccharides (EPS), peptidoglycan-derived muropeptides, teichoic acid, microbial fractions, secreted polysaccharides, cell lysates and pili-type structures (Collado et al. 2009; Sánchez et al. 2017; Wegh et al. 2017; O'Grady et al. 2019; Slavin 2013; Markowiak and Śliżewska 2017).

5.9 Conclusion

The symbiotic relationship of microbiome with the host has been known for centuries. The gut microbiota is considered as an organ because of its role in metabolism, and its genes impact on various host metabolic pathways. Gut microbiome plays a significant role in maintaining human and livestock health and imparting immunity to prevent diseases. The progression in knowledge of gut microbial ecology and mode of action of probiotics has resulted in rising numbers of probiotic strains used in human and animal nutrition. Probiotic use in daily food consumption can become a lifestyle choice for marinating better health. Similarly, probiotics have a great potential in personalised nutrition for various chronic diseases. The microbial population of the gut in different environments modulates both physiological and psychological disorders. Therefore, there is a need to analyse the effects of meditation on microbiome modulation to manage various chronic disorders. The efficacy of probiotics is highly variable. More studies are required to address the concerns about efficacy and safety of probiotics. Therefore, relevant research on probiotics designed to identify the associated risk and capacity building of regulatory experts are various aspects towards one health approach. Therefore, international harmonisation of guidelines for use of probiotics is essential. This approach will prevent any unsuitable micro-organisms being used as probiotics and achieve health and nutrition goals. Moreover the probiotics will play an effective role in fighting hunger and malnutrition by utilising traditional knowledge and will fulfil the SDG goal.

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Enhancement of Probiotics for Functional Food

6

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Abstract

Probiotics have acquired an unavoidable role in human life which upon consumption in sufficient quantity confer numerous therapeutic benefits. Probiotics evidently affect the immune modulation and aid in the prevention of different types of cancers, including colon, breast, gall bladder, etc. Lactic acid bacteria (LAB) are recognised probiotics because they offer a vast range of benefits mainly in host nutrition and health. The most prominent probiotic strains of LAB include members of the genus *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, and *Enterococcus*. Microencapsulation provides complete protection and an incredible tendency for shielded probiotics to overcome all of the challenges of the harsh gastric environment. Well developed and established practices of microencapsulation involve emulsion, spray-drying, and extrusion. Cell encapsulation is emerging as a viable alternative for incorporating probiotics into a range of food matrices. The international market for nutritional and probiotic foods are rising exponentially. Bacteriocins, immunomodulatory compounds, antimicrobial compounds, aromatic compounds, enzymes, and short-chain fatty acids are all produced by probiotic strains with different beneficial effects in the food industry to human health.

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Keywords

Functional food · Encapsulation of probiotics · Benefits of probiotics · Bacteriocins · Gut microbiota · Lactic acid bacteria

6.1 Introduction

6.1.1 Probiotics

Probiotics have been recorded for a series of remedial and curative properties such as to improve the defence mechanism of the immune system, reduce the serum cholesterol, and prevent colon cancer and urinary tract and gastrointestinal infections and in the treatment of atherosclerosis, atopic dermatitis, rheumatoid arthritis, and more (Diez-Gutiérrez et al. 2020). Due to the advancement of probiotics in therapeutics, acceptance of probiotics is expanding (Chin-Lee et al. 2014), and it is estimated that the industry growth will reach 7% annually (Jackson et al. 2019). Lactic acid-producing bacteria (LAB) are the most common genera to be used. Mainly lactobacillus, bifidobacterium, streptococcus, and enterococcus come under these genera. Traditional fermented dairy products including lassi, Maasai milk, curd, kefir, kurut, or butter milk are the common hubs of these bacteria (Ezzatpanah 2020). Curd which is a fermented food, being used for diarrhoea in India that was recommended by the Ayurvedic traditional system of Indian medicine much before the acceptance of the microorganisms existence and being used and still using live microorganism and fermented food since Vedic and pre-Vedic era in for health benefits in India (Singhi and Baranwal 2008). As the circle of knowledge about the human microbiome and its function expands, the range of new discoveries of potential probiotic taxa is also increasing (Veiga et al. 2020). The isolation and characterisation of new potential range of probiotic taxa with full genome sequencing in affordable range and powerful cultivation methods gave rise to an opportunity for the development of next-generation probiotics with many possible health benefits (O'Toole et al. 2017). Approximately 10^8–10^9 CFU (colony forming unit) of daily dose is recommended to be an active probiotic during passage through the gastrointestinal tract (Attri et al. 2021; Dong et al. 2013; Hou et al. 2003; Liu et al. 2019).

The term "probiotics" is a Greek word which means "for life" (Jobby et al. 2020). The definition of probiotics according to the World Health Organization (WHO) is "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Mack 2005). However, Lilly and Stillwell first defined probiotics in 1965 as "substances secreted by one microorganism which stimulates the growth of another microorganism" (Lilly and Stillwell 1965; Setta et al. 2020). Sperti coined the word probiotics to describe tissue extracts that stimulated microbial growth (Mihich 1972). Later it was coined to explain viable microbes (Parker 1974).

6.1.2 Functional Foods

The term "functional food" was first coined by Japan in the early or mid-1980s. They referred functional food as processed foods that, in addition to being rich in nutrients, comprise ingredients that prevent diseases and aid specific body function though there is no universal definition (Arihara 2014; Swinbanks and O'Brien 1993).

6.2 Four Generation of Probiotics and Maintaining the Viability of the Probiotic Strains

The viability of the bacteria is the major issue in all the production stages.

6.2.1 First-Generation Probiotics

The viability of the bacterial probiotics was extremely low, just in between 7 and 30% due to usage of live form and/or lyophilised bacterial cell without any protection or encapsulated in microcapsule. The acidic and alkaline environment in the gastrointestinal tract negatively influenced the bacterial survival rate as no cover was used on the bacterial cells (de Vos et al. 2010; Klayraung et al. 2009). Some bacteria get adapted to the environment of the gastrointestinal tract, for example, *Bifidobacterium bifidum* showed 30% survival rate higher than *Lactobacillus acidophilus* with 10% of survival rate (Bezkorovainy 2001). Another major issue was the shelf life of the probiotic strains, for example, *Bifidobacterium longum* viability was preserved only for two weeks in fermented dairy products (Takahashi et al. 2004).

6.2.2 Second-Generation Probiotics

The viability and survival rate increase up to 80% in the second generation of probiotic development. The lyophilised probiotics were encapsulated with polymeric capsules or tablets with synthetic, semi-synthetic, or natural filters (Klayraung et al. 2009). In an experimental model, the shelf life increased to 6 months of the probiotic strain, and the survival rate increased to 90% after incorporating sodium alginate as tablet filter (Klayraung et al. 2009). The rapid one-time and premature release of probiotic strains in the proximal gastrointestinal tract, before reaching the colon, was the major issue with second-generation probiotics. Consequently, beneficial bacterial metabolites like bacteriocins were degraded too quickly, reducing the effectiveness of probiotics (Attri et al. 2021; Salas-Jara et al. 2016).

6.2.3 Third-Generation Probiotics

Further improvement of probiotics solved the issue of one-time release by destroying the microcapsule gradually to release the probiotic strain in metabolically active state (Burgain et al. 2011; de Vos et al. 2010; Salas-Jara et al. 2016). Different forms like an external coating framework, a cross-linking structure, and matrix structures can be used to construct the microcapsule. Polysaccharides and proteins form the matrix structure, which protects the bacteria from the effects of the environment factors. Polysaccharides provide a physical barrier, while proteins provide a chemical barrier to shield microorganisms from the stomach's acidic state (Liu et al. 2019). Alginate (alginate shows low acid resistance) or carrageenan microcapsules coated with chitosan, whey protein, or poly-1-lysine polymer, which is robust in the stomach's acidic environment, form the exterior coating structure. However, chitosan's use is restricted due to its inhibitory effect on some probiotic strains (Groboillot et al. 1993).

6.2.4 Fourth-Generation Probiotics

The probiotic strains remain encapsulated, but they are now existing in biofilm. Fourth-generation probiotics lead to improved viability and shelf life without any need for special modifications, as well as the release of a substantial amount of CFU in the colon (Attri et al. 2021; Salas-Jara et al. 2016).

6.3 Biofilm Creation and Quorum Sensing (QS) of Probiotics

In two forms a bacterial cell can survive: first is in single cell form which is planktonic form and another in biofilm form. Biofilms are irreversible binding of bacterial strains to the living substrate or to each other. Consequently, change in phenotype occurs. The process of formation of biofilm goes through several stages (Donlan and Costerton 2002; Stoodley et al. 2002). Biofilm is an ordered accumulation of microorganisms residing inside an extracellular polymeric matrix that they create and that is irreversibly bound to the fetish or living surface and cannot be removed unless rinsing is done quickly (Costerton et al. 1994; Hurlow et al. 2015). The attachment stage of a biofilm to the surface results in the formation of extracellular polymeric substances (EPS). The formation of an exopolysaccharide matrix, which provides strength to the interaction of the microorganisms in the biofilm, determines whether a microbial biofilm can form an inanimate or solid surface (Branda et al. 2005; Costerton et al. 1995; Miron et al. 2001). Various bacterial strains show social behaviours and communication among the neighbouring bacterial strains through quorum sensing (QS) mechanism in the course of biofilm formation (Naves et al. 2010).

Quorum sensing (QS) is a bacterial cell-to-cell communication system that uses hormonelike small organic compounds called auto-inducers (AIs) to coordinate gene expression and detect cellular density. Based on population density, bacteria use

these signalling molecules to control the expression of virulence factors, biofilm growth, the production of secondary metabolites, and communications with the host and other microbes. Signalling molecules attach to new bacterial receptors during the quorum sensing process, resulting in the gene transcription within a single bacterial species as well as between different bacterial species, allowing intraspecies and interspecies contact (Barzegari et al. 2020). Biofilm formation is a useful characteristic developed by the strains of probiotic strains including *Lactobacillus* as it provides the long-term viability and persistence in the host mucosa. Moreover, biofilm-forming probiotics strains can prevent pathogenic bacterial biofilm formation by the synthesis of exopolysaccharide (Salas-Jara et al. 2016). Probiotics within the biofilms are more resistant to the adverse effects in the gastrointestinal tract including antibiotics, antimicrobial agents, and other environmental factors inside the GI (Algburi et al. 2017; Gandhi and Chikindas 2007; Pepoyan et al. 2020; Tabak et al. 2007; Zhang and Mah 2008).

In a study on *Lactobacillus rhamnosus GG (LGG)* strain, it was found that the strain was more stable in encapsulated form in a biofilm at low and high temperatures than a planktonic bacterial strain in the same capsule, but when exposed to hydrogen chloride, there was no difference in the survivability of strain (Cheow and Hadinoto 2013). But some bacterial strains showed improved survivability in some studies in the acidic and alkaline environment of the stomach and proximal small intestine respectively when present in the form of biofilm as opposed to the planktonic form. Those bacterial strains include *L. acidophilus* CCM 4833, *Bifidobacterium longum* CCM 4990, *Bifidobacterium breve* CCM 3763, *L. reuteri* HFI-LD5, *L. rhamnosus* HFI-K, *L. fermentum*, and *L. Plantarum* (Aoudia et al. 2016; Grossova et al. 2017; Klopper et al. 2019). *B. subtilis* NCIB3610 can protect other probiotic strains, especially *L. plantarum*, from the human GI tract and the adverse external environment by forming bacterial biofilms (Yahav et al. 2018).

6.4 Mechanism of Action of Probiotics in the Gut

The probiotic exhibits several mechanisms by influencing the metabolic activity, immune system, functioning, and composition of the microbes in the gut. Microbiome modulation in the gut environment has emerged as a promising strategy for improving host health or as part of a new therapeutic approach. By producing bioactive compounds and regulating the host metabolic activity and immune system, this technique shields the host body from a wide range of infections and diseases. By activating various pathways, these bioactive molecules control the host's health.

Mechanism 1 Probiotic strains enter the gut environment after oral administration, where they initiate the synthesis of quorum sensing molecules. These molecules form the biofilm, a lipopolysaccharide-based protective layer that serves as a growth substrate for beneficial bacteria colonisation while preventing pathogenic bacteria colonisation (Dobson et al. 2012; Mukherjee and Ramesh 2015).

Mechanism 2 The probiotic strains activate naive T cells by activating dendritic cells which activates B cells. The activation of B cells results in the production and releasing of secretory IgA. The IgA now stimulates humoral response and helps in destroying pathogenic bacteria. Immune modulation followed by cytokine production occurs when naive T cells differentiate into mature T cells (Derrien and van Hylckama Vlieg 2015).

Mechanism 3 The bacteria produce bacteriocins and antimicrobial peptides to eliminate the pathogenic bacteria; besides, in the intestinal epithelium, secretory IgA, mucus, and bacteriocin are the primary defence mechanism to protect the host from the invading pathogens (Corthésy et al. 2007; Derrien and van Hylckama Vlieg 2015).

Mechanism 4 Low-pH environment in the gut promotes the probiotic strain survivability and better growth. The strains produce organic acids as well as short-chain fatty acids that help them to lower the pH inside the gut. The organic acids produced by the bacteria are lactic acid and acetic acid, and short-chain fatty acids are butyrate, acetate, and propionate. Another advantage of lowering the pH is this type of environment is unfavourable for pathogenic bacteria (Schepper et al. 2017).

Mechanism 5 Biochemical reaction helps to regulate the metabolism of the host by producing nutrients and growth factors such as precursor of enzymes and vitamins mainly B complex, especially vitamin B12 which humans do not produce, and vitamin K that aids in the growth and establishing the beneficial bacteria inside the gut environment. Then the short-chain fatty acids regulate G protein-coupled receptors. The production of interleukins get triggered after propionate and butyrate which are short-chain fatty acids that bind with their specific receptors. Propionate binds with G protein-coupled receptor 43 (GPR43 receptor) to release IL-10 that aids in the control of inflammatory responses. Butyrate in order to promote β-oxidation and oxygen consumption binds with peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and produces IL-10 (Kota et al. 2018; Vitetta et al. 2015). Oxygen consumption reduces the level of oxygen inside the lumen of the gut that results in an anaerobic condition which is unfavourable to the pathogenic bacteria (Cani 2018).

6.5 Strategies for the Enhancement of Probiotics

6.5.1 Selection of Better Probiotics

Selection of probiotics is a crucial and one of the most important steps towards providing a safe and quality probiotic to the end users. To select a potential probiotic, many rough criteria should be observed before its commercial use. Selection of new probiotic strains is a major challenge to constitute a research on those new species like *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, or *Faecalibacterium*. the

criteria is to select a potential strain change and evolve in a better and quality path. It is better and also recommended to isolate probiotic strain from targeted animals only; for instance, the probiotics to be used for humans should be isolated from breast milk or the human gastrointestinal tract. Some criteria to look at are as follows: How do probiotics tolerate the stress inside the intestinal tract? How adhesive is the strain in the intestine? Does the strain release any antimicrobial compounds? What are the immunomodulatory functions of probiotics associated with the host? What technology should be used to increase the viability of the organism? (Attri et al. 2021; O'Brien et al. 1999; Ouwehand et al. 1999; Sanders et al. 2014; Tripathi and Giri 2014).

6.5.1.1 Stress Tolerability of the Strain

In the digestive tract, several digestive enzymes such as lysozyme, amylase, pepsin, and chymotrypsinogen affect the viability of the strain. A strain should be resistant to or should have tolerance ability against acid and bile. Internal body temperature can also affect the viability of a strain and can alter the metabolic activity, so a probiotic should be mild heat shock tolerant. Further studies about the tolerance of the strain say that a bacterial strain should tolerate 0.3–2.0% of bile concentration and pH range of 2–5 (Ogunremi et al. 2015; Psani and Kotzekidou 2006).

6.5.1.2 Adhesive Property of Probiotic Strain to the Human Intestine

The adhesion capability is one of the most important criteria to be a potential probiotic. It ensures that the bacteria are inside the GI tract and establish a better relationship with the epithelial cell of the host with that proliferating and increasing the density and biomass without being washed away (Attri et al. 2021).

6.5.1.3 Antimicrobial Property of a Probiotic Strain

A probiotic strain should have the antimicrobial ability to show competitiveness and fight and survive against a strong pathogen inside the intestine. Prebiotics secrete several compounds such as lacticines, bacteriocin Abp-118, alyteserin-1a, bacteriocin sakacin A, lactic acid, and antibodies to prevent the adhesion of a potential pathogen to the epithelial cell that makes a strain suitable to this criteria. Another way to kill a pathogen is to increase the activity of phagocytosis by the leucocyte, increase the trans-epithelial resistance, phosphorylate the protein of tight junctions, and enhance the cytoskeletal activity (Mathipa and Thantsha 2017).

6.5.1.4 Immunomodulatory Response of Probiotics

Probiotics should release immunomodulatory metabolites that can enhance the immune cell functioning and also stimulate the maturation. These probiotic strains also stimulate and enhance the release of immunoglobulins and the production of cytokines, IgA- and IgM-secreting cells (Lammers et al. 2003; Rocha-Ramírez et al. 2017).

6.5.1.5 Functional Criteria

Probiotic strains provide health benefits to the host as an anti-carcinogenic agent; aid in reducing obesity and cholesterol level; act as an antioxidant in diabetes, infant allergies, lactate digestion, and inflammatory bowel diseases; and should act as anti-anxiety agent and antidepressant. Certain enzymes like nitrate reductase, β-glucosidase, and β-glucuronidase are responsible for pre-carcinogenic activation and can be inactivated or suppressed by the probiotic strains; additionally, the production of linoleic acid and inducing activity for apoptosis should also be checked for possible anti-carcinogenic activity (dos Reis et al. 2017; Ewaschuk et al. 2006; Kumar et al. 2013). The probiotics can be studied on animal in vivo to report the efficacy on the reversibility of anxiety and depression and can also be analysed in the inhibition of mast cell activation and lipopolysaccharide breakdown ability for anti-diabetic and anti-obesity activity (Alokail et al. 2013; Niers et al. 2005). In an artificial gastric digestion study, it was shown that 6–8 log CFU/g of probiotic strain was lost and the residual viable probiotic strain count was not enough to exert health benefits (Brinques and Ayub 2011; Hansen et al. 2002; Sabikhi et al. 2010).

An excellent and potential probiotic strain candidate, *L. plantarum* KCC-24, lactic acid bacteria was isolated and characterised from *Lolium multiflorum* (Italian ryegrass) that was resistant to gastric juice and 0.3% w/v bile salt and also showed resistance to hydrogen peroxide. *L. plantarum* KCC-24 survived at low pH and exhibited several important activities like antifungal activity and proteolytic activity against a wide range of strains, susceptibility to several antibiotics, and antioxidant potential (Vijayakumar et al. 2015).

Eleven strains of *Lactobacillus* were studied and examined by researchers in Culture Collection of Dairy Microorganism (CCDM) based on several probiotic properties including the survival or viability of the strain in the gastrointestinal fluid, the antimicrobial activity of the strain, and competitiveness for adhesion on Caco-2 cell with non-toxigenic *Escherichia coli* O157:H7. It was reported that the growth of at least 16 pathogenic strains was inhibited by 3 best-performing strains: the first strain was *Lactobacillus gasseri CCDM 215*, the second strain was *Lactobacillus acidophilus CCDM 149*, and the third strain was *Lactobacillus helveticus CCDM 82* with significant antimicrobial activity. The adhesion of *Escherichia coli* O157:H7 was reported to be strain-dependent (Turková et al. 2013).

6.5.2 Adaptation of Probiotic Strain on Food Matrix and Human Micro-Environment

Probiotic strains have traditionally been chosen based on stress-resistance phenotypes that ensure their sustainability through the gastrointestinal tract within each food matrix. Bacterial cells are subjected to environmental stress during the development, storage, and use of LAB. LAB are exposed to a variety of stressful circumstances especially during fermentation processes, such as low temperature, low pH, and low water flow (Muruzović et al. 2018). Since physical stress is the

most common approach for inducing cell inactivation and improving food sustainability in microorganisms, microorganisms have developed physiological and genetic mechanisms to withstand certain harsh environments in order to live. When it comes to bacteria or spoilage species, this is obviously of major importance to the food industry (Beales 2004; de Melo Pereira et al. 2018). Probiotics have a series of molecular mechanisms in order to respond to environmental stress experienced during manufacturing, absorption, and passage through the gastrointestinal tract. As a result, explicating the mechanisms involved could lead to the development of probiotics with higher and improved viability. One of the methods for improving probiotic viability is stress adaptation. This is accomplished by pretreating or pre-culturing bacterial cells in a sublethal stress environment before exposing them to a harsher or lethal environment (Upadrasta et al. 2013). When compared to the bacterial strain that are directly placed into the very same lethal stress environment, this method helps bacterial strains to evolve adaptive stress responses, resulting in a higher rate of survival (Saarela et al. 2004). For the development of high-tolerance probiotic strains, adaptive approaches to various forms of stress, such as heat, cold, acid, osmotic, oxygen, bile salts, high pressure, and nutrient shortage, have been used (Pénicaud et al. 2018; Sauer et al. 2017). During yoghurt development and storage, acid and osmotic stress, caused by lactic acid processing and the addition of food additives, are the most common stress factors. Recent advancements in post-genomics techniques, such as transcriptomics and proteomics, have given new insights into how probiotics mitigate environmental stresses (Bron et al. 2019; Mohammadi et al. 2012). LAB can be subjected to osmotic stress in their numerous applications in the food and feed sector when large amounts of sugar or salt are applied to the substance. As a result, in order to sustain, they must adapt to such a shift in their climate. One of the possible mechanistic behaviours is the aggregation of compatible solutes (uptake or synthesis) during hyper-osmotic circumstances and their release (or degradation) during hypo-osmotic circumstances. Enzymes can sometimes be stabilised by compatible solutes, which shield them against stress conditions mainly osmotic as well as extreme temperatures, defrost, and drying manufacturing procedures (Conrad et al. 2000; Gouesbet et al. 2001; Panoff et al. 2000). Similarly, based on the concentration of anthocyanins in the food matrix, polyphenolic compounds found in food, such as blackcurrant juice, can inhibit as well as promote lactobacilli formation (Parkar et al. 2014). Probiotics' adaptive stress responses are also linked to changes in physiological characteristics of the strain and structural cell components (Van de Guchte et al. 2002).

Several studies have found that pre-adaptation may help probiotics survive in a food system. Even so, adaptive responses are thought to be extremely strain-dependent and differ greatly depending on the type of stress as well as other experimental conditions (Capozzi et al. 2016; (Alonso García et al. 2019). Osmotic stress is also linked to probiotic survival in food matrices as a stressor. Flow cytometric experiments on lactobacilli subjected to different levels of sugar concentrations, for instance, revealed that osmotic stress reduced probiotic viability (Sunny-Roberts et al. 2007). A study used flow cytometry to analyse probiotic cell

survival in the presence of different NaCl concentrations (0–5%) and to investigate the metabolic status of the cells. When cells were exposed to NaCl concentrations, the severity of cell damage was discovered through double staining and metabolism analyses. They also focused on three probiotic strains' salinity sensitivity and discovered that *Lactobacillus casei* was more salt-resistant than *Lactobacillus acidophilus* and *Bifidobacterium longum* (Gandhi and Shah 2015). Another challenge that the strains can face throughout the production is oxygen stress. In milk and other liquid foods, oxygen dissolves easily, but oxygen permeability through the package can compromise probiotic viability (Shah 2000). According to some research, gradually increasing dissolved oxygen concentrations improved viability in *L. acidophilus* and *Bifidobacterium* sp. cultures (Ahn et al. 2001; Talwalkar and Kailasapathy 2003).

Food pathogens were the first to be studied for acid stress adaptation because it has a direct impact on their survival in acid conditions. While undesirable for pathogenic species, such a mechanism may be successful in modifying probiotic viability in acidic matrices and during their passage through the gastrointestinal tract. Several researchers have examined at lactobacilli possible stress response mechanisms in addition to enhancing their ability to survive and work in industrial applications (Chen et al. 2017a, b; Pérez Montoro et al. 2018).

The impact of heat shock and initiation of stress response was investigated in *Lactobacillus* sp. Additionally to enhance the thermal tolerance of *L. rhamnosus* GG cells, it was introduced to pressure pretreatment at 60 °C. Salt tolerance for 30 min at 0.3 M Nacl of *L. paracasei* NFBC 338 improved feasibility during spray-drying in an another report (Ananta and Knorr 2004; Desmond et al. 2002; Colette Desmond et al. 2001). The use of a non-lethal heat shock helps bacteria to survived a second at quite intense heat stress (Teixeira et al. 1994).

6.5.3 Selection of Better Food Packaging System for Longer Viability

Probiotics sustainability can be affected by the physical properties of packaging materials and packaging techniques. A number of dairy probiotics and other items are kept and packaged in high-oxygen-permeability plastic containers. Since bifidobacteria are anaerobic bacteria with a higher susceptibility to oxygen, processing and packaging with a higher oxygen permeability can compromise their feasibility during storage. Numerous factors, like temperature, moisture content, and the crystalline nature of the film matrix, may influence the permeability of packaging content, impacting probiotic viability (da Cruz Adriano et al. 2007; Korbekandi et al. 2011; Miller et al. 2002).

According to Miller et al., packing yoghurt in a container with materials that have stronger oxygen barrier properties and an oxygen-scavenging agent offers the most suitable environmental conditions for probiotic preservation (Miller et al. 2003).

It was observed that *L. acidophilus* lived best in bottles made of glass than that in the container made of plastic, and it has been suggested that thicker wrapping

substances be included in yoghurt to improve *Lactobacillus acidophilus* strain and bifidobacteria survival. With the addition of glucose oxidase, the survival of probiotics strains in yoghurts packaged in multiple containers made of plastic of different oxygen permeabilities was examined by (Cruz et al. 2013; Shah 2000).

Lower oxygen permeability rates, as predicted, resulted in better probiotic viability, despite higher organic acid production and post-acidification. As a result, probiotic cultures thrive in containers with low oxygen permeability. However, the use of glass containers, which have a low oxygen permeability, raises the cost of probiotic packaging materials. Better packaging solutions, such as the use of oxygen scavengers or absorbents and the use of vacuum packaging or packaging of food with oxygen barrier material, may offer a better cost-effective solution (Tripathi and Giri 2014).

6.5.4 Adding Probiotic Promoters

Different compounds such as sugar, vitamins, minerals, and prebiotics may be added to serve as growth promoters to the probiotic product; these promoters can act as protectants against several processing conditions of, for instance, skim milk powder, lactose, whey protein, and glycerol. In addition, the application of prebiotics as a protective agent to probiotic microorganism is gaining popularity (Gibson et al. 2017). Casein glycomacropeptide hydrolytes (GMP) were investigated for the feasibility as a potential prebiotic in yoghurt by Tian et al. *L. bulgaricus*, *B. animalis* subsp. *lactis* (Bb12), and *S. thermophilus* produced papin GHP during the evaluation of growth performance in the presence of casein glycomacropeptide (GMP) hydrolysates. About four times higher the number of viable counts of *B. animalis* subsp. *lactis* (Bb12) was obtained after adding 1.5% GHP than the controlled with no GHP addition. Moreover, for other strain, an improved growth was reported for *S. thermophilus*, whether lower growth was observed in *L. bulgaricus* (Tian et al. 2015). The viability of bifidobacteria can also be increased by incorporation of fructo-oligosaccharide (FOS) (Akalin et al. 2004). When fructo-oligosaccharides were applied to skim milk, the feasibility of industrial *Bifidobacterium* spp. increased by 55.7% after 4 weeks of refrigerated storage (Shin et al. 2000). The presence of oligofructose (1.5% w/v) as a prebiotic to yoghurt increased the viability of probiotic species throughout the refrigerated storage (Capela et al. 2006). Whey protein hydrolysate (Mccomas and Gilliland 2003). Whey protein concentrate (Dave and Shah 1998) and insulin (Carvalho et al. 2008) were also found to promote the viability of probiotics as a protective compound. Skim milk solid particles have been shown to coat cell wall proteins with a protective layer and stabilise the cell membrane (Ananta et al. 2005). With the incorporation of whey protein isolate (WPI), Cordeiro et al. tested skim milk that had previously been fermented by *P. freudenreichii* or *L. casei* strain. When both strains were faced with acid, bile salts, cold stress, and heat, supplementing with 30% (w/v) WPI enhanced their survival rate in comparison with fermented skim milk without the presence of whey protein isolate (Cordeiro et al. 2018).

Whey protein microgels were also used to improve the viability of *L. plantarum* cells that were freeze-dried. With an improvement in WPI concentration, the protective impact was increased even further (Su et al. 2018). The impact of the carrier matrix should also be considered when choosing probiotic strains for specific purposes. Cells incorporated into hydrocolloids have already shown tremendous potential in terms of bioactive approaches for functional foods. Similarly, new edible film and coating techniques have been shown to increase cell population viability. In a recent study, the survivability of *L. rhamnosus* GG interplay in the presence of selected biopolymers and whey protein concentrate (WPC) as a possible vehicle was investigated (Bambace et al. 2019; Ebrahimi et al. 2018; Guo et al. 2017; Soukoulis et al. 2017). The most successful performing device, according to the authors, is sodium alginate and kappa-carrageenan/locust bean gum. They also found that adding WPI to the mix improved *L. rhamnosus* GG stability even further. Pavli et al. provided a promising example in which high-pressure processing was used to test Na-alginate composite materials as carrier for spreading bacterial strains to centre cut ham steak. Irrespective of the previous high-pressure processing procedure, delivery of probiotics on centre cut ham steak was found to be effective using the films supplemented with probiotics, as viability remained relatively constant at >106 CFU/g during storage period, regardless of storage temperature at 4 °C, 8 °C, and 12 °C (Pavli et al. 2017).

6.5.5 Encapsulation of Probiotics

Probiotics have been linked to beneficial results such as the regulation of the gut microbiota through the suppression of harmful microbes, the construction of anti-carcinogenic substances and the modulation of immune responses and others (dos Reis et al. 2017; Markowiak and Ślizewska 2017; Prakash et al. 2011). Microorganisms with probiotic claims, on the other hand, must be able to colonise and sustain metabolic activity in the human gastrointestinal tract in order to produce beneficial effects (Collins et al. 1998; Saarela et al. 2000). Cell encapsulation can boost probiotic microorganisms' tolerance to adverse conditions while reducing encapsulated microorganisms' cell losses in hydrocolloid matrices. Various probiotic encapsulation methods are currently in use, with particles of various properties being obtained (Kim et al. 2017; Pasqualin Cavalheiro et al. 2015; Rodrigues et al. 2017). Encapsulation has recently become the most widely studied strategy for enhancing probiotic sustainability and bioactive compound delivery. During manufacturing, storing, and gastrointestinal passage, storing the bacterial cultures, enhancing the stability, and handling the probiotic strain become easier after the encapsulation of probiotics. Besides, LAB used in probiotics get protection against many harsh environments such as acidic condition, freezing, and oxygen. The pH, the initial count of cell population, the strain used as potential probiotic, and the food matrix are all factors to consider and have been established as influencing the efficacy of encapsulation in probiotic protection (Ebrahimi et al. 2018; Lee and

Heo 2000; Muthukumarasamy et al. 2006; Ningtyas et al. 2019; Rokka and Rantamäki 2010).

6.6 Material and Method to Encapsulate Probiotics

6.6.1 Material to be Used

For the sustainability and characteristics of the produced particles, identifying the appropriate material for encapsulating microbial cells is critical. The encapsulating agent must not be hazardous, since it has a direct impact on the particle structure. Besides permeability and diameter, also considered as an important factor is the particle structure. Controlled release of the probiotics is an important factor to consider; hence, it should be able to satisfy the situation with protection to environment factor to the strain (Chen et al. 2017a, b; Rathore et al. 2013). During the storage of the particles, cell viability might get hampered due to temperature and moisture content in the environment that leads to lipid oxidation in the cell membrane. As a result, cell with encapsulation survival increases by using products that can retain moisture. When suspended in digestive enzymes, materials that totally release encapsulated cells cannot be ideal for cell defence when passing through the GI tract (Rajam and Anandharamakrishnan 2015). Polysaccharides (cellulose acetate phthalate, alginate, starch, k-carrageenan, chitosan) and natural water-soluble polysaccharide extraction of mucilages and gum as well as proteins (gelatin, milk proteins) and lipids and fats to immobilise probiotics have been used to encapsulate probiotic cells (Bustamante et al. 2017; Cąbuk and Harsa 2015; Calinoiu et al. 2019; de Araújo Etchepare et al. 2016; Mu et al. 2018; Nami et al. 2017; Singh et al. 2017). Their implementation enables the use of milder methods, such as extrusion, which strengthens the encapsulated microorganisms' cellular integrity (Rodrigues et al. 2017).

6.6.1.1 Alginates

Alginates are widely used as encapsulating materials in the encapsulation of microbial cells. However, in the availability of excess monovalent ions and Ca2+ chelating agents, when these polymers are used, porous matrices that are prone to disintegrate are formed. To enhance particle properties, polymers like plant- and seed-derived polysaccharides were mixed with alginate (Chen et al. 2017a, b; Dokoohaki et al. 2019; Rodrigues et al. 2017). It is used in the food and pharmaceutical industry in the European Union and the United States as well as in the studies of encapsulation techniques, alginate has been labelled as safe for use in E 404 and E 401, as well as GRAS 21 CFR 184.1724 and GRAS 21 CFR 184.1187, respectively (Qin et al. 2018).

Alginate is a hetero-polysaccharide that can be extracted from brown algae, M residue or D-mannuronic acid residue, and G residue or L-guluronic acid residue monomers grouped together in an alternate fashion to make the structure of alginate.

β-(1 → 4) bonds and α (1 → 4) link M and G residues respectively (Ching et al. 2017; Donati and Paoletti 2009; Rathore et al. 2013).

The ratio of M to G units, as well as the sum of each of the three blocks in the polymer, can differ depending on the source. Alginates with a larger ratio of G blocks produce more rigid and brittle gels, whereas alginates with a greater proportion of M blocks produce less rigid and more flexible gels (Gandomi et al. 2016). The affinity of anionic blocks in alginate for divalent cations varies depending on the cross-linking cation. Pb > Cu > Cd > Ba > Sr > Ca > Fe > Co, Ni, Zn > Mn is the order in which the cations are decreased. Toxic cations, on the other hand, have a restricted use and must not be used in pharmaceutical or food applications. As a result, the non-toxic Ca2+ ion is commonly used to make calcium alginate gels (Haug et al. 1970; Mørch et al. 2006). When an aqueous alginate solution is dropped into a calcium-containing bath, rapid cross-linking between alginate guluronic units and calcium ions forms gel beads. Because of its ease, non-toxicity, biocompatibility, and low cost, alginate has been commonly used to encapsulate probiotic bacteria (Krasaekoopt et al. 2003; Rowley et al. 1999).

According to Mandal et al., *L. acidophilus* encapsulated in alginate beads had a higher survival rate than free probiotics under various conditions. With increasing alginate concentrations, *L. acidophilus* survival improved proportionately. With the rise in bead size, the viability of encapsulated probiotics in simulated gastric fluid increased (Mandal et al. 2006). Truelstrup Hansen et al. reported that very large alginate beads approx >1 mm can effectively protect probiotics, whereas small beads approx <100 μm have no effect on bacterial protection in simulated gastric fluid (Hansen et al. 2002). However, alginate beads have some drawbacks, such as easy degradation in acidic environments, easy disintegration when exposed to monovalent ions or chelating agents, and difficulty scaling up the process. Furthermore, the obtained alginate beads are extremely porous. Co-encapsulation with other materials, covering the beads with another polymer, or changing the alginate structure with various additives can all help to solve these problems (Gouin 2004; Mortazavian et al. 2008).

6.6.1.2 Chitosan

The membrane on the surface of the alginate beads is formed by the positively charged amino groups of chitosan and the negatively charged carboxylic acid groups of alginate, which blocked the pore of the alginate beads. Several studies have been published on chitosan-coated alginate beads used to encapsulate probiotic bacteria (Dong et al. 2013).

β-(1 → 4) bonds link the D-glucosamine and N-acetyl-glucosamine residue to make cationic polysaccharides that are chitosan and commercially derived from partial deacetylation of chitin isolated from crustaceans (Silva et al. 2019).

The Food and Drug Administration (FDA) has designated it as GRAS, "generally recognized as safe", and it is soluble at acid pH and biocompatible. Furthermore, cations with anionic polymers can have electrostatic interaction under acidic conditions as chitosan has positively charged amine groups with pKa value approximately 6.5 (Qin et al. 2018). Lee et al. investigated at how chitosan-coated alginate

with three distinct molecular weights affected the viability and feasibility of *L. bulgaricus*. The findings indicate that the molecular weight of chitosan has a significant impact on the viability and stability of probiotic bacteria encapsulated in chitosan-coated alginate beads (Lee et al. 2004). The internal gelation technique was used to coat chitosan in alginate beads containing *Bifidobacterium longum* DD98. Under GI condition and elevated temperature environment, the chitosan coating enhanced the longevity of encapsulated *Bifidobacterium longum*. The extrusion encapsulation by alginate-chitosan results in <76% efficiency in *L. rhamnosus* ASCC 290 and *L. casei* ATCC 334. It was also reported that both strains showed different behaviours but was safe in simulated GI condition (Ji et al. 2019; Farias et al. 2019).

6.6.1.3 Gelatin

For probiotic encapsulation, gelatin, a type of protein gum, has been used alone or in combination with other compounds. It's an excellent candidate for collaborating with anionic polysaccharides like gellan gum because of its amphoteric nature. At a pH greater than 6, these hydrocolloids are miscible because they both have net negative charges and repel each other. When the pH is changed below the isoelectric point, however, the net charge of gelatin becomes positive, resulting in a strong interaction with the negatively charged gellan gum (Anal and Singh 2007; Krasaekoopt et al. 2003).

6.6.1.4 Mucilages and Gums

Food industries and drug manufacturers extensively used natural polysaccharides that are water-soluble mainly derived from plants and seeds due to their dietary properties and excipients in administrating drugs. A mucilaginous material noted for its gelling ability and water-absorbing property can be extracted from the seed husks of *Plantago ovata* Forssk (Fernandes et al. 2018). Besides, it can be used as a gelling agent and encapsulation material. Food industries use these polymers as thickening agent and as food additives (Hamdani et al. 2019; Kumar and Gupta 2012; Pereira et al. 2019; Rodrigues et al. 2018; Salehi 2019; Soukoulis et al. 2018).

Mainly three groups are classified for mucilages and gums that are derived from plants and seeds:

Group I: Galactomannans like non-starch endosperm fractions.
Group II: Seed coat mucilaginous components.
Group III: Endosperm cell wall material like mannans, galactomannans, hemicelluloses, glucomannans, and xyloglucans (Otegui 2007; Soukoulis et al. 2018).

The polymers in this material are natural antioxidants and anti-carcinogenic agents, and their use has been linked to positive health outcomes such as improved traditional and alternative lipid markers, which tends to minimise the threat of atherosclerosis and cardiovascular disease (Jovanovski et al. 2018; Patel et al. 2019). Konjac is a plant belonging to the Araceae family and the genus

Amorphophallus that is native to Southeast Asia's subtropical regions. Its tubers can be used to extract a low-cost hydrocolloid gum made primarily of glucomannan, a soluble dietary fibre. By enhancing the stability of encapsulated materials and ensuring that they efficiently accomplish their objectives, for microbial cell immobilisation, as drug carriers and a material to be used for encapsulation, hydrogels can be applied that are konjac-based gum (Devaraj et al. 2019).

6.6.2 Methods to be Used

To encapsulate the probiotic cells, these are the main techniques:

- Spray-drying (Aragón-Rojas et al. 2020; Bustamante et al. 2017; Rajam and Anandharamakrishnan 2015; Santos 2019).
- Spray chilling (Arslan-Tontul et al. 2019; Arslan-Tontul and Erbas 2017; Bampi et al. 2016; Pedroso et al. 2012; Silva et al. 2018a, b).
- Extrusion (Dimitrellou et al. 2019; Kim et al. 2017; Krasaekoopt and Watcharapoka 2014; Rodrigues et al. 2017; Silva et al. 2018a, b).
- Emulsion (Raddatz et al. 2020; van der Ark et al. 2017; Zhang et al. 2016).
- Fluidised bed (Horison and Surono 2020; Silva et al. 2018a, b).

6.6.2.1 Spray-Drying

Spray-drying is a low-cost, fast-processing, high-productivity technique that is widely used in the food sector. In a high-temperature gas, this technique entails atomising a solution containing the encapsulated composites and forming a powder instantly (Ray et al. 2016). Various natural polymers, mostly gum arabic and starches, could be used to encapsulate bacterial strain cells using the spray-drying technique because of their well-known ability to shape spherical particles after drying. Other substances have been used, including fructo-oligosaccharides, inulin, alginates, gums, and mucilages. Polysaccharide-encapsulating materials have good solubility, low viscosity at high concentrations, an elevated glass transition temperature, and fast drying, all of which are desirable characteristics for spray-drying. Furthermore, combining different polymers will increase the encapsulated cells' viability (Arslan et al. 2015; Avila-Reyes et al. 2014; Kingwatee et al. 2015; Rajam and Anandharamakrishnan 2015; Sarao and Arora 2017).

Recently, using spray-drying at inlet temperature of 120 °C and outlet temperature of 5 °C, *Lactobacillus acidophilus La-5* was encapsulated in insulin. The survival rate of the strain became 86.5% after the procedure of encapsulation. Prebiotic skimmed milk containing the bacteria *Bifidobacterium* BB-12 at the mixing temperature of 55 °C, leading to improved encapsulation, found when probiotics were encapsulated in pre-melted skim milk powder at a temperature of 150°, produced more than 70% effective results (Liu et al. 2019).

6.6.2.2 Spray Chilling

Like spray-drying, also, which is performed with tiny droplets, this method has a process that "expands" the material to produce sufficient bubbles. However, encapsulation is usually done by dispersing the encapsulated agent in a molten matrix, solid matrix made up of lipids that is atomised in a chamber where cold air is pumped, allowing the particles to solidify. Despite the fact that spray chilling is not a new technique, it is less widely used than, say, spray-drying or ionic gelling. However, due to the low cost and industrial-scale application, its process conditions are an excellent alternative for encapsulating microbial cells (Favaro-Trindade 2013).

6.6.2.3 Extrusion

As the extrusion technique is easy and inexpensive, the encapsulation technique has become very popular. However, the procedure, the feasibility of the encapsulated cells is low and they are unlikely to be refused because of the usage of it (Wunwisa Krasaekoopt et al. 2003; Rodrigues et al. 2017). This method uses hydrogeloids that are injected into a cross-linking solution through a nozzle to become gels. In acidic mediums, the resulting gel is usually stable, but in an alkaline setting, it disintegrates (de Araújo Etchepare et al. 2016; Favaro-Trindade et al. 2011).

Internal ionic gelation can also be done with two or more different materials that work together as an encapsulating material, leading to better particle properties. This method was used to encapsulate *Lactobacillus acidophilus* LA-5 in pectin, inulin, and rice bran blends. Under artificial gastrointestinal conditions, the particles obtained protected the microbial cells and enhanced encapsulation performance, reaching rates of over 90%. Furthermore, the bacteria remained viable after 120 days of storage at 25 °C. The use of mixed materials, on the other hand, was found to increase particle size (Raddatz et al. 2020).

6.6.2.4 Emulsion

Emulsions are used to increase the solubility, physiological action, and stability of interest compounds in the food and pharmaceutical industry. Emulsion is an imbalance in which the continuous phase particles are distributed in a continuous phase fluid with a stabilising agent that has a higher affinity for the continuous phase than the dispersion medium. Separating the scattered phase droplets from the continuous phase can also be done with the aid of a solidifying agent (Alemzadeh et al. 2020; Zhang et al. 2016).

It may be necessary to use a solidifying agent for dispersing the scattered phase from the continuous phase to achieve homogeneity. The dispersed phase is called an emulsion, and the water is contained within the liquid. An emulsion is called oil-in-water (O/W) if the dispersed phase is made up of oil, and water is the emissive, or a opposite phase if the dispersed phase is. In both cases, two phases alone are all needed to create an emulsifier. Separation steps, such as emulsification in water or in oil, produce two layers, are produced by the addition of a third (Goibier et al. 2020).

6.6.2.5 Fluidised Bed

Coating, granulation, and drying are all done with the fluidised bed process, which involves atomising a thin layer of material that surrounds the particles in a suspension. To reap significant benefits, you simply need to follow a simple formula that produces low costs and high performance. Additionally, lipids, proteins, and polysaccharides can be used as encapsulants (Zuidam and Shimoni 2010).

The procedure requires particles to move continuously inside a heated chamber where the covering materials are supplied with moving air to cover the particles and prevent them from settling. As the substance is pulverised, it coheres with the particle, combining to form a gel on the surface and solidifying in a series of steps before forming a solid, homogeneous sheet. The injection angle (top, bottom, or tangential spray types), material encapsulation (evaporation rate, solidification, and viscosity), and fluidisation airability are effected by the solid particle mobility (velocity), and temperatures in the amount of the cycles, including encapsulating, have a tremendous impact on the properties of the final coating, thereby increasing its quality and reducing waste (Avilés-Avilés et al. 2015; Manojlović et al. 2010).

In this way, probiotic encapsulation necessitates a prior treatment that promotes the formation of a solid particle that can be stabilised and sealed, defining the fluidised bed as a co-encapsulation technique (Chavarri et al. 2012; Manojlović et al. 2010; Ozdal et al. 2019). In the encapsulation technique, researchers discovered that the addition of a mixture of disaccharide (sucrose and trehalose) to the wall material increased cell wall defence, thereby ensuring better encapsulation of *Enterococcus faecium* protection when *Lactobacillus plantarum* bacteria was cultivating and storage was present (Strasser et al. 2009).

6.7 Bioactive Metabolites Released by Probiotics

6.7.1 Bacteriocins

Bacteriocins are antimicrobial peptides that are synthesised by ribosomes on microbes to inhibit the other pathogenic or harmful microorganisms without harming the beneficial microbes; due to which the antimicrobial properties of bacteriocins are commonly used in food as preservatives (Chikindas et al. 2018; Cotter et al. 2013; Zacharof and Lovitt 2012). Two domains of phylogenetic trees produce bacteriocins, bacteria, and some members of Archaea. Both Gram-positive and Gram-negative bacteria produce bacteriocin in the bacterial group (Gillor et al. 2008; Indira et al. 2015, 2018; Indira et al. 2019c). Gram-positive bacteria produce larger amounts of bacteriocin compared to Gram-negative bacteria; additionally more structural variable of bacteriocin can be obtained. Gram-positive bacteria such as lactic acid bacteria or LAB group are identified with higher capacity of producing bacteriocins. These LAB bacteriocins get activated even at nanomolar range, and most of them are non-toxic to eukaryotic cells (Messaoudi et al. 2013). The probiotic bacteria quorum sensing plays a greater role in colonising and producing bacteriocins to establish a defensive environment against the pathogenic microbes. Also bacteriocins prevent

the pathogens from colonising a specific area and limiting the contact with neighbouring cells (Dobson et al. 2012; Mukherjee and Ramesh 2015). Bacteriocins have bactericidal properties with a narrow spectrum of activity and are situated in the cytoplasmic membrane region of bacterial surface receptor binding. Non-toxic peptide bacteriocins are sensitive to and can be degraded by digestive proteases compared to antibiotics. The property of degradation by digestive enzyme makes bacteriocins a suitable candidate to be used as food preservatives (Gálvez et al. 2007; Mokoena 2017; Yang et al. 2014). Their effectiveness can be determined by a variety of factors, including the bacteriocin dosage and standard purification, as well as experimental parameters such as pH, temperature, and the presence of agents that alter the cell wall integrity (Juodeikiene et al. 2012).

There are several advantages to add bacteriocin as a food preservative:

- It minimises the use of chemical preservatives and protects during excessive temperature.
- Reduces the risk of diseases like food poisoning caused by the pathogens present in foods.
- Increases the shelf life and long-term substantiality to a food product.
- Minimises the processing cost by allowing physical treatment in low intensity, results in preserved nutrients, vitamins, and organoleptic property of food.
- Wastage of food due to spoilage can be reduced.
- Fulfilling the consumer demands by providing safe, fresh, and ready-to-eat minimum processed "novel" foods (Messaoudi et al. 2013).

LAB that produce bacteriocins (Ananou et al. 2007; Gálvez et al. 2007, 2008; Leistner and Gorris 1995; Oliveira et al. 2008):

- Aerococcus
- Carnobacterium
- Enterococcus
- Lactobacillus
- Lactococcus
- Leuconostoc
- Oenococcus
- Pediococcus
- Streptococcus
- Tetragenococcus
- Vagococcus
- Weissella

Only a bacteriocin named nisin which is produced by *Lactococcus lactis* has been approved by FDA and is permitted for commercial use (Sobrino-López and Martín-Belloso 2008; Vesković Moračanin et al. 2014).

6.7.2 Polysaccharides

Various genera of probiotic bacterial strain including LAB have the capability to synthesise glucosidic polymers and produce exopolysaccharides (EPS) in a greater amount (Kodali et al. 2009; Mikkili et al. 2016). These secreted molecules can be held covalently bound to the cell surface in the form of a capsule or released into the environment or remain partially attached to the cell surface (Suresh Kumar et al. 2007; Viana de Souza and Silva Dias 2017). Probiotics and EPS of bacteria have generated lots of diversity and also interest over the years due to their health benefits, but commercially, bacterial polysaccharide cannot be used due to the high price as compared to polysaccharide obtained from algae; animals, i.e. starch, pectin, alginate, and galactomannan; and plant sources (Ates 2015; Zannini et al. 2016). However, EPS produced by lactic acid bacteria can be used as emulsifier, thickener, gelling agent, stabiliser, and viscosifier; also, the water retention property of exopolysaccharides benefits certain products like cheese in texture and calorie reduction (Ferrari et al. 2016; Viana de Souza and Silva Dias 2017). In many food manufacturing companies, in situ fermentation is used to produce exopolysaccharides by lactic acid bacteria with probiotic properties, and this is important for the organoleptic properties that it presents because it affects the quality and rheology of fermented foods (Notararigo et al. 2013). *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *and Weissella* genera of probiotics have been reported to produce exopolysaccharides (EPS) (Amari et al. 2013). The exopolysaccharide can vary in different terms, such as composition, as different types of bond can bind different monosaccharides, and molecular weight and structure, as they consist of several degrees and different types of branching and overall structural confirmation. According to these characteristics of EPS, the role of exopolysaccharide can be determined in food. EPS can be classified into two types based on their composition: one is HoPS (homo-polysaccharides) that consist only a single type of monosaccharides, and another is HePS (hetero-polysaccharides) that consist two or more types of monosaccharides. Further, homo-polysaccharides can be divided into four groups based on different carbon-carbon links: α-d-glucans, β-D-glucans, β-D-fructans, and other polygalactans (Pérez-Ramos et al. 2016). EPS have the ability to act like probiotics and can accelerate the growth of beneficial microbes and de-accelerate the biofilm formation of the pathogen; additionally, the β-glucan product has antitumorigenic, antiviral, antioxidant activity immunomodulatory and antiulcer activity (De LeBlanc et al. 2018; El Ghany et al. 2014; Julendra et al. 2017; Notararigo et al. 2014; Nwodo et al. 2012; Peele et al. 2016; Tsai et al. 2014).

6.7.3 Short-Chain Fatty Acids

The probiotic bacteria break down the undigested sugar molecules into short-chain fatty acids such as acetate, butyrate, and propionate; gasses like CH4, CO2, and H2; and some heat (J. G. LeBlanc et al. 2017; Thursby and Juge 2017; Yoo and Kim

2016). Probiotic bacteria form a symbiotic relationship with the human's gut flora and live alongside colonocytes (Chatterjee et al. 2017). Ten percent of the total calorie requirement is produced in the large intestine by SCF. In the colonocytes 60–70% of the total energy is produced by butyrate. In the oxidation of butyrate, it converts to acetyl coenzyme-A with ketone bodies and carbon dioxide and functions as a source of energy for colon and brain cells (Den Besten et al. 2013). Probiotic bacteria can perform a variety of functions in the gastrointestinal tract apart from producing short-chain fatty acids. The development of pathogenic bacteria is suppressed, the immune system and gut physiological parameters are controlled, and epithelial cell growth is stimulated by the production of vitamins and hormones (Mach and Fuster-Botella 2017). SCFAs, especially butyrate, have been shown to be beneficial in the treatment of inflammatory bowel disease, antibiotic-associated diarrhoea, colon cancer, and cardiovascular disease (Chatterjee et al. 2017; Gill et al. 2018; Moss et al. 2018; Tominaga et al. 2018).

6.7.4 Aromatic Compounds

Various compounds are produced by probiotics that can enhance and improve the organoleptic characteristic including the aroma of the food and flavour. Metabolising the carbohydrate, citrate, proteins, and milk lipids can strongly influence the cheese production in maturation by changing the component of milk into aromatic and flavoured compounds (Attri et al. 2021). Due to the action of lysed and intact lactic acid bacteria, aroma is formed in the fermented food, and enzymes present in cytoplasm also releases that remains active even outside the cell the results in production of metabolites in the matrix of fermented food (Lortal and Chapot-Chartier 2005). Different amino acids are catabolised to produce aromatic compounds in the food matrix by the event of proteolysis. Branched chain amino acids like leucine, isoleucine, and valine after catabolism give a sweet and fruity flavour. Some aromatic amino acids like phenylalanine, tyrosine, and tryptophan produce floral flavour after catabolism. Sulphur amino acids such as cysteine and methionine after the event of metabolism meat, garlic and boiled cabbage flavoured compounds are released (Ardö 2006). Bacteria metabolise citrate which is an organic acid found in fermented products under anaerobic and acidic conditions to produce 4-carbon aromatic compounds such as diacetyl, ethanol, acetoin, and 2,3-butanediol through the pyruvate pathway (Smid and Kleerebezem 2014). From citrate some probiotics such as *lactis* biovar *diacetylactis* subspecies of *L. lactis* (Hugenholtz 1993), few from *Leuconostoc* (Hemme and Foucaud-Scheunemann 2004), some of *Enterococcus* (Martino et al. 2016), *Lactobacillus plantarum* (Minervini et al. 2010), *Oenococcus oeni* (Bartowsky and Henschke 2004), and *Weissella paramesenteroides* (García-Quintáns et al. 2008) produce 4-carbon aromatic compounds. Diacetyl follows a synthetic route, and the efficiency depends on the enzyme that converts the citrate to pyruvate, and citrate disappearance rate in the intracellular medium is determined by the concentration gradient (Laëtitia et al.

2014). After that, lactate dehydrogenase (LDH) transforms most of the pyruvate to lactate (Ko et al. 2016).

6.7.5 Diacetyl

Diacetyl has a strong butter aroma and has a yellow colour in its purified form. Diacetyl still has a variety of useful applications such as being used in dairy products, wine preparation, beer, and roasted coffee, and due to its aroma, it is also used in microwave popcorns (Kreiss and Hubbs 2010; Shibamoto 2014).

Some bacteria that come under lactic acid bacteria such as *lactis* biovar *diacetylactis* subspecies of *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Leuconostoc* species metabolise citrate and produce diacetyl. The bacteria release the non-enzymatic form of diacetyl that originates from pyruvate by oxidation reaction (Díaz-Muñiz et al. 2006; Hugenholtz 1993; Tamang et al. 2016). Diacetyl can penetrate the pathogenic bacterial cell membrane and inhibit the pathogen by inhibiting the function of essential metabolites such as preventing the bacteria from synthesising essential proteins that are made from arginine by blocking the catalytic site for the responsible enzyme. Diacetyl is used as an antagonistic agent for this property of inhibition of microbes (Brass and Palmer 2017; Hor and Liong 2014). Some microorganisms inhibit the action of diacetyl, including *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Escherichia coli*, and *Salmonella anatum* (Naidu 2000). Lactic acid bacteria have the greater potential to generate this antimicrobial compounds that are isolated from the milk of goat (Ferrari et al. 2016).

6.7.6 Organic Acids

Organic acids have tremendous ability to enhance the food quality and safety and are highly used by food industries (Hwanhlem et al. 2011). Different varieties of organic acids are produced in the fermentation process such as lactic acid, succinic acid, fumaric acid, acetic acid, and butyric acid (Martín et al. 2009; Özcelik et al. 2016; Paul Ross et al. 2002). These organic acids reduce the pH level below the optimal growth and act as an antimicrobial agent for some of the microbes by inhibiting the metabolic activity (Crowley et al. 2013). Organic acids cause a drop in the pH level, which increases liposolubility of the cell membrane, enabling them to intervene with the cell membrane potential conservation of the pathogenic microorganism. The interference with the cell membrane potential leads to the inhibition of active transport that allows the passage of organic acid to the cytoplasm resulting in reduced intracellular pH and inhibition of essential metabolic function of the pathogen (Martín et al. 2009; Özcelik et al. 2016; Paul Ross et al. 2002).

6.7.6.1 Lactic Acid

Many pharmaceutical, chemical, food, and cosmetic companies mainly use lactic acid. Approximately 70% of the total lactic acid production is used by food

industries (Castillo Martinez et al. 2013; Li et al. 2015). Bacterial fermentation produces approx 90% of food products worldwide, and lactic acid is obtained from the chemical and fermentation processes of bacteria (Vijayakumar et al. 2008). Many lactic acid bacteria, including *Lactobacillus*, *Bifidobacterium*, *Sporolactobacillus*, *Bacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and *Leuconostoc*, produce lactic acid (Naidu 2000). Many fermented products, for instance, fermented milk, dough bread, and other foods such as olives, pickled vegetables, butter, yoghurt, etc., contain lactic acid naturally or as a fermentation product. It is also used to give acid flavour to the foods with tart and sour taste (Datta and Henry 2006). Lactic acid is produced by LAB such as *Lactobacillus* and *Bifidobacterium*, and this has been shown to inhibit *Escherichia coli* O157:H7 (Reis et al. 2012). This effect is also seen in *Enterobacteriaceae*, *Salmonella bareilly*, *Salmonella typhimurium*, and *Listeria monocytogenes*, as well as *Vibrio vulnificus*, *Bacillus coagulans*, *Yersinia enterocolitica*, *Mycobacterium tuberculosis*, *Pseudomonas fragi*, *Aeromonas hydrophila*, *Clostridium sporogenes*, *Enterobacteriaceae*, *Helicobacter pylori*, *Clostridium botulinum*, *Lactobacillaceae*, *Aspergillus* sp., and *Pseudomonas* sp. (Naidu 2000; Mani-López et al. 2012).

6.7.6.2 Acetic Acid

Acetic acid as vinegar is used by almost every country around the world. Acetic acid is derived from the bacterial fermentation substrate mainly the lactic acid bacteria (LAB) (Panda et al. 2016). Food manufacturers add acetic acids in food to control pH level and as a taste enhancement agent (Supharoek et al. 2018). But it has a pungent odour due to which its use has become limited in food products (Malik et al. 2014). In Canada only concentration of 4.2–12.3% of acetic acid is allowed to be used in vinegar (Panda et al. 2016). Acetic acid is more effective in inhibiting pathogenic growth than lactic acid as acetic acid has bactericidal activity at 0.3% and bacteriostatic activity at 0.2%. Also, acetic acid reduces the chance of survival of Gram-positive and Gram-negative bacteria, fungi, and yeasts and is used in a wide range of foods as a preservative (Ray 2004). Excessive intake or usage of acetic acid is not recommended because of its corrosive properties, which can lead to stomach health problems (Supharoek et al. 2018).

6.8 Role of Probiotics in Cancer and an Immunomodulator

6.8.1 Immunology

Probiotics have the ability to modulate the immune response in both animals and humans, not only at the intestinal mucosa level but also at the systemic stage. To modulate their behaviour via the intestinal mucosal immune system, probiotic lactic acid bacteria (LAB) must communicate with the normal gut microbiome as well as the food consumed. When probiotics interact with intestinal epithelium (enterocytes), cytokines and chemokines are generated, which cause immunomodulatory events. Many other studies have also shown that lactobacilli can activate the

intestinal immune system and, as a result, favour the elimination of potentially harmful infectious microorganisms. This can be accomplished by producing specific type A immunoglobulins or activating K cells ("natural killer") (Gill et al. 2001; Kaila et al. 1992; Manzano et al. 2012). Other immunomodulatory actions of these probiotics come from their ability to boost intestinal leucocyte phagocytic activity, encourage increased B lymphocyte proliferation and immunoglobulin A and G secretion, and induce cytokines including interleukin-2, IL-6, and tumour necrosis factor (TGB). *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium breve*, and *Lactobacillus gasseri* are only a few of the probiotics that have been tested to show this action, as well as the generalised activation of B lymphocytes (Attri et al. 2021). Probiotics' role as immune system modulators has only recently been discovered, particularly in cases where mucosal immunity is crucial (Donkor et al. 2010; Koninkx et al. 2010). The stimulation of cells of the innate immune system, like phagocytes and natural killer cells, and the suppression of irregular immune responses are the two main types of probiotic effects on the immune system. Infections and cancers are expected to be inhibited by the former, while inflammatory bowel disorders, asthma, and autoimmune diseases are expected to be inhibited by the latter (Chiba et al. 2010; Rook and Brunet 2005).

6.8.2 Cancer

Use of probiotic therapy for the treatment and prevention has piqued the interest of clinical nutritionists, scientists, and industry, since it has been discovered that probiotics have a beneficial impact on a variety of cancers, including colon and rectum, breast, blood, cervical, prostate, bladder, skin, oesophagus, liver, gallbladder, head, and neck cancers (Dasari et al. 2017; So et al. 2017; Vandenplas and Benninga 2009). Probiotics have the advantage of having no or few side effects compared to other cancer therapies. *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Lactobacillus acidophilus*, and *Lactobacillus paracasei* are isolated strains of fermented milk with antitumor activity. *B. infantis* and *L. acidophilus* were found to be the most successful organisms for the development of a breast cell line when tested (Biffi et al. 1997; So et al. 2017; Vandenplas and Benninga 2009). However, the anti-cancer effect of LAB has only been observed in in vitro studies; further research is needed to validate this activity in humans (George Kerry et al. 2018).

6.9 Conclusion

It is a very significant step in making the immune system friendly to probiotic compounds through study, which looks set to aid in disease control and care. In the future, identifying new varieties and consuming freshly developed probiotics may be a good strategy for maintaining good health. As a result, probiotics may be

recommended as a biotherapeutic option for the treatment of a variety of infections (Indira et al. 2019a, b).

Using encapsulation to encapsulate bacteria, however, instead of drying or storing them, increases the preservation and sustainability of viable cells. Many research, alluding to various encapsulation methods and encapsulating materials, show that different techniques are employed. However, in order to minimise losses during particle processing and application, both the procedure and the encapsulating material must be carefully selected (Rodrigues et al. 2020).

All in all, the industry for bioactive food study is rapidly expanding, and for excellent purpose, there are numerous potential benefits and applications emerging within the field. However, given the ability of various bioactive ingredients in functional foods to interact with one another, as well as the subsequent cascading impact that these interactions could have within the body of the host, there are still many limitations and risks that need to be properly addressed and acknowledged before actively adopting functional foods on a daily and long-term basis.

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Microbial Production of Natural Flavors and Fragrances

7

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Abstract

Due to global warming, with climate changes and significantly decreasing petrochemical resources, an urgent need has arisen to produce bio-based products in a renewable manner. Chemical-based products also cause a serious impact on health and the environment. Chemical synthesis and extraction of products from plants are non-sustainable. The global demand for natural flavor and fragrance is continuously increasing and has shown a high interest in the aroma industry. Plants and microorganisms are the major sources of flavor and fragrance. Due to production in smaller concentrations, isolation and extraction of such value-added chemicals become expensive. These natural products are terpenoids, aldehydes, methyl ketones, etc., which are used in a wide range of domestic products including cosmetics, soaps, fresheners, candles, and foods. Microbial production is an alternative way of synthesis by modifying natural biosynthetic pathways or inserting a novel pathway into hosts. The present

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chapter highlights important chemicals for flavor and fragrance and their engineered production.

Keywords

Flavors · Fragrance · Metabolic engineering · Biosynthetic pathway

7.1 Introduction

Currently, global demand of natural flavor and fragrance is high, and the market is exponentially growing. The global flavors and fragrances market size was valued at USD 20.75 billion in 2018. It is expected to increase at a compound annual growth rate (CAGR) of 4.7%. These are used in food, beverages, perfumes, scent, cosmetics, toiletries, and many more. In India, the flavor market was valued at USD 284.2 million in 2011, which rose to USD 380.6 million in 2014, and is expected to grow at 10% annually. Noh et al. (2019) reported the use of butyl butyrate as a flavoring and fragrance agent in different products such as beverage, perfumes, food, and cosmetics.

Recently, Nies et al. (2020) produced methyl ketones that have been used as flavor, pharmaceutical, fragrance, and agrochemicals. They engineered *Pseudomonas taiwanensis* VLB120 for the production of methyl ketone. The strain was further improved by eliminating the competitive pathway and a yield of 9.8 g Laq^{-1}. This was fourfold higher than the initial strain. A wide range of microorganisms has the ability to produce natural flavors and fragrances. However, these microorganisms cannot be further manipulated due to the lack of molecular biology tools. Therefore, well-studied microorganisms can be used for the production of these chemicals by installing the complete biosynthesis pathway at an industrial scale. Microbial engineering requires a wide range of approaches including synthetic biology (Patel et al. 2018; Bhattacharjee et al. 2020), metabolic engineering (Singh et al. 2020; Gohil et al. 2017), and fermentation technology for large-scale production at competitive prices. This chapter highlights several microorganisms that have the ability to produce flavor and fragrance to fulfill the rising demand of market globally.

7.2 Production of Flavors and Fragrances

7.2.1 Diacetyl

Diacetyl, also known as butanedione or butane-2,3-dione, is an organic compound. It is a yellow- or green-colored liquid with buttery flavor. It is used in alcoholic beverages and also added in foods for buttery flavor. Natural flavor of yoghurt is achieved by lipolysis of milk fat and transformation of lactose and citrate. A number of compounds such as alcohols, acids, aromatic compounds, heterocyclic compounds, esters, hydrocarbons, and many more are available in yoghurt at low

to trace amounts. Other than lactic acid, the natural flavor compounds in yoghurt are acetaldehyde, acetone, diacetyl, acetoin, and 2-butanone (Cheng 2010). Rincon-Delgadillo et al. (2012) used the starter distillates (SDL), which is a major ingredient for the formulation of a wide range of products including cottage cheese, vegetable oil spreads, margarine, processed cheese, and sour cream to enhance the buttery aroma in the products. The buttery aroma is possible because of the presence of vicinal dicarbonyl and diacetyl which is a key component of SDL. Diacetyl (2,3-butanedione) is a product of citrate metabolism of certain bacteria such as *Lactococcus lactis*. A gene *bud A* encoding alpha-acetolactate when knocked out in strain resulted in the inactivation of diacetyl reductases, thereby eliminating chances of loss of diacetyl owing to its reduction (Zhang et al. 2015). Cofactor engineering has also been applied for enhancing production of diacetyl (Wang et al. 2013); deletion of the gene encoding phosphotransacetylase (*pta*) enhances diacetyl production by about 130% (Wang et al. 2019).

7.2.2 Esters

Ester is mainly produced from an organic or inorganic acid. It contains at least one hydroxyl (-OH) group which is replaced by an alkoxy (-O-alkyl) group. Glycerides are fatty acid esters of glycerol which are the main classes of lipids and generate bulk animal fats and vegetable oils. Low molecular weight esters are used as fragrance that is usually found in pheromones and essential oils.

El Hadi et al. (2013) reported that a number of fruits produce a wide range of volatile compounds which contribute to their flavor. These volatile compounds are composed of esters, aldehydes, lactones, alcohols, apocarotenoids, ketones, and terpenoids. These are produced via biosynthesis initiated from amino acid, lipid, and terpenoid, and once the basic molecules are synthesized, a number of modifications such as acylation, oxidation/reduction, methylation, and cyclic ring closure can be done to allow the compounds to impart proper aroma. Menendez-Bravo et al. (2017) redesigned microbial pathways for the production of routinely used consumer products including waxes, esters, fatty alcohols, and fatty acids. Small-chain aliphatic esters are major elements of flavors and fragrances, while long-chain esters are composed of acids and fatty acids esterified to fatty alcohols. These have been extensively used in paints, coating, lubricant formulas, and cosmetics.

7.2.3 Benzaldehyde

Benzaldehyde is the simplest aromatic aldehyde with a single formyl group. Schade et al. (2001) studied that artificial almond oil is one of the signature vaporized compounds for fragrance of carnation flowers. The production of synthetic and natural benzaldehyde per year equals to 7000 tons and 100 tons, respectively. Usually, 80% of benzaldehyde is produced from cinnamaldehyde by retro aldol reaction (Verma et al. 2017). Benzaldehyde is naturally extracted from cassia, bitter

almond oil, cheese, black tea, kernels, fruits, or leaves of apricots, apples, and peaches (Sen 2015). Naturally, benzoic aldehyde is freed from cyanogenic glycoside amygdalin by using β-glucosidase and mandelonitrile benzaldehyde lyase enzymes which gives a cheery and bitter almond taste (Opgrande et al. 2000). Benzenecarbaldehyde is an essential chemical component for the food and chemical industry. This molecule is used for a wide range of value-added chemicals such as production of cinnamic acid, different aniline dyes, aliphatic fragrance, flavoring agents, intermediate for pharmaceutical products, and bee-repellent for harvesting of bees (Wiener et al. 1988).

In insects, phenylmethanal serves as chemical defense (harvester ants) and pheromones (noctuid lepidoptera, trigona stingless bees) (Beroza 2012). It is also reported to exhibit antitumor, antibacterial and antifungal activities. In 1922, Match suggested that a minimum benzaldehyde concentration of 0.2% in saline could induce loosening and antispasmodic effect in involuntary non-striated muscles of the urinary bladder, uterus, bronchi, arteries, and stomach from different animal species. The process of forming phenylmethanal is possible by single-step bioconversion and de novo synthesis by utilizing flora, microorganisms, or even isolated enzymes (Krings et al. 1998). The bacteria *P. putida* and different species of white rot fungi such as *Trametes suaveolens*, *Polyporus tuberaster* (Kawabe et al. 1994), *Bjerkandera adusta* (Lapadatescu and Bonnarme 1999), *Phanerochaete chrysosporium* (Kim 2005), and *Pleurotus sapidus* (Lomascolo et al. 1999) naturally synthesize benzenecarbaldehyde through the de novo pathway (Paula Dionísio et al. 2012). The amino acid degradation pathways beginning from lipid oxidation of phenylalanine give benzoic aldehyde at pH 3. The presence of 13-hydroperoxide of linoleic acid (LOOH) or 4-oxononenal (ON), derivatives of phenylalanine (phenylacetaldehyde), metabolizes into benzaldehyde and releases a fruity-like GRAS flavoring molecule that has shown to be present in volatile fractions of almonds, cherry, tea leaves, and other food products (Hidalgo and Zamora 2019). Microbes *Lactobacillus plantarum* (Groot et al. 1998), *Streptococcus thermophilus* (Dan et al. 2018), and *L. helveticus* (Klein et al. 2001) induce production of phenylmethanal via catabolism of amino acids. From the manganese transport system of *L. plantarum*, it has been concluded that phenylalanine gets converted into benzoic aldehyde by pyridoxal 50-phosphate-dependent aminotransferase (Petrovici et al. 2018). In plants, through β-oxidative and non-β-oxidative pathways, phenylalanine is converted to benzaldehyde. Recently scientists have found a clue step of β-oxidative pathways where synthesis of benzoate takes place, which serves as the precursor molecule for benzaldehyde in a metabolic engineered pathway of microbes (Kunjapur and Prather 2015). The microbial biosynthesis of phenylmethanal from phenylmethanol takes place in methylotrophic yeast, *Pichia pastoris*, in single-step bioconversion via involvement of different enzymatic roles such as alcohol oxidase and formaldehyde dehydrogenase. The solid-liquid two-phase system (TPPB) uses an immiscible organic solvent for isolating benzenecarbaldehyde compounds (Jain et al. 2010).

Genetic recombination of *Escherichia coli* K-12 MG1655 strain resulting in deletion of gene encoding aldehyde reductase and recombinant carboxylic acid

reductase yielded 350 mg/L production of benzaldehyde from benzoate with only 12% conversion to benzyl alcohol (Carroll et al. 2016). After deletion of cinnamyl alcohol dehydrogenases (*YahK* and *YjgB*) gene, accumulation of benzaldehyde in *E. coli* was observed. In pharmaceutical companies, benzaldehyde is used for the formation of precursor compound L-phenylacetylcarbinol which is involved in the synthesis of ephedrine (Kunjapur et al. 2014).

Benzaldehyde is majorly found in plants such as cucumber. Benzaldehyde synthase is the final enzyme in the bio-formation of benzaldehyde from phenylalanine. Baoxiu Liu and researcher showed that in *Cucumis sativus* L., genes cinnamaldehyde:NADP+oxidoreductase *CsaCCR7* (root cytosol-specific), *CsaCCR9* (peroxisome), and *CsaCCR18* (flower cytosol-specific) are in charge for the production of benzaldehyde from benzoyl-CoA. This was the first time when CCR as in benzaldehyde synthase added a new biochemical role in flora (Liu et al. 2019).

Though in the skin benzoic aldehyde quickly converts into benzoic acid, it shows no unpleasant reaction toward skin irritation or sensitization. Artificial almond oil (benzaldehyde) easily diffuses and is absorbed by the skin and the lungs tissue but does not amass in any particular tissue type. After metabolism of C_6H_5CHO, conjugates are formed with glycine or glucuronic acid and discharged in urine. Benzaldehyde is also confirmed by the US Bureau of Alcohol, Tobacco and Firearms for its usage as a denaturant in different denatured alcohol at 27CFR21.151. Natural benzaldehyde can be used in bath preparation (2%), non-coloring preparation (1%), skin care preparation (1–2%), and fragrance preparation in perfumes and colognes at 0.5% concentration (Andersen 2006).

7.2.4 Alcohols

Excessive alcohol production by yeast continues to be a major concern in the field of distilled liquors. Medium-chain alcohols, among others, have a major effect on the final flavor profile of alcoholic beverages even at low concentrations. It is now widely accepted that the synthesis of chemical compounds in yeasts adversely affects the development of alcohol, in particular carbon metabolites. Conversely, it may not be clear how homeostasis of oxygen and carbohydrates could ultimately impact the alcohol levels in fermented beverages. Consequences between the depletion of oxygen by glucose spike mainly on the accumulation of alcohol in liquid culture and on inclusion of glucose (20 g/L) and leucine (9.8 g/L) as aggregants in alcoholic beverages have been tested using *Saccharomyces cerevisiae* from the industrialized Brazilian cachaça type. The alcoholic fermentation compounds as well as the carbon dioxide balance were analyzed in order to relate the results to biochemical evidence. All findings indicate that the aggregation of isoamyl alcohol by yeast may not be impaired by the presence of oxygen in the atmosphere. Elevated conjugated linoleic acid tests showed a recent and unforeseen accumulation of isobutanol, active amyl alcohol, and 2-phenylethanol that may be linked to gene expression of valine, isoleucine, and phenylalanine biosynthesis. From the emission

contexts, findings further demonstrate that the metabolism of isoamyl alcohol, isobutanol, and effective amyl alcohol, even if not 2-phenylethanol, through wild yeast in some kind of stagnant era, indicates the importance of these higher alcohols as both a carbon supply for carrying costs and/or oxidation/reduction of immune function during that period (Espinosa et al. 2015).

Methodologies for improving energy utilization in the fermentation process of ethanol are now based almost exclusively on the development of technology centered on the use of lignocellulose carbohydrate fractions. Some of the so-called "second-generation" innovations require metabolically adjusted production strains, which maintain a high level of catabolic versatility and are homoethanologenic. It may have been suggested that the processing of ethanol at extreme temperatures could promote its production. The expected offspring of *Geobacillus thermoglucosidasius*, *Thermoanaerobacterium saccharolyticum*, and *Thermoanaerobacter mathranii* already constitute the platform technology of many modern biotech companies. All originally proposed achievements in the development of these processing varieties specially focus on the establishment of productive sources of cellular competence, gene removal, or subsequent activation (Taylor et al. 2009).

Renewable energy technology production would gradually be implemented through efforts of societies or industries to fulfill the nation's financial production targets, as well as to overcome the issues of energy production and climate change. Typically, nearly the entire production of bioethanol tends to be from the cultivation of agricultural crops or biomass. There appears to be a great deal of debate over the cost or energy efficiency of this form of biomass-based ethanol production operation. Conversion may have been achieved using a double homologous recombination process that inserts pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*) ethanol-producing genes *Zymomonas mobilis* into the *Synechocystis* PCC 6803 chromosome under the influence of a solid, light-charged psbA II promoter. The PCR-dependent analysis or ethanol performance test is used to test healthy processors. For the analysis of cyanobacterial cultured cells and the production of ethanol, an automated photobioreactor platform for something like analytical preparation and device selection has been developed. The approach described above suggests an improvement in yield of 5.2 mmol OD_{730} unit/L/day with no required antibiotic/selective agent (Dexter and Fu 2009).

7.2.5 Ketones

4-(4-Hydroxyphenyl)-butan-2-one (raspberry ketones) is an important natural flavoring agent (Liu et al. 2020). It shows several activities such as it attenuates cyclophosphamide-induced pulmonary toxicity (Mohamed et al. 2020), controls hyperlipidemia and insulin resistance (Mehanna et al. 2018), de-pigmenting activity (Lin et al. 2011), antimicrobial activity (Gupta et al. 2015), acts as an antioxidant (Mohamed et al. 2018), antiandrogenic activity, etc. (Ogawa et al. 2010). Due to wide usages in soft drink, sweet, and ice cream, it has a high market demand. Plant

gene has been incorporated for microbial production (Beekwilder et al. 2007). Microbial production has also been achieved by using other strategies such as catalytic aldolization of precursors (Feron et al. 2007), pathway engineering and synthetic enzyme fusion (Lee et al. 2016), and construction of synthetic pathways in *E. coli* (Wang et al. 2019).

7.2.6 Lactones

Lactones are defined as cyclic carboxylic esters and are ubiquitous in nature such as saturated and unsaturated γ-lactones (plants) and δ-lactones (animal products). The organoleptic property of aliphatic lactones is used for components of food. The rising demand of natural flavors and fragrance makes biosynthesis of lactone production more enticing. Flavored lactones are formed via chemical synthesis or microbial and enzymatic conversion (Gatfield et al. 1997). Lactone has various tastes and aroma say oily, creamy, coconut-like, fruity, milky, honey, peachy, and others. Different species of fungi can produce lactone from triolein, sebum, oleic acids, lecithin, and Tween 80 as substrates (Shaaban et al. 2016).

Derivatives of lactone serve as a building block in the environment such as enzyme lactonase, epinepetalactone, and food additive glucono delta-lactone. Majorly δ-lactones and γ-butyrolactone serve as structural scaffolds for different active natural compounds (Andrushko et al. 2013). Lactone derivatives are remarkably used as pheromones and flavoring compounds derived from many flowers and fruits (Mori 1984). Lactone of D-gluconic acid (gluconolactone) is used as a food additive, an acidifier, or leavening agent (Feiner 2016). Spironolactone is a steroid lactone that plays a role in diuretic, antihypertensive, and antiandrogen. Macrolides consist of macrocyclic lactone rings that have antifungal and antibiotic properties against *S. pneumoniae*, *Bordetella pertussis*, and *Haemophilus influenzae*. Sesquiterpene lactone (vernolepin) and epothilones are used as anticancer and antitumor drugs. The 16 macrocyclic lactone (avermectin) is used as a drug and pesticide (Heravi et al. 2019). The most important bacterial signaling lactone molecule (*N-acyl homoserine* lactone) is involved in bioluminescence, production of biofilm, expression of virulence factors, and formation of antibiotics (Yajima 2016; Gohil et al. 2018). Even polycaprolactone is extensively used in scaffolding and tissue engineering (Ducheyne et al. 2015).

Fungus *Trichoderma viride* EMCC-107 produced 6-amyl-α-pyrone through solid-state fermentation of sugarcane bagasse and imparted strong coconut fragrance (Fadel et al. 2015). Other coconut lactone flavors can be produced by *Tyromyces sambuceus* and *Cladosporium suaveolens* from ricinoleic acid of castor oil (-γ-decalactone) and linoleic acid (δ-dodecalactone) (Sharma et al. 2020). Lactones used in dairy and milk products have milky, buttery, and coconut flavors produced by microbiological pathways. Lactone also tends to give out stale flavor when used in excess quantities of heated milk (Gupta et al. 2015). *Candida tropicalis*, *Yarrowia lipolytica*, and *Sporidiobolus salmonicolor* yeasts amass δ-dodecalactone by β-oxidation of ricinoleic acid that gives fruity, oily aroma which is used to impart

apricot, strawberry, and peach flavors (Schrader et al. 2004; Longo et al. 2006). The production of γ-decalactone by using *Y. lipolytica* CCMA 02042 and *Lindnera saturnus* CCMA 0243 yeasts through β-oxidation of ricinoleic acid or crude glycerol yields 214.8 mg/L and 512.5 mg/L, respectively (de Andrade et al. 2017). Waché et al. (2001) stated that decreasing the activity of acyl-CoA oxidase helps in collecting a higher concentration of γ-decalactone. In 2011, strain *Y. lipolytica* TA1 was studied to investigate about the enzyme AOX3 encoded by the *POX3* gene which is responsible for a low yield due to breakdown of lactone. By introducing copper resistance gene *CRF1* to *Y. lipolytica locus* of *POX3* gene, it was demonstrated to accumulate 2.9 times (0.531 g/L at 63 h) higher yield of lactone than 0.194 g/L at 57 h in *Y. lipolytica* AS2.1045. That enlightened the troubleshooting for the fragrance producing industry (Guo et al. 2011). Biotransformation using microorganisms such as *C. boidinii*, *Mucor circinelloides*, and *S. ruinenii* produced γ-decalactone yielding 40.9 g/L, 10.5 g/L, and 5.5 g/L from ricinoleic acid, ethyl decanoate, and methyl ricinoleate, respectively (Kourist and Hilterhaus 2015). The production of lactone in cytosol before import of protein into peroxisomes takes place in the presence of *SmOHYp* gene in strain ST8822. The engineered strain ST7584 (Δpox1–6 LaLHY YlPOX2), by deletion of FAA1, exhibited 0.17 ± 0.03 mg/L production of δ-decalactone. The enhancement of δ-decalactone by copying cytosolic *LaLHY* gene titer 1.74 ± 0.3 mg/L suggested that low levels of hydratase activity influence the flux of lactone (Marella et al. 2020). Microbial production of lactones has become advanced in the field of biorenewable molecules (triacetic acid lactone), fragrance and natural flavors, and pharmaceutical intermediate sector.

7.2.7 Vanillin

Vanillin is an important aromatic flavoring compound that is widely used in beverages, foods, perfumes, and medicines (Priefert et al. 2001). Currently, more than 10 tons per year of this compound is produced through chemical synthesis worldwide. Bio-based production of vanillin is achieved through the conversion of lignin, isoeugenol, aromatic amino acids, phenolic stilbenes, and eugenol using bacteria, fungi, plant cells, and engineered microorganisms (Priefert et al. 2001). Shimoni et al. (2000) had stated that natural aroma compounds are major attention to the fragrance and flavor industry. A number of microorganisms have the natural ability to produce these aromatic aldehydes as intermediates in phenylpropanoid degradation pathway. In a study, researchers isolated *Bacillus subtilis* and used that for bioconversion of eugenol into vanillin. In the presence of eugenol, *B. subtilis* produced 0.61 g/L vanillin (molar yield 12.4%) in growth culture, while in cell-free extract, it was observed 0.9 g/L vanillin (molar yield 14%). Similarly, Walton et al. (2000) isolated *Pseudomonas* strains that were able to produce 6 g/L vanillin from ferulic acid. They characterized biosynthetic pathways at the enzyme and molecular-level for the production of vanillin from eugenol via ferulic acid. This study suggests that it can be further expanded toward higher vanillin production at industrial scale.

Krings and Berger (1998) reported that natural aroma compound (vanillin, benzaldehyde (bitter almond, cherry) and 4-(R)-decanolide (fruity-fatty)) demands have been significantly increasing which demands its scale-up to several thousand tons per year. A single-step biotransformation, de novo synthesis, and bioconversion have been attempted for the production of these compounds using microorganisms and plant cells.

7.2.8 Terpenes

Compounds grouped as terpenes have five carbon isoprene units (2-methyl-1,3-butadiene) which are further classified into groups based on the number of isoprene units, such as two (monoterpenes), three (sesquiterpenes), four (diterpenes), six (triterpenes), eight (tetraterpenes), or more (polyterpenes). Terpenes are potential biofuel (Mewalal et al. 2017) and antioxidants (Ng et al. 2000). These can be converted into various important compounds which hold importance in pharmaceutical, agrochemical, and flavor and fragrance industries (Monteiro and Veloso 2004). Apart from that, terpenes are also part of essential oils (Noriega 2020), resin ducts (Rahman et al. 2008), and rubber (Hattori et al. 2008) (Table 7.1).

Limonene is widely used in the food and cosmetic industry. Several strategies have been tested for microbial production such as employing heterologous mevalonate pathway in *E. coli* by incorporation of single plasmid, i.e., pMevT and pMBI (a pMBIS variant) for production (Alonso-Gutierrez et al. 2013). Expression of MmMK with ScPMK, ScPMD, and ScIDI under FAB80 promoter resulted in an efficient midstream module to produce 181.73 mg/L of limonene (Wu et al. 2019).

7.2.8.1 Valencene

Valencene is a biosynthetic product of farnesyl pyrophosphate (FPP) that has a characteristic juicy, orange, sweet, and woody fragrance, and it is also an aroma component of citrus fruits. It contributes to the production of nootkatone which is the most significant and expensive aromatic component of grapefruit (Bomgardner 2012). Since it helps in decreasing somatic fat ratio, it has a higher demand in cosmetics and fiber sectors. The skin of Valencia oranges has a significant amount of valencene. Valencene has significance in the fragrance and flavoring industry and finds application in fruit-flavored beverages, perfumes, chewing gum, personal care, and cleaning products (Waltz 2015; Gupta et al. 2015).

Valencene has a high value in industry, but its extraction is very expensive, and therefore several researches have been conducted for achieving its high yield. In 2014, Beekwilder et al. (2014) engineered the valencene synthase gene (*CnVS*) in *Rhodobacter sphaeroides* strains and also included the expression of mevalonate operon from *Paracoccus zeaxanthinifaciens*. Recombinant *Corynebacterium glutamicum* was constructed having valencene synthase from *Nootka cypress* and genes *erg20* and *ispA* were overexpressed which gave positive results with about 2.41 mg/L valencene yield (Frohwitter et al. 2014). Microorganism such as *S. cerevisiae* is used as gene expression host because it is safe and comes with

Table 7.1 Important terpenes and their plant sources and activities

Sr. No.	Terpene compound	Sources	Activity	References
1	α-Bisabolol	*Plinia cerrocampanensis*	Antimicrobial	Vila et al. (2010)
		Nectandra amazonum	Anti-*trichomonas vaginalis*	Farias et al. (2019)
		Salvia species	–	Sandasi et al. (2012)
		Laserpitium zernyi	Antimicrobial	Popović et al. (2010)
		Eremanthus erythropappus		Santos et al. (2019)
2	β-Caryophyllene	*Psidium guajava*	–	Arain et al. (2019)
		Zingiber nimmonii	Antimicrobial	Sabulal et al. (2006)
		Plectranthus barbatus	Mosquito larvicidal activities	Govindarajan et al. (2016)
		Aquilaria crassna	Antimicrobial	Dahham et al. (2015)
		Murraya paniculata	Antioxidant activity	Selestino et al. (2017)
3	α-Humulene	*Cheilocostus speciosus*	Insecticidal activity	Benelli et al. (2018)
		Syzygium zeylanicum	Larvicidal activity	Govindarajan and Benelli (2016)
4	(+)-limonene	Citrus lemon, *Mentha spicata*	Antibacterial, insecticidal, repellent	Lopresto et al. (2014), Aggarwal et al. (2002), Ibrahim et al. (2001)
5	Linalool	*Croton cajucara*, *Litsea glaucescens*	Antimicrobial, antioxidant activity, anticonvulsant activity, antidepressant	Alviano et al. (2005), Seol et al. (2016)
6	Myrcene	Lemon grass, *Citrus aurantium*	Analgesic activity	Lorenzetti et al. (1991), Bonamin et al. (2014)
7	α-Pinene	*Syzygium cumini*	Anti-*Leishmania* activity	Rodrigues et al. (2015)
8	β-Pinene		Anti-inflammatory and chondroprotective activity	Rufino et al. (2014)

highly recommended physiological properties (Chen et al. 2019; Ozaydin et al. 2013; Asadollahi et al. 2008).

7.2.8.2 Nootkatone

Sesquiterpenoids are highly value-added aroma compounds found in plants such as strawberry (Hampel et al. 2006). (+)-Nootkatone was first reported from *Cupressus*

nootkatensis and is found in grapefruits in trace amount (MacLeod et al. 1964). It is also isolated from *Alpiniae oxyphyllae* (He et al. 2018) and *Cyperus rotundus* L. (Jaiswal et al. 2014).

Multifaceted activities such as anti-inflammatory (Khan et al. 2011; Tsoyi et al. 2011), neuroprotective (He et al. 2018), antiplatelet (Seo et al. 2011), antibacterial (Yamaguchi 2019), and anticancer (Yoo et al. 2020) are exhibited by (+)-nootkatone. Studies also report that (+)-nootkatone acts as repellent to termites (Maistrello et al. 2003), and maize and rice weevil (Mao et al. 2010), shows toxicity against arthropods (Anderson and Coats 2012) and anti-biofilm efficacy against *Staphylococcus aureus* (Farha et al. 2020). Majorly it is used in food (Shaw et al. 1981; Del et al. 1992), cosmetics, and pharmaceuticals (Leonhardt and Berger 2014).

Due to limited biological production of (+)-nootkatone, it is currently produced through chemical synthesis to fulfil market demand. Due to lesser availability of its natural form in the market, it is alternatively being produced in bacteria, fungi, and plant cells. Cell extracts and purified enzymes are used for the conversion of allylic oxidation of (+)-valencene that allows natural production of (+)-nootkatone in high yields (Fraatz et al. 2009). Terpenoid compounds have always played an important role in the aroma industry. Microbial production leads to the production of desired products. Engineered organisms can be utilized for the production of such terpenes at low cost. Various strategies have been implemented to enhance production such as increase in supply of carbon source (Schindler 1982), high flux toward the central C5 prenyl diphosphate precursors (Schempp et al. 2018), expression of a synthetic amorpha-4,11-diene synthase gene, and the mevalonate isoprenoid pathway from *S. cerevisiae* into *E. coli* (Martin et al. 2003).

7.2.8.3 Patchoulol

A frequently used sesquiterpene alcohol in the chemical industry is patchoulol ($C_{15}H_{26}O$). It can be extracted using steam distillation from the leaves of patchouli plant. It is a major component of patchouli oil, and its odor is known for its characteristic pleasant and long-lasting woody, earthy, camphoraceous odor. Therefore, it is a widely used fragrance ingredient in products like soaps, cosmetics, and perfumes (Bauer et al. 1997). The responsible factor of the typical fragrance of patchouli is (−)-optical isomer. Patchoulol shows anti-inflammatory (Jeong et al. 2013; Xian et al. 2011; Su et al. 2016), anti-viral, anti-ulcer, anti-aging (Zheng et al. 2014; Feng 2014), antidepressant (Sah et al. 2011), antitumorigenic, and anti-influenza activities (Jeong et al. 2013; Lee et al. 2020).

Earlier, the predominant commercial source of patchoulol was patchouli plants (Näf 1981). But now, with advancements in research, there are other promising approaches that present cost-effective synthetic routes for producing enantiomeric pure patchoulol (Martin et al. 2003; Besumbes 2004). Also, steam distillation process is energy- and resource-intensive, and therefore for minimizing its environmental footprint and improving production yield, fermentation process for production of patchoulol is used. By converting FPP to patchoulol using patchoulol synthase enzyme (*Pc*PS; PTS, uniprot: Q49SP3) (Deguerry et al. 2006; Munck

et al. 1990) and using different microbial hosts, say *S. cerevisiae* (Gruchattka et al. 2013), the moss *Physcomitrella patens* (Zhan et al. 2014), and the green microalga *Chlamydomonas reinhardtii* (Lauersen et al. 2016), the heterogeneous expression of patchoulol has been achieved. Bacteria such as *E. coli* and *C. glutamicum* also tend to give better results through this approach (Henke et al. 2018).

7.2.8.4 Sclareol

A fragrant amber-colored chemical named sclareol found in *Salvia sclarea* is a type of diterpene alcohol (Lawrence 1986). It has a sweet and balsamic fragrance, alike *Cistus creticus* (Cistaceae) (Demetzos et al. 1999), *Nicotiana glutinosa* (Solanaceae) (Guo and Wagner 1995), and *Cleome spinosa* (Brassicaceae) (McNeil et al. 2010). It not only has its importance in the perfume industry but also possesses properties like antibacterial, antifungal, and growth regulating activities (Demetzos et al. 1999; Ulubelen et al. 1994). Sclareol is the basic component for synthesizing Ambrox (Schmiderer et al. 2008) that ultimately contributes as a better substitute for ambergris (Barrero et al. 1993). Ambergris is secreted by sperm whales which has a musky, sweet earthy odor, a heavily demanded product in the perfume industry. As it originates from an endangered species, its use is controversial.

The annual production of sclareol from leaves and flowers of clary sage (*S. sclarea*) is greatly affected due to several environmental factors; therefore, many researches were conducted to find alternative cost-efficient and scalable enzymatic production platforms for improving it on high yield. *E. coli* and *S. cerevisiae* have been used as alternatives by elucidating and reconstructing sclareol biosynthetic pathways (Schalk et al. 2012; Ulubelen et al. 1994; Cutler et al. 1977). Genetic manipulation in yeast and moss *P. patens* also enhances sclareol production (Trikka et al. 2015; Pan et al. 2015).

7.2.8.5 Steviol

Stevia rebaudiana (sugarleaf) is a naturally produced sweetener like stevioside and rebaudioside (Dan et al. 2018). The leaves consist of 9.1% and 3.8% glycoside diterpenes stevioside and rebaudioside A, respectively (Goyal et al. 2010). Steviol glycoside has zero calorie. Steviol has a methylene bond between C16 and C17, including diterpenoid steviol as a structural compound with three glucose moieties at C 13-hydroxyl/C 19-carboxylic acid group. The methylene bond gives a sweet taste and serves as a pharmacophore for industries (Upreti et al. 2012). Artificial sweetener stevia is used to enhance palatability in the food products, beverages, dietary supplements, and diabetic sugar as it also serves as a heat and acidity stabilizer. It is also used in fermentation, hardening, absorption of soy sauce, pickles, bread, and biscuits. Stevia is also non-cariogenic (Mitchell 2008).

The leaves of stevia when exposed to gamma radiation showed double production of *Reb A* and by treatment of ethyl methanesulfonate resulted in 1.5–2.0-fold increment of genes *Stev* and *Reb A* indifference to control plants. By increasing the gene expression of UGT74G1 (Stev biosynthesis) and UGT76G1 (Reb biosynthesis), it was found that improvement of steviol glycosides take place in EMS and gamma ray-treated plants (Khan et al. 2016). The chameleon enzyme,

glucosyltransferase UGT76G1, aims at the metabolism of rebaudioside D (RebD) and rebaudioside M (Reb M). Reb D and Reb M enhance the sweet profile and decrease the bitter taste compared to Reb A and stevioside (Olsson et al. 2016).

The reduction of steviol glycoside has been observed by downregulating the transcription genes involved in steviol metabolism: *ent-KS1* (*ent-* kaurene synthase 1), *ent-KO* (*ent*-kaurene oxidase), *ent-KAH* (ent-kaurenoic acid 13-hydroxylase), *UGT85C2*, *UGT74G1*, and *UGT76G1* in polyethylene glycol-treated stevia plants (Hajihashemi et al. 2016). Constructing steviol metabolic pathways in *E. coli* increases the production of steviol. Isoprenoid serves as a precursor molecule for steviol biosynthesis in engineered *E. coli* strain. Moon and colleagues concluded that by expressing the UtrCYP714A2-AtCPR2 fusion protein obtained from *Arabidopsis thaliana*, 5′-untranslated region engineering of KS (*ent*-kaurene synthase) and modification of N-terminal KO (*ent*-kaurene oxidase) sequence, steviol production enhanced up to 38.4 ± 1.7 mg/L in batch fermentation (Moon et al. 2020). Stevioside protects against food-borne bacteria such as *Bacillus cereus*, *Klebsiella pneumoniae*, and *P. aeruginosa* (Puri et al. 2011). Steviol glycoside is also used to decrease blood pressure, to maintain blood glucose level in diabetes mellitus, and also to avoid atherosclerosis (Hajihashemi et al. 2013).

7.3 Conclusion and Future Remarks

The flavor and fragrance market is exponentially growing, and its global demand is quiet high. In order to fulfill the current demand, microbial production is an alternative source met through modifying or extending natural pathways in heterologous microorganisms for overproduction of these molecules. Currently, a number of stronger and well-characterized genetic parts including promoter, ribosome binding site, degradation tag, and transcription terminator are being employed for replacing native weak parts with strong part for boosting production of flavor and fragrance. Several flavors and fragrance are already available in the market, and many will be available soon for commercial applications. In the near future, it is anticipated that it will increase and improve the production of molecules to meet various industrial, pharmaceutical, and biotechnological applications.

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Beneficial Effects of Psychobiotic Bacteria, Cyanobacteria, Algae, and Modified Yeast in Various Food Industries

8

Aeshna Gupta and Indra Mani

Abstract

The exploration of microorganisms in various food and beverage industries has been established since time immemorial. They have been used extensively in the food industry to carry out fermentation reactions for the manufacture of bread and different alcoholic drinks using different carbohydrates, for processing different cheese varieties and chocolates, and for converting raw foods into pickles and can also be used as a food source themselves. This chapter briefly highlights the characteristics of various microbial communities namely psychobiotic bacteria, cyanobacteria, algae, and modified yeast. The beneficial effects and applications of these microbial groups in different food industries have been discussed in detail in this chapter.

Keywords

Fermentation · Probiotic · Psychobiotic · Cyanobacteria · Algae · Modified yeast

8.1 Introduction

Since thousands of years, microorganisms have been used in the production of a wide range of foods and beverages that make up a common human diet. Fermentation is among the earliest method of food processing and preservation. This biochemical process not only helps food to be preserved but also increases its nutritional and organoleptic characteristics (flavor, sight, odor, touch). Microorganisms have been utilized in fermentation since Neolithic times. In 7000 BCE, there is evidence

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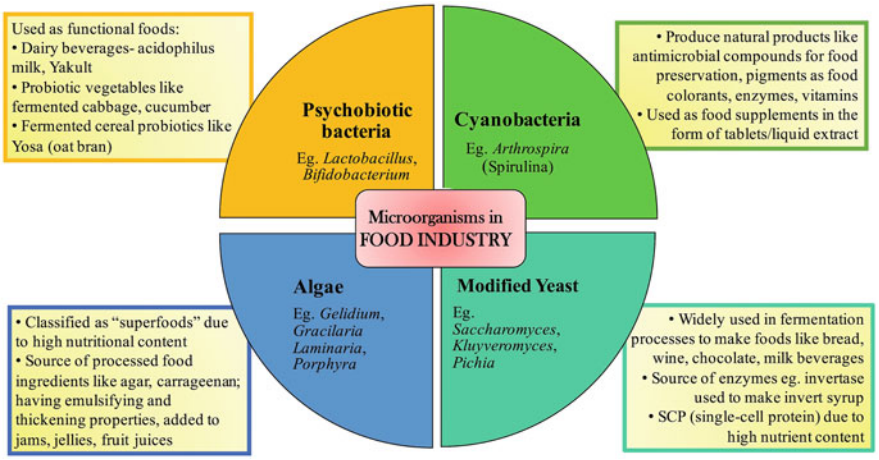

Fig. 8.1 Overview of the beneficial effects of different microorganisms in the food

of ancient Babylonians using fermentation to produce beer. The first documented production of cheese and butter by fermentation dates back to 3000 BCE.

Nowadays, microbes are used in a variety of ways in the food industry. Lactobacilli are needed for the production of foods that utilize lactic acid fermentation, in particular, dairy foods (yogurt, cheese), fermented vegetables (olives, pickles, sauerkraut), etc. (Jay 1996). In the baking and brewing industry, fungi, particularly *Saccharomyces*, have historically been used. *Saccharomyces cerevisiae* is vital in generating revenue from the brewing industry to such an extent that the State of Oregon declared it the official state microbe in 2013 (Semenova et al. 2006). Not only do microbes give foods a fine flavor, texture, and aroma, but they also produce some inhibitory molecules that help in preventing the spoilage of food, thereby improving food storage and safety (Kumar 2016). Overview of the beneficial effects of different microorganisms in the food is given in Fig. 8.1. The following sections discuss the beneficial effects of various microorganisms in the food industry.

8.2 Beneficial Effects of Psychobiotic Bacteria in Food Industry

Functional foods are now a component of everyday diet in the modern world with probiotics and prebiotics being the most significant and widely used functional foods (Nagpal et al. 2012). The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in appropriate concentrations impart health benefits to the host" (FAO/WHO 2002), and prebiotics are non-digestible food ingredients which enable such microbes to grow and function in the gut (Gibson and Roberfroid 1995). Probiotic intake has a number of advantages, like improved intestinal health, regulated by gut microbiota (all microbes and their

genomes inhabiting the intestinal tract). It also helps to stimulate and develop the immune system and enhances nutrient metabolism and bioavailability. The Gram-positive *Bifidobacterium* and *Lactobacillus* families are the most commonly used probiotic bacteria (Mayer et al. 2014; Burnet and Cowen 2013).

The probiotics and prebiotics which influence bacteria-brain relationships are termed as psychobiotics (Sarkar et al. 2016). Psychobiotics confer mental health benefits through interactions with commensal gut bacteria (native bacterial species residing in the digestive tract that exist in symbiotic associations with the host) (Sarkar et al. 2016). They enhance the gastrointestinal (GI) function and the antidepressant and anxiolytic ability by influencing CNS-related processes regulated by the gut-brain axis (GBA) through immune, humoral, neural, and metabolic pathways (Cheng et al. 2019). Species of bacteria which have been utilized for probiotic psychobiotic research include *Lactobacillus helveticus*, *L. casei*, *L. plantarum*, *Bifidobacterium longum*, *B. breve*, etc.

8.2.1 Probiotics as Functional Foods

Probiotics can be utilized as a variety of products like foods, drugs, and nutritional supplements (Song et al. 2012). Following are some applications of probiotics as fermented dairy products and non-dairy food products:

1. *Dairy-based probiotic foods*: Milk products play a vital role in administering probiotic bacteria to humans because they establish an optimal environment to support their growth and viability (Gardiner et al. 1999; Ross et al. 2002; Saarela et al. 2006; Phillips et al. 2006). A number of probiotic dairy beverages are available in the market, e.g., acidophilus milk, Nu-Trish A/B, bifidus milk, Yakult, etc. (Özer and Kirmaci 2010). In addition to fermented milk beverages, yogurt and cheese also serve as probiotic carriers. *Lactobacillus rhamnosus* GG is the most commonly employed probiotic bacteria in dairy beverage production (Succi et al. 2005).
2. *Vegetable-based probiotic products*: Fermented vegetables can serve as a convenient media for probiotic bacteria delivery like *Lactobacillus rhamnosus*, *L. casei*, and *L. plantarum* (Savard et al. 2003). Numerous studies have been carried out to develop probiotic vegetable products, for instance, fermented cabbage, carrots, onions, and cucumbers (Song et al. 2012).
3. *Cereal-based probiotic products*: Cereals are suitable substrates for the cultivation of probiotic strains, and non-digestible cereal matrix elements can also function as prebiotics (Charalampopoulos et al. 2002; Salovaara and Gänzle 2012). Yosa is a snack produced primarily in Finland and other Scandinavian countries from oat bran pudding prepared in water and fermented with lactic acid bacteria (LAB) and bifidobacteria. Because of the oat fiber and probiotic LAB components, it is considered a nutritious food (Wood 1997). Other cereal-based probiotic beverages are *Boza* (Bulgaria, Turkey) made from wheat, rye, and millet and *Pozol* (Mexico) made from maize (Prado et al. 2008).

4. *Meat-based probiotic products*: Fermented meats like dry sausages are the foods to which probiotic applications are limited. Dry sausages may serve as useful carriers of probiotics into the human GI tract as they are non-heated meat products. To ferment the sausage, lactic acid bacteria and staphylococci are utilized as starter cultures (Hammes and Hertel 1998; Tyopponen et al. 2003).

8.3 Beneficial Effects of Cyanobacteria in Food Industry

Cyanobacteria, also known as blue-green algae, are photosynthetic prokaryotes present in nearly every feasible ecosystem on earth (Ferris et al. 1996; Ward et al. 1997; Nubel et al. 1999, 2000; Abed and Garcia-Pichel 2001; Garcia-Pichel and Pringault 2001). While all cyanobacteria perform oxygenic photosynthesis, some may switch to bacterial anoxygenic photosynthesis using sulfide as an electron donor (Cohen et al. 1986). They carry out fermentation during anoxic conditions and in the dark (Stal 1997). Some cyanobacteria even form heterocysts and are capable of fixing atmospheric nitrogen (Capone et al. 2005). Cyanobacteria cells are a viable asset to biotechnology because of their ability to photosynthesize and fix nitrogen, and they are autotrophic (Singh et al. 2016, 2017; Lau et al. 2015).

8.3.1 Natural Products from Cyanobacteria

Cyanobacteria produce numerous molecules that uncover valuable properties with high prospect in sectors including the food industry:

1. *Antimicrobial compounds*: 85 families of metabolites with antimicrobial function are produced by cyanobacteria (Singh et al. 2016). Those with no toxic effects are especially desirable for use in the food industry, where they can be used to clean processing equipment or to preserve food (Sung et al. 2013; Abushelaibi et al. 2012).
2. *Pigments*: Cyanobacterial phycobiliproteins, phycoerythrin, and phycocyanin are colored water-soluble proteins that are used as natural pigments (Galetovic et al. 2020). Food colorants, food additives, and nutrients for human and animal feeds are popular applications of these sustainable, non-toxic pigments. The blue phycocyanin is extracted from *Spirulina platensis* and sold as a natural blue pigment for use in health foods and cosmetics by the Japanese company Dai Nippon Ink and Chemicals. Confectioneries, iced candies, and sherbets are some of the other applications of biliproteins (Branen et al. 2002).
3. *Enzymes*: Cyanobacteria are known to produce a wide range of enzymes that have industrial applications, e.g., protease, amylase, and phosphatase. Proteases are extensively utilized in the food processing industry (Venkatesan et al. 2010).
4. *Vitamins*: Some marine cyanobacteria are important vitamin sources, and they are utilized in large-scale synthesis of commercially useful vitamins like vitamins B and E. *Spirulina* (*Arthrospira*) is a good source of vitamin B12, beta-carotene,

thiamine, and riboflavin. It is commercially available as powder, granules, tablets, or capsules (Watanabe et al. 1999).

8.3.2 Cyanobacteria as Food Supplements

Human food supplements containing cyanobacteria are marketed in a variety of forms like tablets, capsules, and liquid (Radmer 1996). They are supposed to improve the nutritional value of pastas, snacks, candy bars, gums, as well as beverages (Liang et al. 2004). Because of its high protein level and nutritional value, *Spirulina* (*Arthrospira*) is the most commonly used cyanobacterial strain for human nutrition (Desmorieux and Decaen 2005; Soletto et al. 2005). It is considered to be one of the greatest sources of vitamin B12. Its protein content is over 60% and is rich in beta-carotene, thiamine, and riboflavin (Plavsic et al. 2004; Prasanna et al. 2010).

Aerospace agencies like NASA (National Aeronautics and Space Administration), JAXA (Japan Aerospace Exploration Agency) and ESA (European Space Agency) are exploring the use of microalgae to support astronauts' diets. They have also initiated research for the use of Spirulina as a primary food source for long-duration space travel or deep space operations. Evidently, microalgae may play a role in these missions by regenerating oxygen, supplying a sustainable food and fuel source and recycling waste.

8.4 Beneficial Effects of Algae in Food Industry

Algae are chlorophyll-containing plants of the most basic form, without any true roots, stems, leaves, or leaf-like organs. While most algae are autotrophic, meaning they synthesize their own food, heterotrophic and holozoic types are also common. They can be found in a number of environments, but majority are aquatic (Singh et al. 2014). These life forms are recognized among the fastest-growing organisms because they have a short doubling time. They possess various pathways for fixing atmospheric CO_2 and efficiently using the nutrients, turning it into biomass (Sharma and Sharma 2018). Based on cellularity, they can be divided broadly into macroalgae (macroscopic algae), e.g., *Ulva lactuca*, *Sargassum vulgare*, etc., and microalgae (microscopic algae) which include diatoms and dinoflagellates (Singh et al. 2005).

Algae with sophisticated photosynthetic systems can convert absorbed solar energy into other energy forms for food and metabolite processing. Furthermore, they have the potential to be valuable biocatalysts and can be used for enhancing the sustainable production of food (Singh et al. 2017). Therefore, algae appear to be essential elements of the agribusiness system, which includes commercial practices related to food production, processing, and supply, as well as industrial operations linked to the supply of production goods and facilities to agriculture and the agri-food sector (Goldberg 1981; Reardon and Timmer 2012; Clay and Feeney 2019).

8.4.1 Microalgae as Food

Microalgae have made their way into the food industry as food additives in the form of tablets, capsules, powders, and occasionally liquid extracts. They were instantly classified as "superfoods." Microalgae biomass is a rich store of several essential components including vitamins A, C, and E, as well as B1, B2, B6, and B12, along with niacin, biotin, nicotinate, folic acid, and pantothenic acid (Goh et al. 2009). It has high amounts of proteins with necessary amino acids, polysaccharide hydrocolloids, antioxidants like carotenoids and chlorophylls, active enzymes, monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs), and minerals in its nutritional profile (Batista et al. 2012; Goh et al. 2009; Tokusoglu et al. 2003; Priyadarshani et al. 2012). *Monostroma*, a green microalga, is widely harvested in port regions of Taiwan, Korea, and Japan. It is a very nutritive substrate due to its high amounts of calcium, magnesium, and lithium, as well as vitamins and amino acids like methionine. It is usually pressed into sheets and dried before being boiled with sugar, soy sauce, and some other additives to make "nori-jam" (Mouritsen et al. 2019).

8.4.2 Macroalgae as Food

Several coastal countries have historically utilized macroalgae/seaweeds as a food source and for other industrial purposes. *Porphyra* (a red alga) is a common food element because it contains 30–35% proteins, 40–45% carbohydrates, and numerous vitamins (Mouritsen et al. 2019). *Laminaria*, otherwise identified as kelp, is a brown alga that consists around 2% fat, 10% protein, and a large proportion of vital mineral components including iodine, potassium, magnesium, calcium, and iron. This brown alga has a high commercial value because it is one of the key additives in foods known as "kombu" or "konbu," which are widely included in Japanese dishes for making dashi, a soup stock (Mouritsen et al. 2019).

8.4.3 Algae as Processed Food Ingredients

Agar, alginates, and carrageenans are some of the most valuable products that can be extracted from algae and employed in the food industry because of their gelling and thickening effects.

1. *Agar*: Agar, made from the red algae *Gelidium* and *Gracilaria*, has a wide range of uses in food, including frozen foods, desserts, candies, and fruit juices (Ververi and Georghiou 2007).
2. *Carrageenans*: Carrageenan is a water-soluble polysaccharide extracted from algae that is widely used compared to agar, as an emulsifying and stabilizing agent in food. κ- and ι-carrageenans are often added in a variety of foods like

jellies, jams, deserts, and meat products because of their thickening properties (Ververi and Georghiou 2007).
3. *Alginate*: This compound is derived from brown alga and has chelating effect and the potential to produce highly viscous formulations making it a promising option for food and pharmaceutical industries (Ververi and Georghiou 2007).

It is also well established that the polysaccharides found in carrageenans and agar have antibacterial properties (Jönsson et al. 2020). Presently, these algae derivatives are being tested as food additives for food preservation, as components in biodegradable films, and as key elements in active packaging with a variety of purposes such as anti-biofilm and anti-fouling agents (Silva et al. 2020).

8.5 Beneficial Effects of Modified Yeast in Food Industry

Yeasts are eukaryotic unicellular fungi that can be found in abundance in nature. *Saccharomyces cerevisiae*, also known as "baker's yeast," is the most commonly utilized and explored yeast species. Yeasts are chemoorganotrophic organisms, implying that they get carbon and energy in the form of organic compounds. They utilize sugars such as glucose, fructose, and mannose. *S. cerevisiae* conveniently ferments these sugars into ethanol and carbon dioxide (Walker 2009).

Yeasts have been used in conventional fermentation processes to make beer, wine, and bread for centuries. There are many strategies to genetically modify yeast cells using recombinant DNA technology because they incorporate the benefits of unicellular organisms such as ease of genetic manipulation and growth. As a result, modern yeast biotechnology products have an impact on a wide range of economically relevant industries like food, beverages, chemicals, industrial enzymes, and so on (Walker 2009).

8.5.1 Yeast in Fermented Foods and Beverages

1. *Bread*: The most prevalent yeast species in bread and sourdoughs is *S. cerevisiae*. Since the nineteenth century, it has been utilized as a starter culture. Bread production requires the mixing of flour, water, and sourdough (Carbonetto et al. 2018). Usually, *S. cerevisiae* is inoculated into bread dough at a concentration of 2% of the overall ingredients. The yeast cells capture oxygen from the air trapped in the dough during mixing, and within the anaerobic conditions that follow, yeast cell proliferation slows down and fermentation reaction occurs (Hidalgo and Brandolini 2014). Due to fermentation of sugars, yeast cells are used as leavening agents in baked products, resulting in a rise in volume of the bread dough owing to fermentation gases. Therefore, this leads to changes in the product's structure and production of organic acids and volatile compounds that add to the texture and flavor of bread (Heitmann et al. 2018; Hidalgo and

Brandolini 2014). Yeast can also produce glycerol, which improves the texture of bread, particularly when frozen (Heitmann et al. 2018).

2. *Wine*: For hundreds of years, wine has been made from grapes and their microbiota, which are supplied by the vineyard or the winery. *S. cerevisiae* or subspecies such as *S. bayanus* (wine yeast) are the most common yeast species involved in the wine-making procedure, and they transform grape sugars into ethanol, CO_2, and other compounds (Pretorius 2000). During fermentation, *S. cerevisiae* produces two types of esters: acetate esters of higher alcohols and ethyl esters of medium-chain fatty acids (MCFA), both of which attribute to the wine's fruity and floral aromas (Verstrepen et al. 2003; Lambrechts and Pretorius 2000). Recombinant technologies with wine yeast aim to improve fermentastion performance, processing efficiency, and flavor characteristics (Pretorius 2000).
3. *Chocolate*: *Theobroma cacao* beans are the main raw materials for the manufacture of chocolate, but they are bitter and astringent, making them inedible. As a result, they are fermented to lower the amount of polyphenols and alkaloids that cause bitterness and astringency, as well as to produce flavors that define the delicate organoleptics of cocoa and chocolate (Schwan and Wheals 2004; De Vuyst and Weckx 2016; Aprotosoaie et al. 2016). Cocoa fermentation primarily includes yeasts followed by lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (De Vuyst and Weckx 2016; Papalexandratou and De Vuyst 2011; Meersman et al. 2016). At the start of fermentation process, yeast ferments pulp sugars in the anaerobic and low pH (3–4) environment, yielding ethanol and also a variety of flavor compounds that will decide the quality of the end product (Schwan and Wheals 2004; De Vuyst and Weckx 2016; Aprotosoaie et al. 2016; Gutiérrez 2017; Meersman et al. 2015).
4. *Dairy products*: Kefir, a fermented milk beverage, is made by fermenting kefir grains through a symbiotic relationship between bacteria and yeasts found in kefir grains. *Kluyveromyces marxianus/Candida kefyr*, *K. lactis var. Lactis*, and *Debaryomyces hansenii* yeast strains are used in the production of suitable and characteristic kefir sensory qualities (de Oliveira Leite et al. 2013). Koumiss is a fermented mare's milk drink that is mildly alcoholic. It was initially made with a natural mixed starter culture of lactic acid bacteria and yeasts. Many yeast strains including *Candida pararugosa*, *Dekkera anomala*, and *S. cerevisiae* are used in koumiss production for scent, texture, and nutrients valuable to human health (Mua et al. 2012).

8.5.2 Yeast Enzymes

Yeasts, particularly *Saccharomyces* species, are best known for whole-cell biocatalyst and are employed in the food sector for alcoholic drink production as well as bread fermentation (Schaefer et al. 2013; Katz et al. 2003; Steensels et al. 2014). They are also a source of enzymes like lipases, dehydrogenases, and invertase. Invertase is utilized to make invert syrup, which is an equimolar solution of fructose and glucose that is derived from sucrose and used in the food and beverage

industries. Being sweeter and hygroscopic than sucrose, it is mostly used to make soft-centered candies and fondants. Invertase is also used in the production of synthetic honey (Kotwal and Shankar 2009; Kulshrestha et al. 2013).

8.5.3 Yeast as Food Supplement

The development of single-cell protein (SCP), an alternate source of high nutritional content proteins used as a food or feed additive, is a significant industrial application of yeast (Gour et al. 2015). The nutritive benefits of yeast-derived SCP are high, and it contains essential amino acids in addition to sulfur-containing amino acids (Anupamaa and Ravindra 2000). *Candida*, *Hansenula*, *Pichia*, *Torulopsis*, and *Saccharomyces* are some of the yeast species used to make protein-rich food (Nasseri et al. 2011).

8.6 Concluding Remarks

Nature uses microbes to carry out fermentation, and humans have taken advantage of this capacity to produce bread, beer, wine, vinegar, yoghurt, and cheese, as well as fermented fish, meat, and vegetables. Moreover, the microbiota in the human microbiome is critical to our well-being. Therefore, microbiology is essential for food manufacturing, processing, preservation, and storage. Microorganisms, the human microbiome, nutrition, and food safety have all served significant roles in the development of the modern food industry. Furthermore, combined efforts of molecular biology, genetic engineering, biochemistry, and bioinformatics have paved the way for strain improvement which could be used to significantly improve the microbial growth rate, fermentation performance, and production of biomass.

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9 Current Status, Future Challenges, and Opportunities for Improving the Crop Yields Using Microorganisms

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Abstract

There is an incessant increase of demand for foods with the increase of populations, globally. The situation becomes more complex due to decreasing cultivated lands. Therefore, there is a need for developing newer strategies that increase soil fertility or fulfill the demand for foods. Soil fertility might be improved by using various organic, eco-friendly processes and soil inhibiting microorganisms (bacteria, fungi, and viruses). These microorganisms enhance the nutritional value of the soil and improved the translocation ability of the essential nutrients to the plants. Moreover, microorganism enhanced the soil structure through the aggregation of soil particles and maintains their stability. Microorganism has the potential ability to restore the fertility of the soil, thereby enhancing the yield of crops. Interestingly, numerous microorganisms have been observed to be effective against pathogens and insects, thereby easily protecting

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crops and subsequently improving the yield of the crops. In this book chapter, we focus on the current scenario of crop yield and the role of microorganisms in increasing the yield of economically important crops. Finally, we discuss the future opportunity for enhancing the yield of growing crops with the help of microorganisms.

Keywords

Microorganisms · Entomopathogenic fungi and bacteria · PGPR · Microbial-based biofertilizers

9.1 Introduction

Presently, the demand for food increases with the increasing global population mainly in a developing country, where there is lack of fertile land area. Fertility of the soil is one of the key factors for the production of crops. Therefore, soil health preservation and conservation are essential for sustainable agricultural production. Small-scale farmers face a lot of problems in producing crops such as non-availability of fertilizers, lack of hybrid or improved variety of seeds, lack of advanced technologies (machinery), and improper crop protection measurements in developing country (Tilman et al. 2011; Timmusk et al. 2017). Moreover, agricultural land continuously decreases with increasing urbanization and industrialization due to increasing the population, globally. On the other hand, fertility of the land might be decreased with various aspects such as continually using agrochemicals and higher accumulation of metal ions within the soil that inhibit or kill essential microorganisms as well as prevent the production of phytohormones within the plants, thereby increasing the fertility of soil as well as decrease the yield of crops. The phytohormones are essential for the growth and development of the plants that enhance the yield of crops. These requirements might be fulfilled by smart agricultural technologies, smart irrigation, and enhanced fertilizer translocation efficiency within the plant that might be beneficial for the yield of crops as well as soil fertility. Recently, nano-fertilizers, nano-carriers for delivery of micronutrients, and use of phytohormones, pesticides, and insecticides can enhance the fertility of the land, thereby increasing the yield of crops. However, applying them several times in one crop limits their applicability (Döös 2002; Fróna et al. 2019; Ashfaq et al. 2017; Kumar et al. 2018; Omar et al. 2019; Irsad et al. 2020; Mohammad Ashfaq 2020; Mohammad Ashfaq 2017; Tilman et al. 2011). In this context, hybrid seed and smart fertilizers might be used as potential agents that improved the yield of crops.

The hybrid seeds (cross-pollinated seeds) and smart chemical fertilizers (metal-based fertilizer and nano-fertilizers) might become alternative tools for the improvement of crop yield. The hybrid seeds and smart chemical fertilizers are effectively used for the growth and development of the plants that fulfill the requirement of foods. Moreover, excessive uses of pesticides with chemical fertilizers develop microbial resistance against pesticide that enhances soil contamination. However,

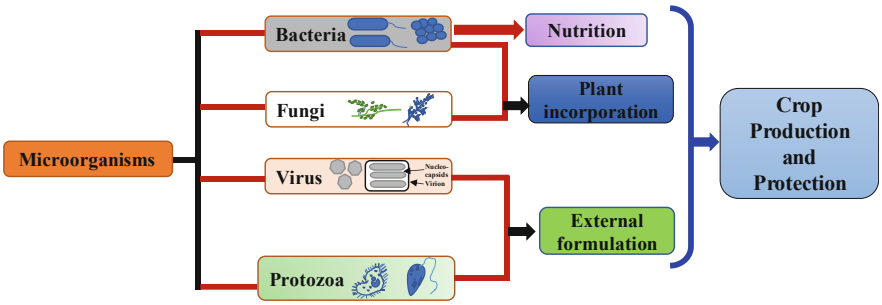

Fig. 9.1 Schematic representation of the microbial strategies in crop improvement

a multi-dimensional problem associated with hybrid seeds and smart chemical fertilizers limits their applicability that affects soil vigor, decreases the fertility of the soil, decreases the nutritional value of crops, and kills the beneficial microorganisms (Chaparro et al. 2012; Bhatt and Nailwal 2018). In this context, microbial agents might be a suitable alternative that resolves various issues associated with smart chemical fertilizers.

The demand for food might be fulfilled by utilizing microorganism or microbial agents in a suitable manner that enhances the fertility of soils as well as controlling the pest, thereby improving the yield of crops. Numerous microbial agents include bacteria, fungi, protozoa, viruses, and archaea, which are usually found within the plants, soil, and animal guts for their survival. These microorganisms improved the fertility of the soil, as well as the yield of crops (Gibbons and Gilbert 2015; Marchesi and Ravel 2015). Interestingly, the diversity of the microorganism is associated with the soil eco-system and can survive even in adverse conditions. However, the changes in the environments might be altering the microbial population, species, and effectiveness (Forchetti et al. 2007; Orozco-Mosqueda et al. 2018).

Despite various negative aspects, microbial agents have been extensively used in organic farming that effectively control disease management and increase the fertility of the soil compared with that of the synthetic molecules of the pesticides (Vaidyanathan 1987; Müller et al. 2016; Hussain et al. 2018). Usually, fertile soils have better diversity of microorganisms such as bacteria, protozoa, algae, Actinomycetes, molds, nematodes, and worms. The co-existence of microbes with plants leads to the interaction in the agro-ecosystem. These microorganisms might be beneficial in plant nutrition and protection against pests and diseases (Avis and Bélanger 2002). Figure 9.1 shows the schematic representation of the microbial strategies in crop improvement.

Usually, around 17 essential macros (C, H, O, N, P, K, Ca, Mg, S) and micronutrients (Fe, Mn, Mo, Zn, B, Ni, Cu, Cl) are required for the growth and development of the plants. Among all of them, around 11 nutrients are present in the soils. These nutrients increase the fertility of the soils. Moreover, fertility-enhancing microorganism like rhizobacteria is present in the root zone of the plants. The rhizobacteria enrich the soil structure by the production of phosphate, glucose,

dehydrogenase, antibiotics, increase the solubilization of the mineral phosphate, and stabilization of soil aggregates (Haas and Keel 2003; Miller and Jastrow 2000). These microbes are essential to promote and enhance the biological processes of the plant and soil persistence (Singh et al. 2016). The rhizobacteria can improve plant growth, regulate biochemical pathways (through the involvement of direct and indirect mechanisms), manipulate plant hormonal signaling, inhibit the activity of pathogenic microbial strains, and enhance the bioavailability of soil-borne nutrients (van der Heijden et al. 2008; Mendes et al. 2013; Verbon and Liberman 2016). The microorganisms can significantly enhance the nutrients uptake, transform root morphology, and trigger the microbial population. It makes microbial diversity a prime factor in creating the organic and inorganic nutrients in their available form to the plants (Lawlor 2004). The phenomenon is well known and has been incorporated as an important tool or component of IPM (integrated pest management) for crop production and protection (Antoun and Prévost 2006). On the other hand, these microbes also represent a promising biological suppression of several devastating pests and soil-borne diseases. Several beneficial microbial strains are in use for the commercial production of effective microbial products (biopesticides) with established success of improving crop productivity (Lindemann et al. 2016; Vessey 2003; Lucy et al. 2004; Mustafa et al. 2019).

In general, most of the microorganisms are beneficial or required in nature that play an utmost role in the ecosystem and fertility of the soil. Moreover, microorganism is advantageous to human life because it can extensively be used in agriculture biotechnology, food processing, genetic engineering, protection of crops against the pathogen, and treating municipal wastes. However, some of the microorganisms are pathogenic that cause various diseases and disorders to humans, animals, and plants. It has also been reported that microorganisms may influence the photosynthetic activity of plants and are regarded as most crucial for sustainable crop production. This book chapter focuses on the past, present, and future of crop improvement strategies using beneficial microorganisms.

9.2 Microbial Strategies for Crop Improvements

The microbial agents can considerably increase the nutrient uptake, transform root morphology, trigger the microbial population, manipulate signaling of phytohormones, enhance the solubilization of the mineral phosphate within the soil, and stabilize soil aggregates, thereby improving the yield of crops. Usually, there are various strategies used for the improvements in the crops: (1) gene incorporations within the plant for growth and pest control (transgenic plants) and (2) external formulations such as biopesticidal formulations and plant nutrition (microbial fertilizers).

9.2.1 Transgenic Plants

The transgenic plants have genomes that are modified through the genetic engineering process by the addition or removal of a specific gene. Significant research has been done so far for the development of molecular biology and genetic engineering technologies that produce an enormous variety of transgenic plants (from dicots to monocots plants) with various agronomic traits such as drought tolerance, and pest resistance, thereby improving the yield of crops. With the help of genetic engineering techniques, we can easily produce ideal agronomic traits, quality, and higher yields of crops. Moreover, these transgenic plants can produce protein for the pharmaceutical industry. Several efficient and modern techniques are being utilized to transfer the gene into the selected plant. Tobacco was the first genetically modified plant developed in 1983 and investigated in 1986 as having herbicide-resistant potential in France and the United States (Carter and Shieh 2015; Zhang et al. 2003; Hau-Hsuan et al. 2017; Abbas 2018). The following steps have been involved in the formation of genetically engineered crops. Figure 9.2 shows the transformation of gene using *Agrobacterium*.

1. Gene identification and its location for the plant traits is the first step.
2. Designed genes to be transferred into a plant.
3. Transformation of plants. Usually, there are two types of transformation process: (a) gene gun process and (b) the Agrobacterium process.
4. Selection of transformed tissues.
5. Regeneration of whole plants under controlled environmental conditions (Fiester 2006).

Numerous genes such as *Bt* cry genes, Cry1A, Cry3A, Cry1Ab, and Cry1Ac have been used to produce transgenic plants (Sanahuja et al. 2011). The plant incorporation makes the plants unpalatable for harmful insect pests. It utilized the harmful bacterium incorporated in plants using genetic engineering. Genetic engineering or recombinant DNA technology refers to the identification and insertion of the useful gene to bring desired changes in the target organism and the outcome called a modified organism or transgenic organism.

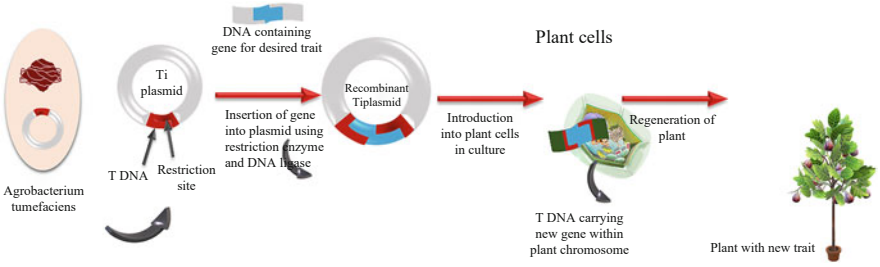

Fig. 9.2 Transformation of gene using *Agrobacterium*

9.2.1.1 *Bacillus thuringiensis*

Bt has been an extensively utilized microbe in crop improvement as plant-incorporated protectants and as external formulations. *Bt* is a spore-producing bacteria and can remain active in the environment even in non-suitable conditions for its survival and development. *Bt* can be found in soil, infested insect, storage, and the environment. This bacterium is of various shapes and different molecular weights, ranging from 30 to 142 kDa as strain HD-1 (*B. thuringiensis* var. *kurstaki* (*Btk*), isolate HD1 (contains Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins); *B. thuringiensis* var. *kurstaki* (*Btk*)-isolate HD73 (contains Cry1Ac); and *B. thuringiensis* var. *aizawai*-isolate HD137 (contains Cry1Aa, Cry1B, Cry1Ca, and Cry1Da). These Cry proteins are water-soluble, belonging to the δ-endotoxin class of bacterial proteins, form crystals in the host body, and cause toxicity to insect pests. Additionally, *Bt* also produces several toxins such as hemolysins, enterotoxins, phospholipases, and chitinase. However, the actual significance of *Bt* lies in Cry proteins, profoundly toxic to a variety of insect pests, viz., order Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera, Orthoptera, and Thysanoptera. Upon ingestion by insects, the crystals of Cry protein get solubilized by the alkaline environment of insect midgut and subsequently proteolytically converted into lethal toxic fragments. However, the actual process is still not understood properly (Roh et al. 2007). In the recent past, several strain-specific commercial *Bt*-incorporated crops have been developed against important insect pests of agriculture and medical importance (Table 9.1).

Table 9.1 Genetically modified crops and their target insect pests

Crops	Bt toxin	Target pest	Reference
Maize (*Zea mays*)	Cry1Ab	*Diatraea saccharalis, D. grandiosella*, *Ostrinia nubilalis*, *Spodoptera frugiperda*	Trisyono and Chippendale (2002), Zhang et al. (2017)
	Cry1Ac	*Chilo partellus, S. frugiperda*	Sharma et al. (2010)
	Cry1F	*O. nubilalis*, *S. frugiperda*, *D. grandiosella*, *D. saccharalis*	Gaspers et al. (2011), Siebert et al. (2012)
Cotton (*Gossypium* spp.)	Cry1Ac	*Helicoverpa armigera, H. zea, Pectinophora gossypiella, S. exigua*	Dhurua and Gujar (2011), Qiu et al. (2015)
	Cry2Ab2	*H. armigera, H. zea, Heliothis virescens, P. gossypiella*	Dhurua and Gujar (2011)
Soybean (*Glycine max*)	Cry1Ac	*S. litura*	Yu et al. (2013)
Sugarcane (*Saccharum officinarum*)	Cry1Ac	*D. saccharalis*	Gao et al. (2016)

9.2.2 External Formulations

9.2.2.1 Biopesticidal Formulations

Biocontrol research is gaining importance across the world due to its positive impacts; earlier experimental or field findings reveal its performance and excellence in utilizing in crop improvement. Several isolates are being done, and mass production is being prepared at a large scale. There are increasing demands for alternative and safer methods compared to synthetic pesticides. The goal behind the use of these formulations is safe for human health and the environment. The composition of microbial-formulated products is biocontrol agents and other compatible ingredients to develop effectively and survival (Fravel et al. 1998; Schisler et al. 2004). From an efficacy point of view, effective formulation involves a precise knowledge of biological control organism, insect pest, pathogen, environment, and their interaction with other associated organisms. Before launching any biocontrol agents, the biology of isolated microorganisms needs to be studied and experimentation is needed to be done to observe the efficacy of tested microbial formulation and develop a suitable and applicable formulation. Alteration of a media component, amount of nitrogen, and carbon growth conditions such as temperature, pH, and incubation period can donate the production of resistant to stable potential spores (Schisler et al. 2004). Each strain has its distinctive characteristics that will affect the stability and effectiveness. The knowledge of biocontrol agents can lead to developing innovative ideas to increase the efficacy of the microbial formulation. Environmental condition is also a considerable factor that should be suitable or favorable for the growth of biocontrol agents, and it is noticed that microbes have preferred range of moisture and pH for survival and others functions.

9.2.2.2 Bacteria-Based Formulation Against Diseases and Insect Pests

It is necessary to manage plant diseases and pests for securing food grains and maintaining balance for a healthy and rapidly increasing population. Several management tactics and approaches are being employed or adopted to minimize the losses caused by insect pests and pathogens. According to the earlier finding, it is reported that plant pathogenic disease can be suppressed or reduced through the application of potential microorganisms. The interaction between plant pathogens and plants may be complex; it may exudate some allelochemicals, provide suitability for the competition of space, and accelerate the host defensive mechanism and predation. However, some strains have the potential to manage plant pathogens when it is applied in certain agro-climate and various cropping pattern, and some of them may have efficacy to a broad range of plant pathogens. Among bacterial formulation, genus *Bacillus* has more importance than others. The application of *Bacillus* is increasing at a faster rate because of its broad-spectrum use as a biocontrol agent and ability to be multiplied for mass production as well as resistance to survive even in adverse environmental conditions. The spores of *Bacillus* can survive for a longer duration, enhance secretion of metabolites, and reduce plant pathogens. *Bacillus* spp. release exo-polysaccharides that are highly effective in inhibiting the movement of several toxic ions and helps in maintaining the ionic

balance, improve the water movement, and prevent the growth of pathogens (Verschuere et al. 2000; Tan et al. 2016; Radhakrishnan et al. 2017; Hashem et al. 2019; Shafi et al. 2017).

9.2.2.3 Mode of Action of *Bacillus thuringiensis*

The widely used bacterium, *Bacillus thuringiensis* (*Bt*), generally reproduced by spores and crystalline protein along with bacterium cell is called endotoxin. Endotoxins are ingested or penetrated within the host body and affect the survival of target insects. After ingestion of these spores through gut or integuments, spores get activated caused toxicity in alkaline conditions of insect's stomach (Schünemann et al. 2014). Further, spores are moved and blended with insect blood (hemolymph) and multiplied in numbers. Several strains are found to be caused by mortality in a variety of harmful insect pests. Strains which are found effective in reducing insect pest population are called as Entomopathogens. Symptoms after ingestion of spores have been observed, i.e., change in the gut, lead to disruption in their development, nutrient consumption, degenerative transformation, loss of appetite and gut paralysis, several physiological disorders, and paralysis. These symptoms are observed on targeted or tested susceptible insects after ingestion of *Bt* spores and crystals, leading to death (Bravo et al. 2007; Schünemann et al. 2014).

9.2.3 Entomopathogenic Virus

Viruses are the most plentiful and diverse biological objects on the planet. Viruses belonging to 18 different families are known to infect invertebrates and insects (Wu et al. 2020; Taboada et al. 2018; Jiwaji et al. 2019). *Baculoviridae* is the most important family that primarily infects the Lepidoptera. Viruses are useful agents having entomopathogenic properties against a variety of insects and are applied due to their high host specificity and safer to humans and animal health as well as non-target beneficial insects such as pollinators, predators, and parasitoids. Therefore, it is advisable to treat crops even before the harvesting of crops because of the least chance to develop stable resistance. Baculovirus is itself a component of nature. These are highly compatible with other methods and suitable for IPM strategies (Haase et al. 2015; Jehle et al. 2006).

Baculoviruses are occluded, double-stranded DNA (dsDNA) viruses and characterized by the presence of occlusion or inclusion bodies (OBs). Virus particles could be isolated and identified as an infectious viral agent. The baculoviruses are classified into two genera, nucleo-poly-hydro viruses (NPVs) and granuloviruses (GVs) based on size their shape and size (Slack and Arif 2007; Hilton 2008; Kroemer et al. 2015; Sallam et al. 2013).

9.2.3.1 Mode of Actions

The virus is a non-motile microorganism; it may reach the target with the support of vectors or naturally infested food. After ingestion, the baculovirus particles replicate within the host body and affect the host body. The infected larvae show pale swollen

bodies and are moribund. The larvae of *H. armigera* and *A. albistriga* crawl to the top of the twigs (negative geotropism) on which they feed. The larvae of *Spodoptera* and *Helicoverp*a are nocturnal; the initial signs of baculoviral infection are gradual changes in the color and luster of the integument. Infection of the epidermis caused the host to appear soft and rupturing of the cuticle is ruptures and discharge fluid from the body and due to starvation and desiccation eventually larvae get dies (Kumar et al. 2011).

9.2.4 Entomopathogenic Fungi

Entomopathogenic fungi refer to the manufacturing of potential formulations from naturally available fungi that have been used against several devastating insect pests. Microbial control is being used due to its eco-friendly, wider acceptability, virulency, and least toxic property. Potential fungi are effectively used in stimulating plant growth and reducing pest density as biocontrol agents. Fungi are used in minimizing or regulating pest density called mycopesticides. Entomopathogenic fungi have considerable potential as insect pathogenic agents by infecting lepidopterous larvae, sucking pest, viz., aphids and thrips, and many cosmopolitan insects, which are of great concern in agriculture worldwide.

Beauveria bassiana, the anamorph stage of *Cordyceps bassiana*, is a facultative cosmopolitan entomopathogen with an extremely broad host range. First, it was isolated from the larvae of silkworm by Agostino Bassi de Lodi; the fungus grows as white mold producing single-celled, haploid, and hydrophobic conidia. RNA sequence transcriptomic study revealed the startling ability to adapt to varied environmental niches including survival outside the insect host (Singh et al. 2015). Thus, the ecological habitat of these fungi extends from the simple insect-host interaction to a broader perspective including plant.

9.2.4.1 Infection Mechanism

B. bassiana attack on insect host. Beauvericin is a toxic compound that is found to be effective to cause mortality on the target pest. Infection begins via attachment of conidia to the host cuticle, and germination and penetration take place with the help of hydrolytic enzymes (e.g., proteases, lipases, chitinases); mechanical pressure and other factors are also involved in the entire process (Mascarin and Jaronski 2016; Litwin et al. 2020; Dannon et al. 2020). These spores blend with hemolymph, proliferate, and exploit nutrients as well as several antimicrobial peptides that are produced during colonization. They are involved in host immune suppression and depletion of nutrients that lead to the death of the host.

9.2.5 Metarhizium Anisopliae

The taxonomic revision of the genus *Metarhizium* based on phylogenetic analysis of rRNA gene sequence data and reported that only four species—*M. anisopliae,*

M. album, M. flavoviride, and *M. taai—exist. M. anisopliae* and *M. flavoviride* have a wide geographic distribution. The cosmopolitan distribution of these species has enabled them to be exposed to a broad diversity of environmental factors and insect host species. There are two varieties in *M. anisopliae*. *M. anisopliae* var. major (Johnston) Tulloch has long conidia of about 10.0–14.0 μm in length (range 9.0–18.0) and is common. The short conidial type, with conidia of 5.0–8.0 μm (range 3.5–9.0), has a diverse host range. The species *M. flavoviride* var. *flavoviride* Gams and Rozyspal with conidia of 8.0–9.0 μm (range 7.0–11.0) and *M. flavoviride* var. *minus* with conidia of 4.5–5.5 μm (range 4.0–6.5) long) are confined to grasshoppers and locusts (Pantou et al. 2003).

9.2.5.1 Mode of Action

This fungus acts in two ways. The first is a pathogenic phase, which begins when the conidia come into contact with the epicuticle of the host integument or are ingested. The conidia adhere and germinate forming a germ tube that penetrates directly or grows over the surface of the epicuticle. Successful penetration depends on the inherent capabilities of the germ tube and the physiological state of the host and involves mechanical and enzymatic processes. The germ tube penetrates by lysing both the epicuticle, a layer characterized by a cross-linked network of lipids and protein, and the procuticle, a layer of chitin and protein. Active digestion and absorption of cuticular components occur, colonizing host tissues and producing elongated hyphae and/or hyphal bodies (blastospores). Before the extensive invasion of the host tissues, the insect dies as a result of the production of an array of secondary metabolites called destruxins which affect on transport channels involved in muscle response and cell-membrane integrity (Aw and Hue 2017).

9.2.6 Verticillium Lecanii

This fungus has been developed and commercialized against aphids, whiteflies, thrips, and red spider mites. The fungal spores are dried to form a wettable powder formulation, which can be mixed with water to produce a suspension suitable for immersion of plant cuttings. Strains of this fungus are highly pathogenic to several crucifer pests, yet are harmless to honeybees. Mass production of conidia can be prepared on the different substratum and the type of growing medium affects the production of conidia (Faria and Wraight 2007).

A complex process is involved in reaching the spore to the target site:

1. Spore attachment to the insect cuticle
2. Spore germination and colonization
3. Penetration through germ tubes
4. Multiplication of blastospores and attack of the target tissue
5. Release of the fungus through the production of conidiophores

9.2.7 Trichoderma as Significant Bioagent in Reducing Phytopathogens

Trichoderma is an essential genera of antagonistic microorganisms for the control of a large number of phytopathogen in agro-ecosystem. It is a cosmopolitan soil fungus, which is frequently on soil from the plant root system (Begum et al. 2010; Howell 2003; Mbarga et al. 2012). The secondary metabolites secreted by *Trichoderma* spp. have proven their role in suppressing the growth of pathogenic microorganisms and stimulating plant growth (Contreras-Cornejo et al. 2015a, b; Kubicek et al. 2001; Kullnig et al. 2000). Besides, the interaction between plant and *Trichoderma* spp. successfully regulate root architecture and increase the length of lateral and primary root that result in the effectiveness of nutrient uptake by the plant. *Trichoderma* spp. characterized as antagonistic strains do not particularly target only pathogenic organisms but also the other microorganisms (Cai et al. 2013; Naseby et al. 2000; Yedidia et al. 2001; Ros et al. 2017).

Purification of *Trichoderma* spp. is essential to observe and present many ways or techniques. Nevertheless, the monosporic culture is suggested by *Trichoderma* on culture media as PDA or *Trichoderma*-specific medium. Once it is isolated and grown on culture media for further clear-cut identification, *Trichoderma* can be applied as a seed treatment, soil application, and root dip methods. Products that are commercially marketed commonly contain one or more *Trichoderma* species such as *T. viride*, *T. virens*, and *T. harzianum*. Important commercial formulations are available in the name of Sanjibani, Guard, Niprot, and Bioderma. These formulations contain 3×10^6 cfu/g of the carrier material.

9.2.7.1 Mechanism of *Trichoderma*

Antagonist microorganisms, such as *Trichoderma*, reduce growth, survival, or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion. The primary mechanism for biological control by *Trichoderma* spp. acting upon the pathogens is first to recognize and invade towards plant pathogenic fungal-like species through cell wall disruption and absorption of released nutrients known as mycoparasitism and induce the resistance of plant towards diseases by root architecture alteration during the interaction with the pathogen (Kumar et al. 2019; Harman et al. 2004; Omann et al. 2012). Figure 9.3 shows the *Trichoderma* activity in promoting plant growth and helps in controlling plant disease.

9.3 Nutrition (Microbial Fertilizers)

Soil microbes are an important component of agro-ecosystem as they supply essential elements to the diversity of plants and they play a significant role in nutrient management and for the betterment of plants. In recent years, it is being observed that the toxicity of inorganic fertilizers leads to the deposition of toxic substances in soil and the food chain eventually affecting the entire environment and human health

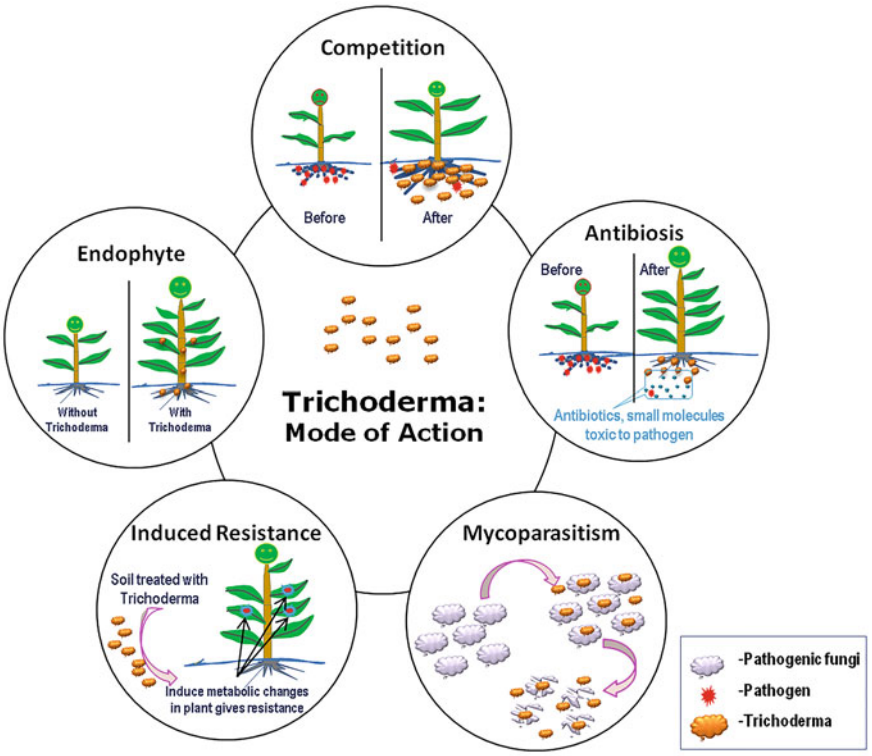

Fig. 9.3 *Trichoderma* activity in promoting plant growth and helps in controlling plant disease. Image taken with the permission (Waghunde 2016). Copyright © 2020 Author(s) retain the copyright of this article under Creative Commons Attribution License 4.0

globally. Beneficial microorganisms are considered an excellent alternative to meet the demand for nutrients due to their safer applicability and eco-friendly approaches. There is less chance of resistant development against pests and pathogens. Bio-molecules which are used to incorporate nutrients are known as biofertilizers. They trigger the mobilization of nutrients and bioactivity of the microbiome. Several formulations are being employed for sustainable crop production (Jacoby et al. 2017; Kumar and Gopal 2015; Rashid et al. 2016).

9.3.1 Plant Growth-Promoting Rhizobacteria (PGPR)

Several microbes are dwelling or welling in soil, and the rhizosphere is the site of perpetuation for a variety of beneficial microorganisms such as bacteria, fungi, virus, algae, and protozoa. All the rhizosphere microbiomes are not beneficial; some of them may be pathogenic for the plants as well as human beings. These beneficial microorganisms are effective agents in improving growth and protect against

pathogens. PGPRs help in promoting plant growth, i.e., phosphate-solubilizing bacteria (PSB), *Azospirillum*, *Pseudomonas,* and *Rhizobium*. PGPRs promote growth through producing plant growth regulators such as gibberellins, auxin, and cytokinin and supply biologically fixed nitrogen, *Pseudomonas* is extensively used in seed treatment and soil application in an array of crops. The genus *Azotobacter* is a gram-negative and nitrogen-fixing bacteria that have a high rate of respiration (Kumar and Dubey 2020; Compant et al. 2019; Igiehon and Babalola 2018; Pascale et al. 2020). Bacteria isolated from the soil in 1901 from Holland and different species such as*, A. paspali, A. salinestri, A. armeniacus, A. beijeriickii, and A. nigricans. Azospirillum* also has been recorded to fix the nitrogen and growth-promoting activity. AMF (Arbuscular mycorrhizal fungi) are beneficial in improving plant health as they enhance absorption of nitrogen, phosphorus, potash, copper, and iron to the root. Several phosphate-solubilizing bacteria (PSB) are significant in controlling metabolic pathways. PSB also helps in solubilizing inorganic phosphorus. Some soil-inhabiting bacteria are found as a promising agent in nitrogen fixation from the atmosphere which forms a symbiosis with leguminous crops (e.g., rhizobia) (Ahemad and Khan 2012; Zahran 2001). Rhizobia establish symbiotic relation with the root of leguminous plants, and there is a need for the establishment of a link between host and symbiont, leading to the formation of the nodules where rhizobia make its colonies as intracellular symbionts.

9.4 Future Challenges and Opportunities

Genetically modified (GM) crops offer an effective solution to ensure food security throughout the world. GM crops enhanced production, but still, there is less adaptability towards genetically engineered crops because of high cost of seeds and resistant development of insect pests against *Bt* crops. Cry1Ab-resistant strain of *Ostrinia nubilalis* may provide help in identifying the resistant gene. Although GM crops help in improving plant health and enhanced yield effectively, few farmers are aware of the fact of microbial pesticides and GM crops; they need to be trained and communicated by the extension workers for the maximum adoption of microbes in growing crops. Sophisticated technology requiring costly laboratory equipment and average funding by concerned agencies is also a great matter of concern for the preparation of commercial formulation and application.

Potential microbes can be employed or incorporated as an ecologically sound practice to pest and pathogen management as well as can target the inaccessible pests and pathogens viz., forest and aquatic pests. The public and farmers should understand the utility of beneficial microbes, and their mass production is required for sustainable production. Other possible microbes can be investigated for future use in promoting the use of microbes and integration of microbial formulation with existing farming methods is necessary. Microbes mitigate all the adverse impacts of synthetic pesticides being a significant tool. The efficacy of other available microbes should be explored as a bioactive agent.

9.5 Conclusion

In the current era, feeding the exponentially growing population from available land resources and maintaining the natural or ecological balance are a challenge. Microorganisms offer diverse pathways in enhancing plant growth through the mobilization of nutrients and suppression of destructive pests and pathogens. Additionally, the two-way approach of utilizing these beneficial microbes is a vital aspect in achieving sustainable plant production and protection. The identification, isolation, and mass multiplication for proper and effective utilization to enhance crop productivity are necessary. However, after decades of research, effective utilization of microbes is yet to be achieved. Therefore, focused continuous investigations are required to fill the gap of knowledge in this particular area.

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Part III

Role of Macromolecules and Micromolecules in Food

Diverse Role of Enzymes in Food and Dairy Industry

10

Muhammad Usman Khan, Nalok Dutta, Shaheer Arif, Muhammad Sultan, Muhammad Ahmad, and Mohammad Ali Shariati

Abstract

As long as there have been people, enzymes and microorganisms have been a part of food preparation. Innovative enzymes with extensive applications and specificity have been created by technological advancements, and we are still in the process of exploring new areas of application. Many foods can be enhanced with the use of microorganisms including bacteria, yeast, and fungi and their enzymes, resulting in an improved taste and texture. These industrial benefits are substantial. Several advantages, such as the ease of production, cost-effectiveness, and consistent nature of microbial enzymes, make them the preferred source of enzymes rather than those obtained from plants or animals. We examine recent advances in enzyme technology in the food industry in the following review. The

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broad range of applications for enzymes in food processing is discussed, as is their microbial source and the wide range of enzymes that are used.

Keywords

Microbial enzymes · Food and dairy industry · Brewing · Starch processing · Baking industry · Juice clarification

10.1 Introduction

Microbe-mediated food fermentation has been practiced since ages and is being implemented at a large scale nowadays in various industrial scale fermentation processes (Soccol et al. 2006; Vladimirovna et al. 2016). Enzymes derived from microbes play a pivotal role in food industries owing to their enhanced stability compared to biocatalysts originating from plants/animals. Microbial enzymes can be readily isolated through simple microbiological methods mediated by fermentation which are economically viable and can overcome time and space constraints (Zinina et al. 2020). Process modification and optimization are brought about by the efficacy and consistency of the microbial biocatalysts (Zinina et al. 2016). Enzymes derived from a vast array of microbial sources are implemented in the design of optimized bioprocesses in various industries ranging from pharmaceutical, food and beverage, pulp and paper, textile, and leather industries (Gavrilova et al. 2019a, b; Chernopolskaya et al. 2019; Nesterenko et al. 2016). The microbial biocatalysts are eco-friendly options for performing enzyme-mediated reactions at ambient/mild conditions of temperature and pH with significantly high turnover rate. More than 150 industrial bioprocesses have utilized the full potential of enzyme-mediated process optimization with more or less 500 products being developed in the process (Kumar and Singh 2013). Among the top revenue incurring industries utilizing enzymes, food and beverage industries rank the highest raking in a revenue of nearly $1.8 billion in 2016 which is predicted to cross $2 billion by the end of 2020 (Patel et al. 2016). The chief usage of enzymes in food and beverage sectors of the industries is implemented in the control of physical, chemical, and rheological properties of the foods (Aguilar 2008). Engineering novel biocatalysts by site-directed mutagenesis or directed cloning offers excellent opportunities towards economical and environmental biotransformation mediated by synthetic/natural enzymes. The food processing enzymes are employed in various sectors of the food industry ranging from but not limited to packaging, baking, brewing, dairy products manufacturing, processing of emulsified/non-emulsified oils and fats, and fruit juice preparation and in fermentation industries (Homaei 2015; Sharipova et al. 2017; Belookov et al. 2019; Tretyak et al. 2020; Ashan et al. 2020). The food enzymes are mostly employed for (1) preprocessing of foodstuffs, (2) formulating effective methodologies of production, (3) refining and packaging, and (4) storage (Ghorai et al. 2009; Porta et al. 2010). The first two enlisted processes are carried out to eliminate the excess amount of a particular component of the foodstuff in question

to impart quality and additional clarity to the resulting products. The refining process and downstream clarification of fermented beverages are economically unviable and involve large work force with generation of a considerable amount of disposal resulting in eventual loss of the product. This can be overcome by the use of specific enzymes like amylases, pectinases, tannases, proteases, etc. which tend to address the bottleneck vis-à-vis stimulate the entire biocatalytic process, thereby improving the product quality and the production value. This battery of enzymes has been instrumental in seamless processing of several food products (fruits/vegetables). Moreover, the engineered/synthetic enzymes accelerate the overall extraction process efficiency producing commercially viable and economically feasible end product (Hasan et al. 2006). The industrial potentialities of extensively studied enzymes in the food industry are displayed in a tabular form in Fig. 10.1. Figure 10.2 represents a graphical representation of potential enzyme applications in the respective food and dairy industries.

This review article brings to light the state of art applications involving food-processing enzymes with special focus in dairy- and food-related industry. A detailed discussion has been made on the aspects of protein engineering and immobilization approaches of the enzymes to improvise on the catalytic activities with newer capacity to broaden their application spectrum in food industries.

10.2 The Role of Enzymes in Food Industry

10.2.1 Diversified Role of α-Amylase, β-Amylase, Pullulanase, Glucoamylase, and Transglutaminase in Starch Processing Industrial Setups

α-Amylases (EC 3.2.1.1) are categorized as endo-1,4-α-d-glucan glucanohydrolases which bring about the cleavage of the 1,4-α-d-glucosidic linkages between the adjacent glucose units in linear amylase and amylopectin chain of starch. The α-amylases belong to the family of GH-13 (glycoside hydrolase group of enzymes) and require metal ions (mostly calcium) for unhindered enzyme activity and catalytic stability (Gurung et al. 2013). Amylases have multilayered role as biocatalysts with diversified usage in industrial applications as additives in processed food industries, detergents, wastewater treatment, biopulping, bioremediations, and molecular biology. These enzymes account for about 30% of the world's enzyme production (Dutta et al. 2017).α-Amylases, upon being added to the dough during baking, brings about transformation of the starch to smaller dextrins followed by yeast-mediated fermentation. α-Amylases bring about improvements in the overall quality of the bread by upgrading the quotient of taste and the color of the bread crust (Van der Maarel et al. 2002). α-Amylases are indispensable in the manufacturing of branched dextrins which are furthermore used in the manufacturing of rice cakes and powdered forms of foods (Aiyer 2005). Amylase has potent application in the starch industry wherein it is used for bringing about conversion of starch into glucose and other reducing sugars. Amylase-mediated starch conversion incorporates the sequential

Food Enzymes and their implementation in the industrial sectors		
Industry	**Enzyme type**	**Function**
Starch processing industry	**α and β amylase**	transformation of starch to smaller dextrins(glucose)
	pullulanase	cleave α-1,6 glycosidic bonds in industries specialized in grain processing
	glucoamylase	removal of glucose molecule from the non reducing end of the starch molecule releasing monomers, β-glucose.
	transglutaminase	improves dough quality by increasing the triglyceride content and altering the rheological properties of the bread.
Bakery industry	**protease**	processing of gluten, bread texture and heightening flavour.
	lipooxygenase	utilized for their ability in bleaching the carotenoids pigment in flour
	xylanase	help in mitigating the water requirement with formation of a stable dough
	α-amylase	improves in the quality of the bread in terms of softness and volume
Juice industry	**pectinase**	extraction, clarification and liquefaction of fruit juices
	tannase	clarifying agent in wines and fruit juices
	naringinase	debittering agent in fruit juices and beverages
Brewing industry	**β-glucanase**	hydrogenation of 1,3 β-glycosidic bonds between glucose molecules
	α-amylase	increase the carbohydrate yield in biofermentation
	protease	exposes the starch molecule for fermentative processing
Dairy industry	**lipase**	ripening agent of cheese
	lactase	hydrolysis of lactose content in milk into glucose and galactose
	catalase	eradicate peroxide from milk

Fig. 10.1 Food enzymes and their potential application in the industries

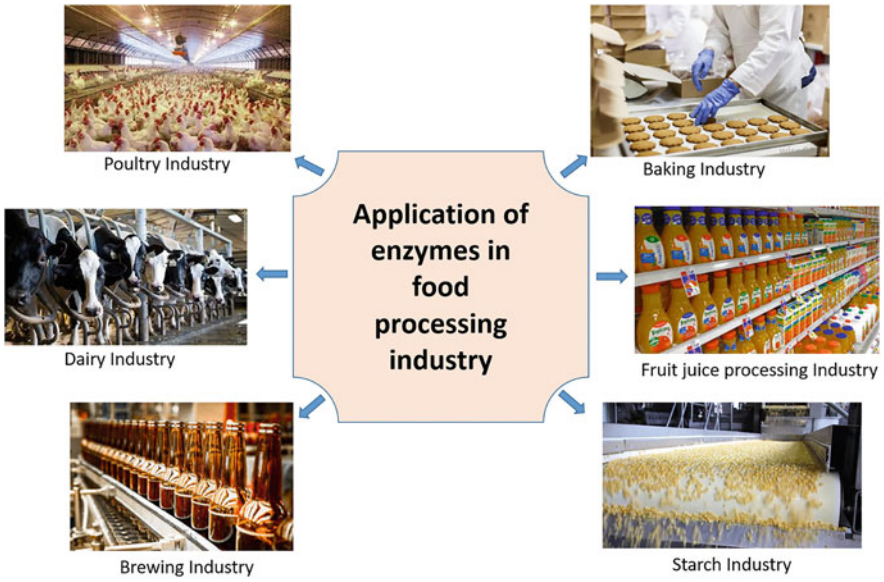
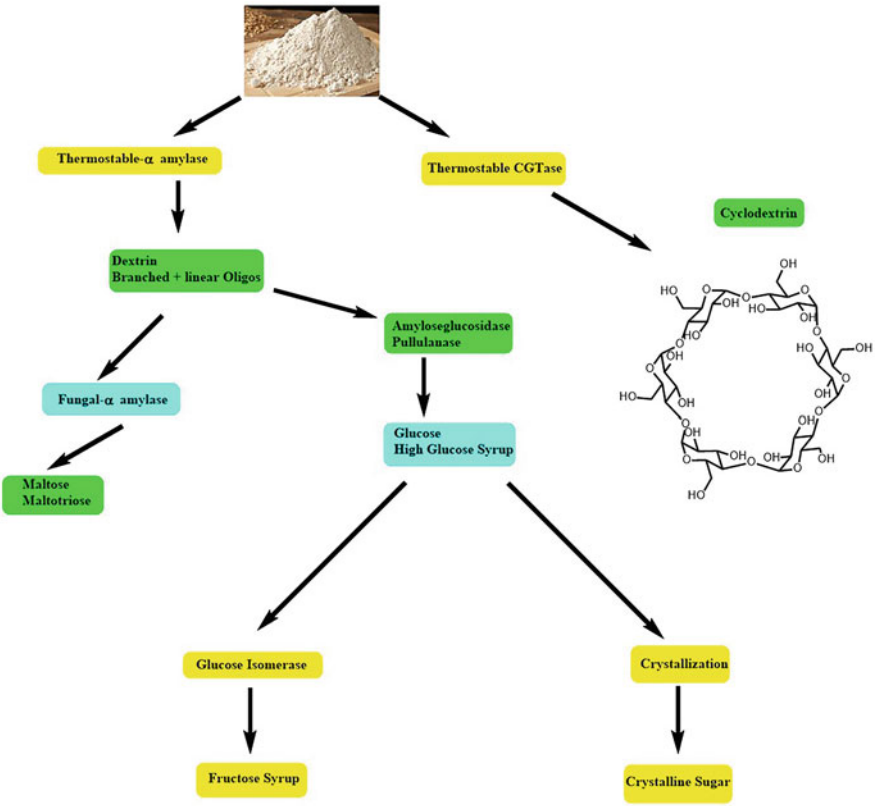


Fig. 10.2 A pictorial representation of potential enzyme applications in the industry

steps of (1) gelatinization, (2) liquefaction, and (3) hydrolysis. A gelatinous suspension is formed because of starch breakdown in the first step of the process. The second step involving liquefaction promotes hydrolysis which impacts in reduction of viscosity. In the last part of the process, saccharification helps in the seamless release of glucose and maltose brought about by the thermostable α-Amylases from *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *or Bacillus stearothermophilus* (Raveendran et al. 2018; Van Der Maarel et al. 2002). Starch is processed downstream to fermentable sugars and ethanol by the action of α-amylases. α-Amylases are also used for clarification of fruit juices supplemented by cellulase and pectinase for economic feasibility and attaining a greater yield. Biocatalyst appended lab-based micro/macro reactors have been applied for carrying out uninterrupted starch transformation by pretreating the starch with α-amylase.

Glucoamylases (EC 3.2.1.3), a class of saccharifying enzymes, are mostly derived from fungus aids in the removal of glucose molecule from the non-reducing end of the starch molecule releasing monomers, β-glucose. The fungal strains that secrete this enzyme are comprised of *Aspergillus niger*, *Aspergillus awamori*, and *Rhizopus oryzae*. All of these strains have been widely characterized and engineered for facilitating biotechnological applications at mild temperatures as they lose their native structure and functionality at adverse conditions of temperature and pH (Coutinho and Reilly 1997). Glucoamylases have been rightfully implemented in diversified applications ranging from food processing and preparation of glucose and fructose syrups and preparation of sake, soya sauce, and light beer [with low calorific value and zero alcohol beer] (James et al. 1996; Blanco et al.

2014). Biocatalyst appended lab-based micro/macro reactors have been applied for carrying out uninterrupted starch transformation by pretreating the starch with α-amylase.



Various enzyme effecting starch and their products

Transglutaminases (EC2.3.2.13) belong to the cluster of enzymes that bring about the formation of isopeptide bonds between macromolecules (proteins) (Kieliszek and Misiewicz 2014). This property of the transglutaminase enzyme is being implemented in bakery to produce improved variety of the dough by increasing the triglyceride content and altering the rheological properties of the bread. Moreover, transaminases catalyze deamination reactions in absence of free amine groups with water acting as the acyl group acceptor (Kuraishi et al. 2001).

Pullulanase (EC 3.2.1.41) is a debranching enzyme pertaining to a unique kind of glucanase (an amylolytic exoenzyme) that brings about complete breakdown of pullulan. This enzyme is produced by a large number of microorganisms in the form of *Klebsiella* sp. and *Bacillus* sp. (Nair et al. 2007; Zareian et al. 2010). Microbial pullulanases have been utilized across various industries owing to their

ability to cleave α-1,6 glycosidic bonds in industries specialized in grain processing biotechnology and downstream production of ethanol and sweeteners.

10.2.2 Role of Protease, α-Amylase, Lipooxygenase, and Xylanase in the Industrial Processes Pertaining to Bakery

Proteases (EC: 3.4) belong to a group of enzymes which are associated with catalyzing the hydrolytic peptide bonds in proteins and higher peptides. They are further subdivided into six groups of exopeptidases (EC: 3.4.11–19), namely aminopeptidases, dipeptidases, dipeptidyl peptide hydrolases, peptidyl dipeptide hydrolases, serine carboxypeptidases, and metallo-carboxypeptidases [acts on the polypeptide chain ends] and endopeptidases (EC:3.4.21–99) [mainly acts on the inner moiety of the polypeptides] which are further classified into six specific groups of protease enzymes based on the type of catalytic residue present at the active site, namely serine protease, aspartic protease, cysteine protease, metalloprotease, glutamic acid, and threonine protease (Rao et al. 1998; Dipasquale et al. 2009). Both classes of proteases are extensively used in the seamless manufacturing of diverse products ranging from breads, wafers, choux pastry, and waffles. Moreover, protease is also utilized for the processing of gluten, bread texture, and heightening flavor (Mohapatra et al. 2003; Goesaert et al. 2005; Aguilar 2008). Nowadays, protease is being also used towards replacement of bisulfite for mitigating the disulfide bonds in the gluten.

α-Amylases are instrumental in decreasing the dough viscosity with a resultant improvement in the quality of the bread in terms of softness and volume (Van der Maarel et al. 2002).

Lipoxygenases are non-heme iron-containing enzymes that bring about dioxygenation of PUFA in lipid molecules which consists of a cis-1,4-pentadiene. Lipoxygenases are mainly utilized for their ability in bleaching the carotenoid pigment in flour which takes place simultaneously with oxidation of the intervening fatty acids (Mcdonald 1979; Stauffer 1991). Lipoxygenases increase the rheology and mixing properties of the dough (Cumbee et al. 1997). This is manifested by the oxidation of the gluten thiol group, which leads to firming the texture of the gluten.

Xylanases (EC:3.2.1) are enzymes produced by a host of microorganisms ranging from fungi to bacteria which cleave xylan, a chief component of hemicellulose. The three distinct classification of xylanases are: exoxylanases, endoxylanases, and β-xylosidases. These enzymes work in unison in breakdown of xylan. Endoxylanases (EC 3.2.1.8) effect the cleavage of β-1,4 bonds of xylan backbone. Exoxylanases (EC 3.2.1.37) bring about hydrolysis of the β-1,4 bonds in xylan near the non-reducing ends with the subsequent release of xylooligosaccharides. Again, β-xylosidases promote the cleavage of xylobiose and xylooligosaccharides for the eventual release of xylose (Sukumaran et al. 2010). To this end, they are utilized for whole baking of rye and dry crisps in the bread industry. The *Trichoderma*-, *Bacillus*-, and *Aspergillus*-derived xylanases are extensively applied in the bakery industry to increase the fineness of the dough texture and volume vis-à-vis with a

longer shelf life of the dough. Xylanases help in mitigating the water requirement with formation of a stable dough (Nair and Shashidhar 2008; Bajaj and Singh 2010; Sanghi et al. 2010; Sharma and Chand 2012; Mandal 2015).

10.2.3 Role of Pectinases, Tannases, and Naringinases in Juice Industry

Over the past decade, the interest in maintaining healthy lifestyles among the present generation has increased manifold with its direct effect on the food and the beverage industry. As a consequence, there has been an escalated production of fruit juices, beverages, and nectars in the recent times which promotes good health. Among various groups of enzymes that are being routinely employed in the beverage industry for the production of a variety of products, pectinases, cellulases, and tannases deserve special mention.

Tannin acyl hydrolase (EC 3.1.1.20) or tannase is an extracellular hydrolase that facilitates hydrolyzing ester and depside bonds in tannic acid and gallates with glucose and gallic acid as byproducts (Lekha et al. 1997; Mahapatra et al. 2005). Tannases are frequently encountered in bacteria isolated from soils with dense vegetation vis-a-vis in rumen and gut microbiota (Iwamoto et al. 2008; Jimenez et al. 2014). Till date, two different types of tannases encoded by two different sets of genes have been reported. Of these, the extracellular tannase is encoded by tanA gene containing a signal peptide with a molecular weight of ~66 kDa. Tannases.

of this category are constituted by the genesets TanASl from *Staphylococcus lugdunensis*, TanALp from *Lactobacillus plantarum*, and TanASg from *Streptococcus gallolyticus* (Noguchi et al. 2007; Jimenez et al. 2014). Again, studies revealed the occurrence of ~50 kDa tannases encoded by tanB genes in *L. plantarum*, *S. gallolyticus*, and *Streptomyces sviceus* (Iwamoto et al. 2008; Jimenez et al. 2014). The growing demand for tannase in the global market has led to its commercialization by companies across the world. To name a few, they are Biocon, Sigma–Aldrich Co., Wako Pure Chemical Industries, Ltd., and Novo Nordisk. Moreover, tannase also has potential applications in the form of clarifying agent in wines, fruit juices, and a number of beverages (Yao et al. 2014; Sharma et al. 2016; Amin et al. 2017a; Amin et al. 2017b)]. Tannase enzyme obtained from fungal strains has slow rates of tannic acid degradation and is not suitable for industrial applications (Selwal et al. 2010; Govindrajan et al. 2016). However, the bacterial tannases obtained from *Lactobacillus*, *Staphylococcus*, and *Klebsiella* can break down gallotannins and ellagitannins impacting production at an industrial scale (Noguchi et al. 2007; Figueroa et al. 2014). Tannase-mediated production of fruit juices aid in increased yield of fruit juice with improved clarification and pulp liquefaction with lowered viscosity. A research study also highlighted the decreased turbidity in the fruit juice content when treated with an enzyme concoction of tannase, laccase, peroxidase, and pectinase (Sharma 2013). Tannase treatment of fruit juices renders lowered bitterness to the end product with improved juice (Beniwal et al. 2013). Tannase helps in eradication of high tannin contents in fruit juices reducing sediment formation and

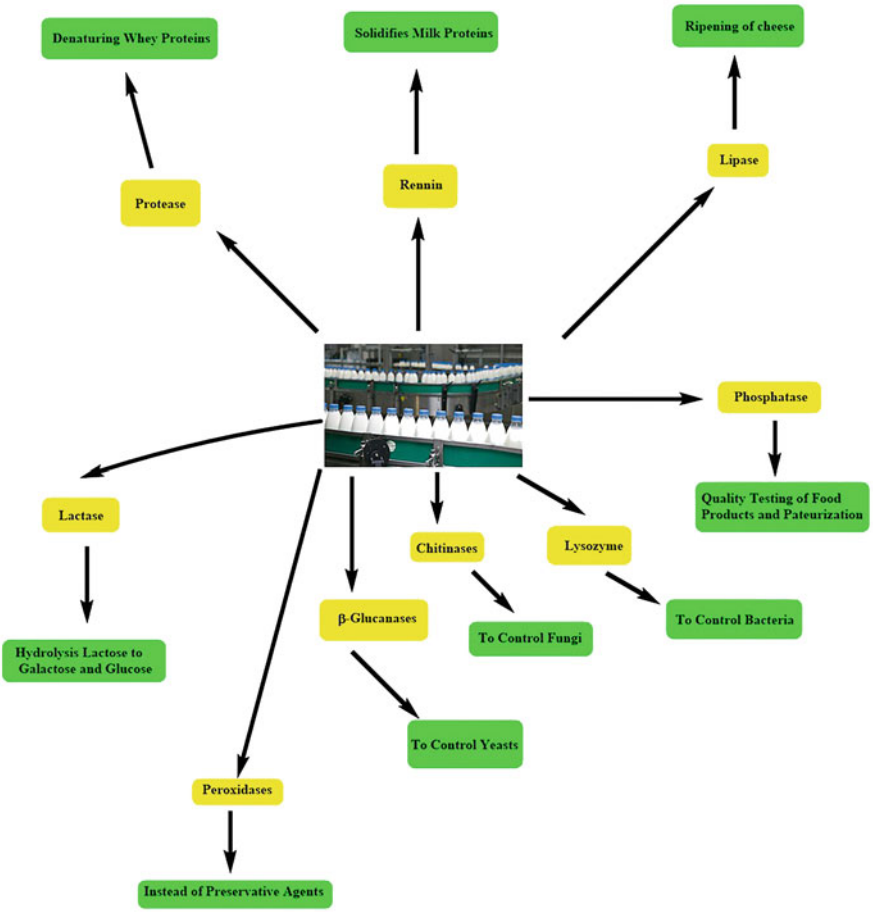
the resultant astringency thereby eradicating bitterness of the fruit juices during long-term storage. Tannase-treated fruit juices and beverages like black tea can be well-maintained for longer periods without clouding exhibiting superior quality (Beniwal et al. 2013). In an interesting finding, Rout and Banerjee (2006) proclaimed that tannase pretreatments triggered 25% degradation of tannin content, whereas combinatorial pretreatments of gelatin and tannase steered appx. 50% tannin depolymerization in pomegranate juice. Pectins are the principal constituents of foodstuff in the form of vegetables, fruits, and cereals. Pectins are acidic and high molecular weight structural polysaccharides. D-Galacturonic acid is a major component of pectin with rhamnose being a minor constituent of the pectin backbone. Other neutral sugars in the form of arabinose, galactose, and xylose are also present in the side chains. Pectinolytic enzymes are either acidic or alkaline in nature, and they are able to hydrolyze pectins. Henceforth, they are utilized towards extraction, clarification, and liquefaction of fruit juices and wines (Kumar and Singh 2013; Blanco et al. 2014). Pectinases have been widely applied for processing of various fruits ranging from different kind of berries to apples and oranges for improving the overall quality, shelf life, and proliferation of the juice taste (Kohli and Gupta 2015). Again cellulase enzymes have been reported for their ability to successfully depolymerize lignocellulosic biomaterials for production of value-added products (Sharma et al. 2016).

Naringinase (EC 3.2.1.40) has been used tremendously in the juice and beverage industry owing to its unique ability to degrade the flavanone glycoside naringin, the bittering agent which is found in citrus fruits (Puri et al. 2000). Naringin upon degradation forms naringenin and rhamnose owing to the biocatalytic actions of the enzymes α-rhamnosidase and β-glucosidase. Fungal isolates in the form of *Fusarium*, *Penicillium*, and *Tricoderma* are the chief producers of naringinase (Puri et al. 2012). Naringinase plays an important part as a debittering enzyme in fruit juices in both free and immobilized forms. (Zhu et al. 2017).

10.2.4 Role of Amylase, Protease, and β-Glucanase in the Brewing Industry

Amylase, β-glucanase, and proteases emerge to be the chief enzymes of the brewing industry (Johnson 2013). The brewing industry facilitates effective enzyme-mediated batch culture fermentation operations for optimized processing of the feedstocks. Commercially obtained enzymes are being utilized at a larger scale to optimize the overall production feasibility since the microbial enzymes are difficult to generate and procure at a grand scale. In the brewing process of beer, efficient hydrogenation of 1,3 β-glycosidic bonds between glucose molecules is brought about by β-glucanase. In the malting process of beer, α- and β-amylases are implemented for proper break down of starch into simpler sugar molecules (dextrins, maltose, and glucose) (Guerrand 2017; Sammartino 2015). α-Amylase is utilized to increase the carbohydrate yield in biofermentation during processing of light beers following malting. Proteases are employed for the process of brewing to facilitate protein breakdown in the malting process (Hui and Sherkat 2006). The enzymes in

question mitigate the viscosity in beer following protein degradation to amino acids which acts as valuable substrate promoting yeast growth (Hui and Sherkat 2006; Lei et al. 2013). Proteases bring about hydrolysis of the cell wall and associated softening of the kernel during mashing which exposes the starch molecule for fermentative processing.



Different enzymes in dairy industry

10.3 Diversified Role of Food Enzymes in the Dairy Industry

10.3.1 Role of Lipase, Lactase/β-Galactosidase, and Catalase

Enzymes play a pertinent role in processing of food in the dairy industry. The chief enzymes that are involved in this process are lipase, esterase, catalase, and lactase which catalyze reactions ranging from transesterification to hydrolysis facilitating

important biological reactions pertaining to dairy processing. Lipase is being utilized in the dairy industry as a ripening agent of cheese (Ray 2012). At present, the cumulative market supply of appx 90% lipase enzymes is derived from microbial strains (Guerrand 2017). This enzyme is implemented on a vast scale at the dairy industries. Lipase derived from *Penicillium camemberti* and *Aspergillus niger* improve the quality and texture of camembert and cheddar cheeses, respectively (Aravindan et al. 2007). Phospholipases catalyze the degradation of phospholipids into fatty acids and lesser lipids. They are categorized into classes A, B, C, and D based on the reactions they catalyze (Borrelli et al. 2015). Phospholipase A1, A2, and B promotes breakage of the carboxylic ester bonds of phospholipids with simultaneous replacement with an acyl group through transesterification processes (Choudhury and Bhunia 2017; Guerrand 2017). Phospholipases C and D with polar head groups are known as phosphodiesterases since they can identify phosphodiester linkage. During processing of cheese, owing to maturation of the peptides in the milk proteins, a bitter taste is rendered to the ripened cheese. Phosphodiesterases help in the cleavage of these bitter peptides thereby reinstating the flavor of cheese (Budak et al. 2018; Maitan-Alfenas and Casarotti 2018; Kolok et al. 2018).

Lactase/β-galactosidase (EC 3.2.1.23) of microbial origin catalyzes the hydrolysis of lactose content in milk into glucose and galactose thereby facilitating consumption of the same by lactose-intolerant individuals. The commercial strains of lactases are derived from bacterial and fungal strains in the form of *Aspergillus niger*, *Aspergillus oryzae*, and *Kluyveromyces lactis* (Ozturkoglu-budak et al. 2016; Rehman et al. 2016).

The psychrostable strains of β-galactosidase are utilized towards increasing the creaminess of the ice creams effected by hydrolysis of lactose. This hydrolysis step also aids in increasing the sweetness of the products. Lactase is also instrumental in the lactose fraction hydrolysis in whey, a byproduct obtained during cheese manufacturing of which appx. 75% is lactose. This high lactose content generated via whey production is associated with high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Khider et al. 2004; Johansen et al. 2002). Lactase also helps in generation of galactooligosaccharides at the onset of lactose hydrolysis via transglycosylation reactions, which can be used as prebiotics (Gibson et al. 1994).

Catalase (EC 1.11.1.6), a tetrameric enzyme, is predominantly found in aerobic organisms and is an efficient degrading agent of H_2O_2. Microbial strains of *Aspergillus niger* and *Micrococcus luteus* are efficient producers of catalase enzyme. Catalase is seamlessly implemented in the dairy industry to eradicate peroxide from milk (Sîrbu et al. 2011). However, catalase has restricted use in cheese production.

10.4 Conclusion

The vast machinery of synthetic and natural enzymes is being utilized across various sectors in the food industry owing to the great potential they offer. Seamless process control, optimized process determination, high yield, and economic feasibility are the main criteria which made them gain such a headway in the industrial processes. The genes transcribing the enzymes can be bioengineered with increased enzyme production to facilitate various physiochemical processes associated with food processing. The future research studies will reap the benefits of current ongoing catalyst research around the globe and will untap the potential of these eco-friendly biocatalysts in the near future.

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Recent Trends in Microbe-Based Food Hydrocolloids

11

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Abstract

Polymers have become an irreplaceable part in countless living applications. With rapid technology shift and focus on the food industries, the demand of synthetic polymers has significantly increased. However, the recalcitrant nature and its associated ecological problems have led the researchers to substitute the synthetic polymer with bio-based polymers. Biocompatible and biodegradable properties make biopolymers commercially important and sustainable. Among the different classes of biopolymers, the microbial polysaccharides are vital for food industry. Microbial polymers like celluloses, gums, pectin, starch, agar, and alginates have gained significant attention in food industries as these polysaccharides are used as composite materials in manufacturing of industrial product like hydrogels, nanoparticles, and micelles. The advantage of easy manipulation, easy availability of raw material, and low cost makes them ideal industrial candidate for high commercial production. The present chapter focuses on the different microbe-based biopolymers and its application in food industry.

Keywords

Microbes · Polysaccharides · Biopolymers · Food industry · Hydrocolloids

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11.1 Introduction

Polymers are large molecules mostly composed of the synthetic compounds linked with a chain of blocks. The polymers are compounds representing unique properties with type and characteristic of bonding pattern. These polymers can be also twisted, bended, and moulded in the unique form depending on the product design and its commercialization. Polymers represent diverse set of products from representing flexible compounds like rubber to tough compound like glass or epoxides. Polymers are usually formed in three-dimensional, two-dimensional, and one-dimensional networks. Long building block chains are made of the components like various biomolecules in different combination (Elias 1997). Depending on the structure and molecular arrangement, the polymers are classified in different classes in brief. Most common classes are composed of hydrocarbons. Due to arrangement of the carbon atoms and its long chain arrangement, it makes the backbone of the polymer. This class of polymers enlists the most useful commercial compounds like polyethylene, polypropylene, and polybutylene. Other class of polymers mostly manufactures are those whose backbone is composed having elements other than carbon, for example, nylon. Nylon are the polymers with the repetitive chain of the nitrogen atoms. Other examples include the polyesters and polycarbonates which contain oxygen instead of the carbon as backbone. These all are also class of inorganic polymers having the silicon and phosphorous elements as backbone (Belgacem and Gandini 2011).

Polymers are used in almost all the corners of living niche, starting from the fancy grocery bags, smart phones, computers, food packaging, and membranes for the water filtration and also designing novel drug delivery system. The synthetic polymers are the hazardous and also not environmentally friendly. The extensive use of the synthetic polymer has caused large-scale environmental hazard. Globally, search of different polymers from natural resources is carried out by researchers and different environmental agencies. Apart from the hazardous problem, the raw materials for synthetic polymers are also non-renewable sources like petrochemicals (Farris et al. 2009; Laine et al. 2013). Therefore, the biobased products including biopolymers have been foreseen as substitute for chemical-based macromolecules (Cutter 2006; Satyanarayana et al. 2009).

The macromolecules of biological/natural origin possess distinct advantages like renewable resource, biocompatibility, and ecological safety (Prashanth and Tharanathan 2007). Further, biopolymers have shown increased shelf life which has numerous uses in the commercial industries (Mensitieri et al. 2011). The monomeric structure present in the biopolymers makes it more thermal stable, flexible, gas resistant, and biodegradable.

Many researchers have classified the polymers based on the source and its production method:

A: Polymers which are isolated from the animal or vegetal biomass
B: Polymers produced from the renewable biobased monomers
C: Polymers produced from the microbial origin (Ruban 2009; Nampoothiri et al. 2010)

Among all this bio-based polymers, microbial origin polymers are renewable, easy to produce in environmentally stable conditions.

11.1.1 Microbial Polysaccharides

Microbial polysaccharides have emerged as the important biopolymers with its novel and distinct properties. The microbial polysaccharides like Arabic gum, xantham gum, and carrageenan have proven as potential substitute for the chemically synthesized polymers (Milani and Maleki 2012; Shit and Shah 2014) (Fig. 11.1).

Microbial polysaccharides are generally extra or intracellular in nature. Mainly in food industries, the extracellular polysaccharides are used. The extracellular polysaccharides are usually attached to the microbial surface in forms of sheaths making films or in forms of capsules (Madhuri and Prabhakar 2014; Nwodo et al. 2012). This extra or intracellular polysaccharides are water soluble and easy to

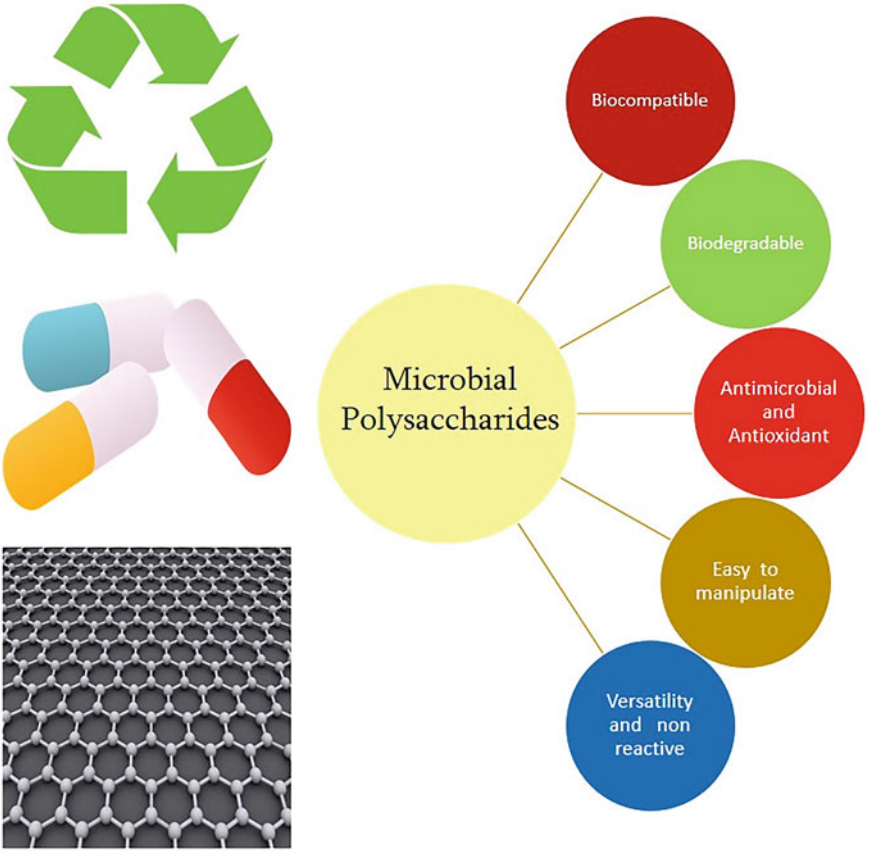

Fig. 11.1 Characteristics of polysaccharides from microbial origin

extract in the liquid medium (De Philippis et al. 2000). Rheological and biocompatible properties make the microbial polysaccharides ideal candidate as biopolymer production. The unique properties of microbial polysaccharides are its use as thickeners, gelling, and stabilizing agents in food industry (Lovegrove et al. 2017). Major advantages of the microbial polysaccharides for industrial applications include the following:

1. Mass production of the microbial polysaccharides is cheap and not region or climate specific.
2. Optimization and growth conditions can be easily manipulated.
3. Strain improvement can be easily done for enhanced production.
4. It has cheap raw materials and easy downstream processing.

The presented chapter gives overview of the diverse types of microbial polysaccharides used in the food industries.

11.2 Biopolymers from Bacteria

11.2.1 Curdlan

Curdlan or curdlan gum is a macromolecule composed of glucose subunits with β-1,3 glycosidic linkage. It thus belongs to (1, 3) β-glucans class of molecules. A single curdlan molecule may have around 135 glucosyl residues and an average molecular weight ranging between 5.3 × 104 and 2 × 106 (Harada et al. 1968; Zhang and Edgar 2014). Like many other polysaccharides, it is also not soluble in water or neutral aqueous solutions at room temperature. Its high molecular weight, linear chain structure, and other properties impart many characteristic features to this compound. It can form complex three-dimensional structures due to orientation of hydrogen bonds between the linear chains (Lo et al. 2007).

The best thing about curdlan is its thermoresistant gel formation upon heating. Unlike most other gelling agents, which require additional conditioning step post heating, e.g. cooling for agar, agarose, carrageenan, gellan gum, gelatin, etc., and calcium addition for alginate and low methoxyl pectin (Lo et al. 2007). Curdlan exhibits no toxicity, no carcinogenicity, and neither adverse effect on reproduction of animal. The US Food and Drug Administration (USFDA) approved its use in December 1996 (Spicer et al. 1999).

Curdlan gels find many applications in food industry owing to its distinct fractures like moisture holding capacity and absence of taste, odour, and colour. Curdlan is used in Japan, Korea, and Taiwan to make large variety of commercial food products (Spicer et al. 1999). It is used in manufacture of jellies, meat analogue, edible fibre, noodles, and dairy products, etc. The inherent property of high molecular weight of curdlan has resulted its usage as dietary fibres (Divyasri et al. 2014; McIntosh et al. 2005).

Curdlan is added for upsurging the quality of food items, viz. noodles and tofu owing to its exclusive stability and flexible consistency after freezing and thawing cycle. It can be also used in manufacture of cheap consistency food products due to its water-insoluble nature. Many researchers have demonstrated the addition of curdlan in potato starch noodles could enhance the texture of the noodles, syneresis, firmness, as well as tensile strength while lowering the cooked weight (Nishinari and Zhang 2004; Nishinari et al. 2009; Zhang et al. 2002; Wang et al. 2010).

Curdlan is produced by fermentation of *Alcaligenes faecalis* and Agrobacterium and is extracted as extracellular polysaccharide by alkaline extraction. Zhao et al. (2016) have modified native curdlan and have prepared a solubilized form of curdlan displaying very slight change in rheological properties. The results show that carboxymethyl curdlan can be employed as thickening and gelling agent in food industry. Further chemical modifications like sulphation, phosphorylation, amination, esterification, etc. are the much promising methods to enhance its solubility as well as bioactivity (Jin et al. 2006; Suflet et al. 2011). These chemically modified curdlan derivatives have been examined for therapeutic uses owing to its robust bioactivities like antimicrobial, anti-cancer, anti-HIV, and immunomodulatory properties (Ichiyama et al. 2013).

11.2.2 Xanthan Gum

Xanthan is a polysaccharide with complex structure. Xanthan gum was first reported in the 1960s and was further approved for commercial practice in the 1970s (Huang et al. 2010; Wustenberg 2014). The architect of the molecule can be divided in two parts, backbone and branches. The backbone is similar to the cellulose, composed of series of β-D-glucose with (1 → 4) linked. The branches are trisaccharide unit of β-D-mannopyranosyl—(1 → 4)—β-D-glucuronopyranosyl - (1 → 2)—6-O-acetyl-α-D-mannopyranosyl molecules, attached to C_3 carbon of every other β-d-glucose of backbone (Xu et al. 2015; De Jong and van de Velde 2007; Mukherjee et al. 2010; Rodd et al. 2000). This structure makes xanthan soluble in hot and cold water and gives it unusual stability to acid, alkali, and heat.

Xanthan is mainly used to make rapid-hydrating water-soluble hydrocolloids. Commercially, it is produced mainly by aerobic submerged fermentation of *Xanthomonas campestris*. The type and character of xanthan gum varies and depends upon microbial strain, carbon and nitrogen source, amount of oxygen, concentration of inorganic ions in medium, as well as temperature and pH of fermentation. Xanthan gum is finally recovered from fermentation broth by alcohol precipitation.

Xanthan gum solutions have diverse viscoelastic properties making a weak gel. Hydration of xanthan gum depends upon stirring speed and time, chain length, ionic strength, temperature, etc. At low temperature, xanthan forms rigid structure, but with the increase in temperature, the conformational change of solution turns to it a more flexible form. The polymer is resistance from pH 3 to 10 and temperature resistant to 0 to 100 °C in water-based solvents (BeMiller 2018). These properties

are unique and most advantageous for food industry for imparting enhanced flavours, texture, dressing, viscosity, and consistency. Food products using xanthan gum include cake and bakery product mixes, chocolate drinks, salad dressings, soy milk, mayonnaise, pasta, pizza and taco sauces, cheese, cream and chocolate spreads, pizza vegetable pattie, pizza, fudge, chocolate syrups, honey-roasted peanuts, meat, chicken, poultry products, and barbecue (BeMiller 2018). The advantage of using xanthan is its wide range stability to pH and temperature and resistance to enzymes like amylase, proteases, and celluloses, which may be present in food products which otherwise can breakdown thickeners like starch, cellulose, CMC, or methylcellulose.

11.2.3 Gellan Gum

Gellan gum is a polysaccharide made up of a repeating unit tetrasaccharide, containing 2 D-glucose residues and 1–1 residues of D-glucuronic acid and L-rhamnose (Sarkar et al. 2017; Jana et al. 2013; Wang et al. 2016a, b; Duan et al. 2015). Each repeating unit may contain about 1 glycerate and ½ acetate molecule attached to the glucose moiety (Duan et al. 2015; Morris et al. 2012; Kang et al. 2015; Fernández-Ferreiro et al. 2017; Sonje and Mahajan 2016; Salunke and Patil 2016). Depending upon the level of acetylation, the gellan gum can be divided into two broad categories: high acyl gellan gum and low acyl gellan gum. The average molecular weight of gellan gum can be about 500 kDa (Duan et al. 2015).

Products made from gellan gum are usually divided into two categories: low and high acyl polymer. The categorization depends on the acetate groups linked with the sites of macromolecules. The polymer with low acyl side links forms the less flexible and stiff gels compared to the flexible and suspendable gels from the high acyl side link macromolecules (Chakraborty et al. 2014; Rosas-Flores et al. 2013).

Gellan gum can tolerate heat and acid stress in the production process. It is biocompatible, biodegradable (Jin et al. 2006; Pacelli et al. 2015, 2016), spongy (Jana et al. 2013; Salunke and Patil 2016; Chakraborty et al. 2014; Goyal et al. 2011), and non-toxic (Salunke and Patil 2016; Prezotti et al. 2014). Being an anionic polysaccharide, it can make polyelectrolyte cationic polymers like chitosan (Vilela et al. 2015). Gellan gum is resistant to the enzymatic action and not degraded by acidic environment (Nag et al. 2011).

Gellan gum is approved by the US Food and Drug Administration and European Union to be used as ingredient (Xu et al. 2015; Zia et al. 2018), and it is used for dough stabilization, water-based gels, jams, confectionery products, fabricated foods, dairy products like yogurt, milkshakes, ice cream, jellified milk, etc. as emulsifier, stabilizer, binder, gelling agent, lubricant, coagulant, thickening, and film former agent (Danalache et al. 2015; Zhang et al. 2017; Fernández-Ferreiro et al. 2015; Bonifacio et al. 2017; Chang et al. 2012; Sonje and Mahajan 2016; Zia et al. 2018). Gellan gum has an advantage of acid tolerance, stability, and producing strengthened gels over commercially used classical crystalizing mediators (Lorenzo et al. 2013).

Due to poor stability under physiological conditions, the gellan gum needed modification and addition of stabilizing ingredients. A MeGG (methacrylated gellan gum) hydrogel, chemically modified hydrogel, was reported to have applications as stabilizing agents and as scaffold in tissue engineering (Coutinho et al. 2010; Zia et al. 2018). The hydrogels had adjustable and better mechanical and physical properties as native ones (Gantar et al. 2014). Gellan gum has be blended and mixed with several other natural polymers like polysaccharides, proteins, etc., and synthetic polymers for enhancing stability, mechanical strength, sustained release, desired binding, and other properties are found to have promising results and wider applications.

11.3 Bacterial Biopolymers for Food Packaging

Plastic packing materials are a big cause of environmental pollutions. Their non-biodegradable nature results piling of waste plastic increasing day by day. They cannot be reused or recycled as they are often contaminated by food materials and biological substance; also it is neither convenient nor economical. Bioplastics on the other hand have an advantage of being eco-friendly and healthy option as compared to conventional plastics. Several types of bioplastic find application in food packaging, surgical and medical material, drug packaging, agriculture, textile, toys, etc. Several biopolymers like polylactic acid (PLA), polyhydroxy butyrate (PHB), polyhydroxy alkaloids (PHAs), etc. have been made extensively for food packaging in replacement to harmful plastic packaging materials.

11.3.1 Polylactic Acid

Polylactic acid (PLA) is hydrolysable aliphatic polyester of lactic acid. It is semi-crystalline in nature and can be produced by direct polymerization of lactic acid. Lactic acid is produced by microbial fermentation using corn, starch, sugar, or other feedstocks (Garlotta 2001). Lactide is a cyclic chiral di-ester derivative of two moieties of lactic acid. Lactide can be present in two forms L-lactide and D-lactide. PLA has can be divided into three types based on its structures: PLLA, PDLA, and D, L-lactide (poly L-lactide, ploy D-lactide and D,L-lactide) (Tang et al. 2004). For general packaging use, PDLA is mostly employed containing 90% L-lactide. The problem with PLA packaging films is its low thermal stability, high production cost, crispness, and noise due to the PLA crystallinity and stiffness. PLA degrades by the hydrolytic breakdown of backbone ester groups. Moreover, the rate of degradation is subjected to its molecular weight, crystallinity, morphology, rate of water diffusion into the polymer, and stereoisomeric content (Janorkar et al. 2004).

To deal with these issues and to make a better polymer, PLA can be blend and mixed with other natural and artificial polymers for enhanced stability, elasticity, strength, and durability. These additives or ingredients intercalate and fill the PLA matrix structure to produce better PLA composites. Several ingredients like starch

(Yokesahachart and Yoksan 2011), other polymers (Yu et al. 2006), plasticizers (Pillin et al. 2006), nanoclays (Bordes et al. 2009), carbon nanotubes (Wu and Liao 2007), etc. have been blended into the PLA matrix. Blending PLA with other bioplastics such as thermoplastic starch (TPS), PHA, PCL, PBS, (PBAT), etc. has been studied and reported by many authors (Asian et al. 2000; Liu et al. 2005; Noda et al. 2004). The subsequent blended polymer showed improved ductility and robustness.

11.3.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are biopolyester polymers that are produced by bacterial fermentation of sugars or lipids (Galliard 1987). They have straight chain hydroxyalkanoates. PHAs have a wide range of monomers that deliver PHAs with flexible and different characteristics.

Different types of PHAs have different properties depending upon their chain length, molecular weight, and other features. This is generally induced by amount-deficient conditions like nutrient stress but under high carbon availability (Halami 2008). PHAs are one of the primary biopolymers used in different fields of application depending on operational alterations and change in distinct characteristics. They are also biocompatible and biodegradable properties which do not deteriorate the environment and are degraded by the other living organisms (Fabra et al. 2014).

PHA polymers and copolymers have moisture, carbon dioxide, oxygen, and flavour blocking properties. It also has higher hydrophobicity as compared to other biopolymer such as starch (Koller 2014). Thus, it would be a good packaging material for packaging of fresh fruits, meat, salads, various convenience food, dairy products, bakery products, etc. Among all PHAs, polyhydroxybutyrate (PHB) is the most thoroughly studied and used one. PHB matches with polypropylene in most mechanical traits, except the brittleness (Rai and Roy 2011; Sudesh et al. 2000). It is commercially a class of biological polymer and used for the manufacturing of the composite materials (Peelman et al. 2013). PHB has been reinforced and tested with materials like vanillin, eugenol and pediocin, sporolipid, paper, and cellulose (Xavier et al. 2015; Narayanan et al. 2013; Solaiman et al. 2015; Cherpinski et al. 2018; Cyras et al. 2007) and showed sustained release of flavour, antifungal compounds, controlled release, enhanced coating, and increased mechanical strength for composite films for food packaging. PHB is produced using microbial fermentation of *Cupriavidus necator*, *Ralstonia eutropha*, *Bacillus flexus*, etc. PHBs are purified by solvent extraction from microbial cell biomass and dried before further processing.

11.4 Plant-Based Biopolymers Used in Food Industries

Plants are the major constituent of the biosphere and have been used for basic needs for living, i.e. food, clothing, and shelter of human beings since ancient time. Apart from basic needs, plants are also utilized in medicines, cosmetics, and many more other purposes related to health and wealth of the human. Over a time period of human civilization, upliftment and modernization, and exaggerated population growth rate, human beings are overexploited the plant resources for obtaining their basic requirements as well as luxurious demands. Although human beings are consuming numerous plant-based biopolymers for their daily life, this section focuses on applications of four plant-based biopolymers (pectin, gum, gluten, and starch) in different food industries into manifold approaches (Fig. 11.2).

11.4.1 Pectin

Pectin is the major constituent of plant cell walls and provides structural integrity, strength, and flexibility and acts as an outward barrier towards protecting the plants from the external environment. Pectins are branched heteropolysaccharides comprising of long chains of galacturonan moieties joined with other neutral saccharides, i.e. arabinose, galactose, rhamnose, and xylose. Structural analysis of pectin revealed that it is a polymer comprised of a chain-like configuration of approximately 100–1000 saccharide units. Due to the occurrence of different types of sugar molecules with different levels of methyl esterification, pectin does not have a defined structure and hence does not definite molecular weight. Pectin is a commercially important entity, and the global market size of pectin is estimated as USD 1.0 billion in 2019 and is projected to grow at a compound annual growth rate (CAGR) of 6.5% to reach a value of 1.5 billion USD by 2025. Mostly, commercially viable pectins are extracted from citrus (citrus peel owns 20–30% pectin) and apple fruits (apple pomace owns 10–15% pectin); however other plant species such as sunflower head, mango peel, soybean hull, sugar beet pulp, peach pomace, banana peel, chickpea husk, etc. also largely been used for pectin extraction (Vanitha and Khan 2019).

Chemically, pectins are classified into three types, i.e. Homogalacturonan, Rhamnogalacturonan I, and Rhamnogalacturonan II. Homogalacturonan is a polymer of galacturonic acid, comprising galacturonic acid residues as long as 72–100% of the overall sugar moiety along with other sugar molecules. Pectins extracted from Cashew apple, citrus, and sunflower are the examples of Homogalacturonan pectin. Rhamnogalacturonan I is a heterogeneous polymer that is consisting of rhamnose and galacturonic acid residues. Pectin extracted from apple, citrus, mung bean, and kidney bean is an example of Rhamnogalacturonan I pectin. Rhamnogalacturonan II is a highly conserved pectin which constitutes about 10% of the total pectin polymer. This polysaccharide is made up of α(1,4) connected galacturonic acid residue units with 12 separate glycosyl residues as side chain like D-rhamnose, apiose,

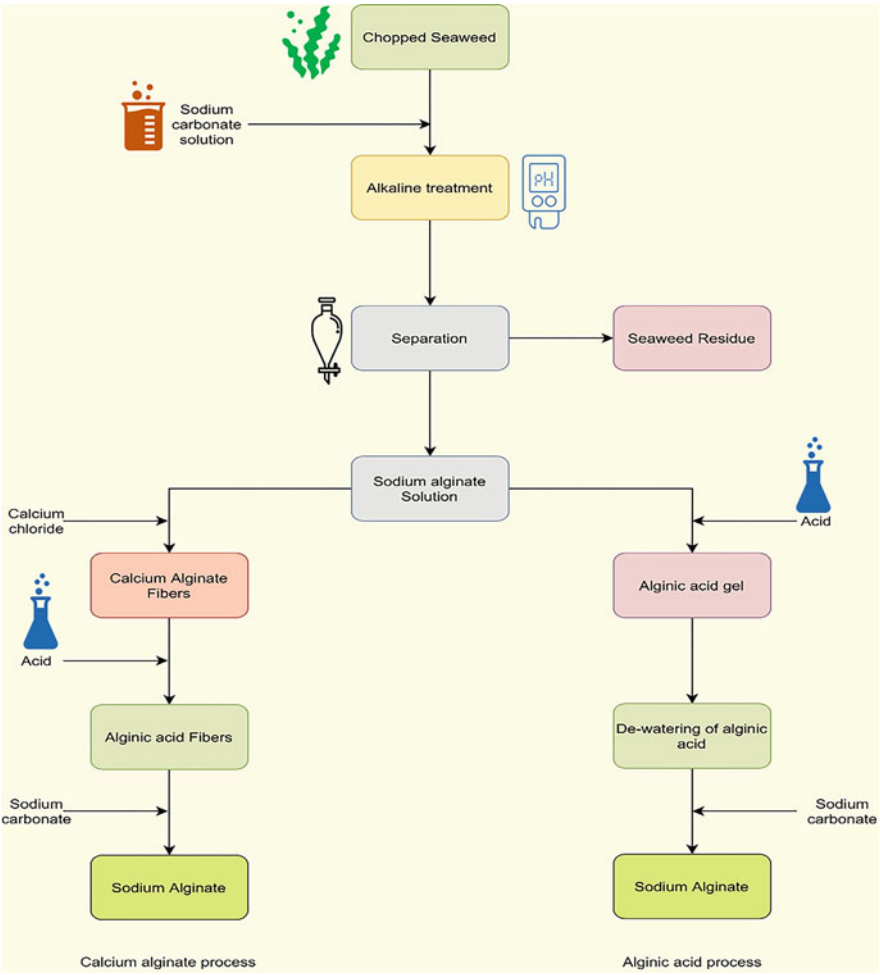

Fig. 11.2 Sodium alginate production

D-galactose, L-galactose, galacturonic acid, L-arabinose, xylose, and L-aceric acid (Zhan et al. 1998; Vincken et al. 2003; Voragen et al. 2009).

Due to easy availability, commercial feasibility, and physicochemical properties, pectins are extensively used in several food industries. Pectins are natural hydrocolloid, and due to gelling ability, it is used as viscosity enhancer during food processing. Pectin is used as a best-emulsified agent, and examples of pectin-based emulsified marketed available products are meat products, bakery products, desserts, sauces, low-cholesterol mayonnaise, low-fat cottage cheese, low-fat drinking yogurt, low-fat drinking dairy products, etc. (Méndez-Zamora et al. 2015). As a gelling agent, pectins are used in the preparation of jams, jellies, and marmalades. High-methoxyl pectin is generally used in the preparation of standard jams, high-quality

tender confectionery jellies, fruit pastes, etc. However, low-methoxyl pectins are used for the preparation of low-calorie food products such as milk desserts, jams, and jellies (Chassaing et al. 2015). Pectins are also used as biodegradable food packaging material that serves as moisture, oil, and aroma barrier and reduce the respiration rate and oxidation of food. Pectin along with other food-grade emulsifiers is also used in the preparation of edible films that are used in fresh and minimally processed fruits and vegetables, foods, and food products (Bourtoom 2008). Pectin-based biofilms are used for extension of shelf life, maintaining moisture and vitamin contents of avocado fruits, fresh-cut melon, fresh-cut mangoes, sapota fruits, papaya slice, pineapple slice, etc. Nowadays, antimicrobial films have been developed using pectin along with lysozyme to prevent food poisoning and spoiling due to microbial contaminations of certain food and food products (Bayarri et al. 2014; Silva et al. 2015).

11.4.2 Arabic Gum

Arabic gum/acacia gum is dried exudate, edible gum obtained from the plant *Acacia senegal* and *Acacia seyal* (Phillips and Williams 2000). Arabic gum is a branched-chain polysaccharide that exists as a calcium, magnesium, or potassium salt of Arabic acid. Gum Arabic contains the sugar units such as L-arabinose, L-rhamnose, and D-glucuronic acid and backbone structure consisting of β (1,3)-linked D-galactopyranosyl units. Arabic gum comprised of 39–42% galactose, 24–27% arabinose, 12–16% rhamnose, 15–16% glucuronic acid, 1.5–2.6% protein, 0.22–0.39% nitrogen, and 12.5–16.0% moisture which varies according to the source, climatic conditions, and age of the tree. The gum has been used in many food industries make it commercially valuable being. Arabic gum has been commonly used as a food stabilizer, thickener, and emulsifier agent in several food industries. The global gum Arabic market size was valued at USD 396.1 million in 2019, and it has been forecasted to reach USD 603.25 million by 2025 (CAGR of 6.17%). Arabic gum (10%) has been successfully used as an edible coating on tomatoes at ambient storage conditions which had delayed the ripening process and prolonged the shelf life. The composite combination edible coating of Arabic gum (10%) with chitosan (1%) on banana extends the shelf life of the fruits over a month without any quality compromise. The said composite combination of Arabic gum and chitosan has also been used as bio-fungicide in banana and papaya fruits during cold storage. Arabic gum in combination with soybean gum has been extensively used in the storage of apples, cucumber, and other fruits and vegetables to enhance the shelf life and quality of the cold stored fruits and vegetables (Maqbool et al. 2011; Al-Juhaimi et al. 2012; Ali et al. 2013; Sahaa et al. 2017).

11.4.3 Guar Gum

Guar gum is a galactomannan derived from the seeds of the leguminous plant *Cyamopsis tetragonolobus*. The seeds of the plant are made of the germ (43.47%), endosperm (35.42%), and hull (14.17%). The endosperm of the seed is separated, grounded to a fine powder, and sold commercially as guar gum. Guar gum is a polysaccharide, composed of linear D-mannose units joined by β (1,4) glycosidic linkages. D-Galactose side chains are linked to the mannose units by 1,6-linkages and galactose to mannose ratio of approximately 1:2. Due to viscosity property at lower concentrations, guar gum is used as a thickener, binder, and emulsifier agent in food industries. The gum is enlisted as an economically important material, and the global guar gum market size was estimated at USD 604.6 million in 2019 and expected to grow at 4.4% CAGR to reach USD 785.2 million by 2025. Examples of uses of guar gum are edible coatings on tomato, Japanese persimmon, and cucumber which lead to delayed ripening of tomatoes and enhance shelf life of cucumber and other vegetables and fruits (Ghosh et al. 2014; Sahaa et al. 2017).

11.4.4 Gluten

Gluten is a group of seed storage proteins found in cereal grains (wheat, rye, barley, and oats), and gluten-containing grains are widely consumed as staple food. Generally, "gluten" pertains only to wheat grains and is the combination of two naturally occurring proteins called gliadin and glutenin. Amino acids present in both gliadin and glutenin help the two proteins to form hydrogen bonds with each other and hence stabilize the structure of gluten. These two proteins bind to each other to form a network that supports dough and allows bread to be light and fluffy. The bread wheat comprises of 75–85% gluten of the total protein. Wheat germ agglutinin is a carbohydrate-binding protein, found mostly in the wheat kernel (de Punder and Pruimboom 2013). The global wheat gluten market is projected to grow with a significant CAGR of 8.22% and reaches a market value of USD 1.48 billion by the end of 2025. Gluten is widely used as a binding and enriching ingredient in processed meats, i.e. in beef, pork, mutton, chicken, turkey meat, and fish. It is also used for making batter, pasta, and common ingredient of pizza toppings, salad toppings, veggie burgers, and sandwich fillings. In processed-meat products, gluten is an excellent binder in poultry rolls, canned "integral" hams, and other food products. Gluten improves slicing characteristics and minimizes cooking losses and mimics the mouthfeel, chew, and taste of meat. Gluten is also used in the preparation of synthetic cheese, fruit puree, and soy sauce in different food industries (Van and Schueren 2002; Marchal et al. 2002; Day et al. 2006; Biesiekierski 2017).

11.4.5 Starch

Starch or amylum is the most plentiful homopolymeric branched carbohydrate consisting of numerous glucose units joined by glycosidic bonds. Starch is composed of two kinds of polysaccharides, amylose and amylopectin, exclusively composed of D-glucose residues with α(1,4) glycosidic linkages in a linear amylose and α(1, 4) glycosidic linkages along with ∼5% of α(1, 6) branched glycosidic linkages in amylopectin. Starch is an insoluble, biodegradable, and thermoplastic carbohydrate polymer. This polysaccharide is produced by almost all green plants during the photosynthesis process as an energy storage entity. Starch is the utmost common carbohydrate found in staple food (wheat, rice, maize, potatoes, and cassava) of the human diet, and the whole world population is depending on these five grains for their primary energy sources. Due to its easy availability and extraction from the source, economically feasible, and easy modification on physical and chemical properties, it is the first preferred choice and hence most used in the different food industries for numerous food processing applications. Generally, commercially marketed starches have been extracted from the five easily available raw food materials, i.e. corn, potato, rice, tapioca, and wheat. The global industrial starch market size is estimated at USD 87.93 billion in 2019, and it will reach USD 87.93 billion in the next 5 years at a CAGR of 6.6%. The above said five starches have been categorized as gluten-free starch which suits well for people who are allergy to gluten (Martinez-Garcia et al. 2017; Li et al. 2018; Yazid et al. 2018).

Starch is the major biopolymer utilized naturally as food by the living organism as compared to other existing biopolymers ever. Due to easy availability of starch and diversity in food applications, it has been utilized by most food industries for the preparation and processing of different types of food and food products. Some common examples of starch-dependant foods are bread, cookies, pasta, pastries, noodles, and breakfast cereals. The primary sources of starch are rice, wheat, maize, potatoes, and cassava. Rice starch has been used as emulsion stabilizers and improved gel properties with the addition of hydrocolloids for the preparation of many foods and food products (Sun et al. 2017; Shrivastava et al. 2018). Corn and wheat starch help in increasing fibre content in cake productions, corn syrup production, thickener in infant formula, breadfruit, and cookies formulation (Gilbert et al. 2013; Sitohang et al. 2015; Majzoobi et al. 2016; Santoso 2018). Due to more adhesive and gelling characteristics, potato starch has been used for the preparation of potato starch bio-film and used in several food industries. Tapioca starch has been used as a thickener in fruit fillings and enhancer of soup quality and for the preparation of gluten-free jasmine rice bread (Wongsagonsup et al. 2014). Overall, starch has been used in baked products, i.e. bread, pies, samosas, wafers, biscuits, and sausages. It is extensively used in confectionery (to produce candy, sweets, and sweetmeat), gravies, soups, sauces, tomato paste, ketchups, mayonnaises, salad dressing, ice cream, spreads, beverages, making of pasta (spaghettis, macaroni, etc.), and puddings (custard, pap, others). It is also used as a flavour encapsulating agent for battered meat and meat products, refrigerated and frozen foods, and many

other innumerable applications in numerous food products (Mason 2009; Ackar et al. 2015; Korma et al. 2016; Egharevba 2019).

11.5 Biopolymers from Seaweeds

The demand for use of seaweed-based biopolymers in food industry has significantly increased in the recent century. Although the use of seaweeds has dominated traditional food and cuisine industry in Asian region for centuries but owing to the modern food processing methods and equipment, there is upsurge growth of seaweed-derived biopolymers globally. In year 2018, 32,386 tonnes of aquatic seaweeds were produced globally (FAO 2020). The global commercial seaweed market income is predicted to cross USD 85 billion by 2026, and rising consumption of seaweed as a food ingredient will propel commercial seaweed industry growth to attain 12% CAGR over the forecast timeframe between 2020 and 2026, according to a research report by Global Market Insights Inc. (Global Market Insights 2020). The three major dominating biopolymers (hydrocolloids) in the food industry (Alginates, Agar and Carrageenan) are discussed here (Fig. 11.3).

11.5.1 Alginates

Alginates or alginic acids are obtained from brown seaweed, i.e. *Phaeophyta* genus such as *Macrocystis pyrifera*, *Laminaria hyperborean*, *Laminaria digitata*, *Ascophyllum nodosum*, etc. Alginic acid is made up of linear polymers of

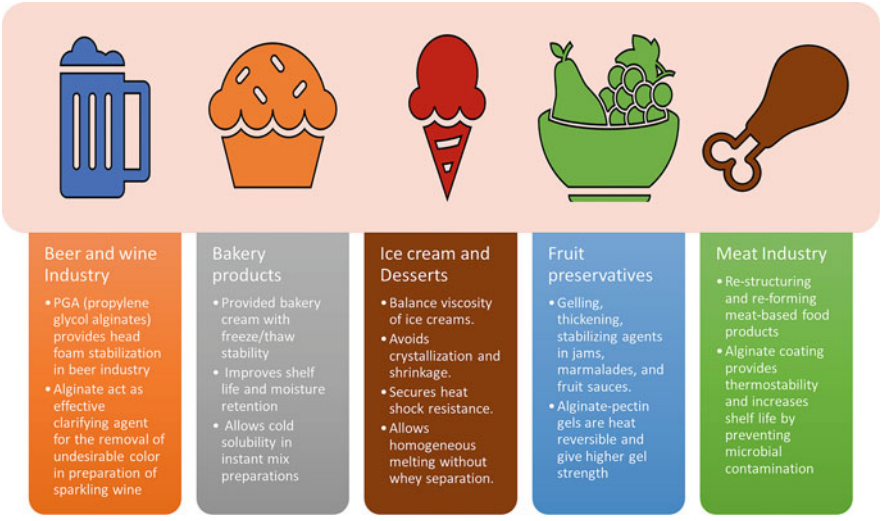

Fig. 11.3 Alginate polymers and its application in food industry

α-l-guluronic acid (G) and β-d-mannuronic acid units (M) connected by 1 → 4 glycosidic bonds; both these M and G units are alternately arranged (Olatunji 2020) (see Fig. 11.4). The way of arrangement of these M and G units and the overall ratio, M/G, vary from one species of seaweed to another, and hence the "alginates" extracted from different species are not necessarily the same. Moreover, alginates with a higher G content are known to provide a stronger gel, and such alginates have a low M/G ratio (Liu et al. 2019). The alginates have ability to rapidly form hydrogels in existence of divalent cations (Mg^{2+}, Ca^{2+}, etc.) at room temperature. The gelation of alginate is brought by the cross-linking property of the alginate polymers with divalent cations such as Mg^{2+}, Ca^{2+}, $Fe^{2+,}$ Ba^{+2}, Sr^{+2}, or even trivalent ions such as Al^{+3} (Tonnesen and Karlsen 2002). The divalent and trivalent ions participate in the interchain ionic bonding in G-blocks of the polymer thereby inducing a 3D network denoted as "Egg Block" structure, resulting in gelation (Cao et al. 2020; Costa et al. 2018) (see Fig. 11.4). Monovalent ions such as Na^{+} are known to compete with the divalent ions; for junction sites of G residues, hence Na^{2+} are known to disrupt or depolymerize the gelation process. The gelation attribute is widely exploited in various industrial application. Ca^{2+}–alginate gels are used in microencapsulation and immobilization of various compounds, enzymes, cells, etc., owing due to its gelling, biocompatibility, and biodegradable properties (Orive et al. 2006a, b). The chelating and gelling properties of alginates have widely attracted its use in food industry as thickeners, emulsion stabilizer film, and coatings (Stephen and Phillips 2016).

11.5.1.1 Alginate as Thickeners

The thickening attributes of alginate are exploited in preparation of sauces, syrups, and ice cream toppings. In bakery industry, the pie is filled with alginate gels to make it thicker. Also, in pastry preparation, the softening effect occurring due to liquid fillers is reduced by addition of alginates. In addition, the alginate coating protects the baked goods by providing non-sticky layer from the icings and semisolid toppings (Glicksman 1986). Alginates are mixed with water-in-oil-based emulsion sauce such as mayonnaise to prevent separation of oil and water (Li et al. 2020; Yang et al. 2020). However, under acidic conditions the sodium alginate becomes insoluble due to low pH and disruption of polymers. Hence in such cases, propylene glycol alginate (PGA) is used to provide stability in mild acid conditions. Alginates are well recognized to enhance the smoothness, consistency, and sheen of yoghurt; in addition, PGA is also employed for the stabilization of milk proteins under acidic pH, as in case of yoghurts. Propylene glycol alginates are known to increases foam stability and are readily used in beer industry (Stewart 2016). Some fruit drinks are acidic and have fruit pulp; hence it is desirable to keep this pulp in suspended form. In such case, addition of PGA in acidic pH can avoid the sedimentation of the pulp. Similarly, in chocolate milk, the cocoa can be kept in suspension by an alginate/phosphate mixture, along with carrageenan. A small amount of alginate (0.05–0.1%) can impart thickness and sustain foam stability of the whipped cream. In wine industry, sodium alginate is used as an effective clarifying agent for the removal of undesirable colour and in preparation of white wine. Moreover, alginate beads are

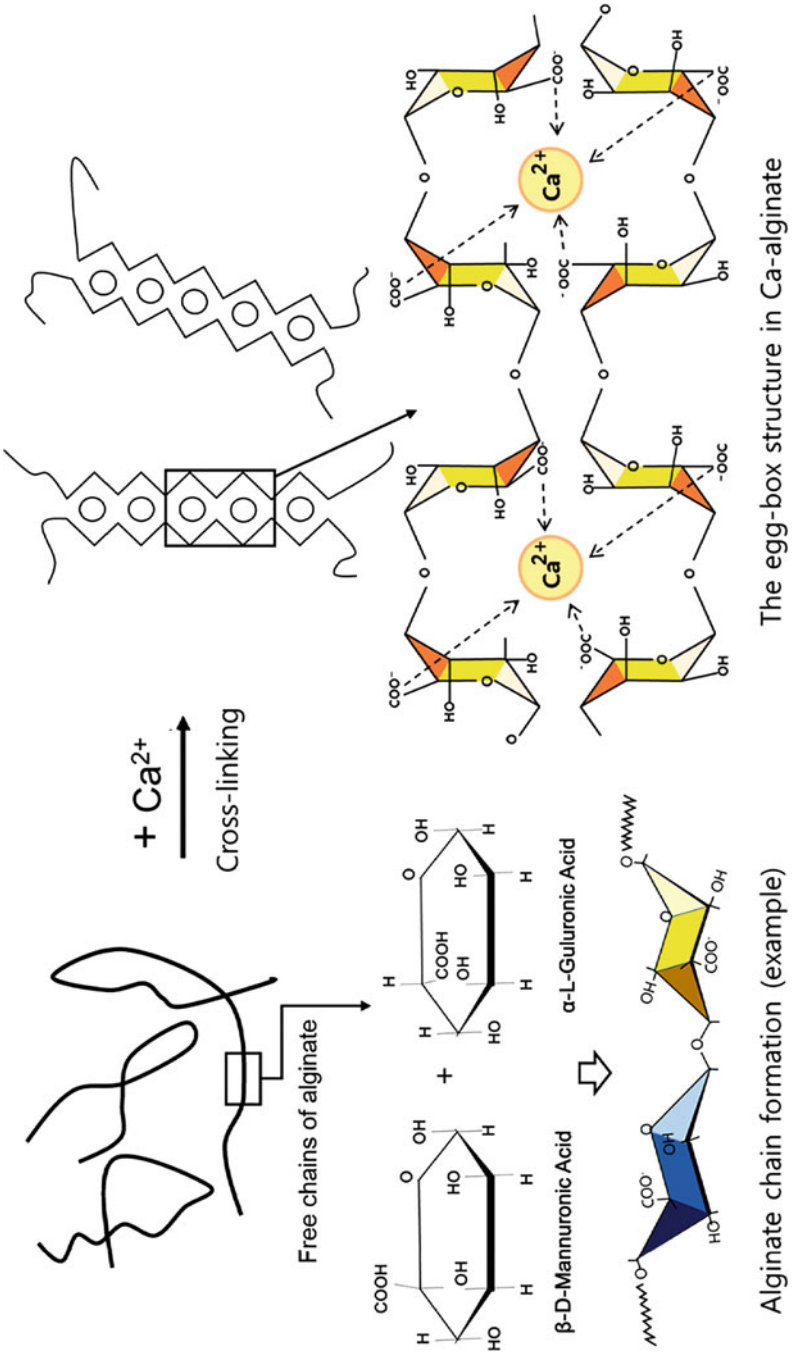

Fig. 11.4 Cross-linking of alginates and calcium cations in "egg box model" adapted from Park and Lee (2017)

used for encapsulation and immobilization of yeast cells in wine industry (Benucci et al. 2019).

11.5.1.2 Alginates as Gelling Agent

Alginates have several applications owing to the gelation properties. The gelling attribute of alginate is exploited in preparation of artificial cherries since 1946 (Syplie 1946). A desirable flavoured and coloured solution of sodium alginate is dropped, in a calcium solution. The calcium ions enter the alginate drop and replace Na^{+} ions, resulting in cross-linking of Ca^{+2} ions and converting it to gel matrix, which is further hardened in calcium solution (Lee and Rogers 2012). Since this process does involve heating and melting of gels, hence it has widespread use in confectionery industry. Moreover, alginate cherries are firmer compared to gelatin jellies. Apart from flavoured solution, the fruit-based gels are also prepared by employing fruit syrups. Alginate gels are also used in restructuring or reforming meat-based food products. Meat pieces are bonded and restructured by using gelling properties of alginate to provide a desirable shape such as nuggets, roasts, meat loaves, steaks, etc. (Kim et al. 2020). The binder powder such as sodium alginate, calcium carbonate, lactic acid, and calcium lactate is readily used in meat-based industry. In some cases, soy protein concentrate, whey protein, garlic, herbs, condiments, anti-browning agents, etc. are also blended along with the meat to impart flavour and act as coating thereby increasing shelf life by preventing microbial contamination (Molayi et al. 2018). Moreover, thermostability ability of alginate coating also facilitates the microwaving of alginate-based food.

11.5.2 Carrageenan

Carrageenan are well-known mixed biopolymers obtained from the Rhodophyta genus (carrageenophytes) of algae. The first reported use of seaweeds (Irish moss, *Chondrus*) containing carrageenan in foods and medicines, as fertilizer, and to modify milk texture was recounted about 600 years ago from the residents of County Carraghen, on the southern Irish coast (Loth 1993). However, the extraction and isolation of the active polysaccharide was reported in 1837, and its refinement and purification using solvent precipitation (alcohol) was done as early as 1871 was the first to coin the name "carrageenin" for the extract from Rhodophyta alga (*Chondrus crispus*). Apart from *Chondrus crispus* (Iris moss), the other well-known species used for extraction of carrageenan includes *Eucheuma* spp., *Kappaphycus*, *Furcellaria fastigiata*, *Gigartina* spp., *Hypnea* spp., *Iridaea flaccidum*, *Phyllophora rubens*, and *Rhodymenia palmata* (Qin 2018). Principally, carrageenan is known to possess linear sulphated polysaccharides with a galactose unit with alternating α (1–3) and β (1–4) bonds. Commercially, the three main classes of carrageenan are kappa (κ), iota (ι), and lambda (λ) occurring due to alteration in the sulphate moiety. κ -carrageenan has one sulphate moiety per disaccharide, ι-carrageenan has two sulphate moieties, while λ-carrageenan has three. Furthermore, ι-carrageenan and κ-carrageenan are used as gelling agents, and λ-carrageenan is used as thickening

and viscosifier agent in food application due to its excellent rheological properties (Bagal-Kestwal et al. 2019; Kozlowska et al. 2018). Apart from pure form, different types of hybrids also have been used in food industry, such as diastereomer of κ/ι-hybrids and λ/ι-hybrids. The κ/ι-hybrids are collectively used in food industry, specifically in dairy applications (Geonzon et al. 2020). Carrageenans are readily soluble in polar solvents while however are insoluble in non-polar solvents (Olatunji 2020). The water solubility attribute is due to the presence of sulphate groups and the type cationic salts such as sodium, potassium, calcium, and magnesium. Furthermore, the 3,6-anhydrogalactose and ester sulphate moiety are known to influence hydration, gel strength, texture, melting, and setting temperatures. The κ- and ι-carrageenan have excellent gelling properties owing to presence of 3,6-anhydrogalactose units, whereas absence of 3,6-anhydrogalactose group in λ-carrageenan results in its nongelling properties. In case of κ-carrageenans, K^+ ions are known to play crucial role in gelation. As the concentration of K^+ ions is increased, the subsequent gel structure becomes strongly aggregated and results in syneresis (moisture on the gel surface) (Bui et al. 2019; Mangione et al. 2005). While in case of ι-carrageenans it is generally seen, the Ca^{2+} initiates gelation and imparts rigidity and stiffness owing to hydrophobic interactions (Pan et al. 2017). All the three types of carrageenan are regarded as safe by the food industry (E407) (Food Standards Agency 2020).

11.5.2.1 Carrageenan in Dairy Foods

The prime applications for carrageenan are in the food industry, especially in dairy products. A very minute proportions (0.01–0.05%) are added to cottage cheese to prevent separation of whey and hardening of cheese (Makhal et al. 2013). Similar amount is also added to ice cream to control smoothness, grain size, and ice crystal growth. Furthermore, in chocolate milk, the cocoa is maintained in suspended form by supplementing it with κ-carrageenan, since κ-carrageenan is known to provide a mild thixotropic gel which provides stability for cocoa unless shaken vigorously (Zhu et al. 2020). In instant chocolate powders, λ-carrageenan is blended along with milk powder to impart stability. λ-carrageenan is also employed in liquid coffee whiteners to avoid the separation of fat (Kapchie et al. 2018). A combination of λ-carrageenan and κ-carrageenan is blended with natural whipping cream to maintain the lightness, ensuring to trap minute air bubbles (Kováčová et al. 2010).

11.5.2.2 Carrageenan in Water-Based Foods

Gelatin has a wide application in food and beverage segment. Primarily, gelatin is obtained from hydrolysis of collagen fibres (extracted from skin, bones, and connective tissues) of animals. However, owing to the safety and concern associated with bovine spongiform encephalopathy (mad cow disease) and foot-and-mouth disease, attempts have been made to find appropriate replacements for gelatin in food industry. Carrageenan obtained from seaweeds serve as excellent substitutes of gelatin (Oladzadabbasabadi et al. 2017). Various permutation and combination of carrageenan with locust bean gum, konjac flour, corn starch, etc. provides a range of melting and non-melting gels, with numerous textures, suitable to meet the

requirements of water-based jellies and food products (Campbell and Hotchkiss 2017). Furthermore, the shelf life of refrigerated mousse desserts, based on carrageenan, is more in comparison to gelatin. In addition, carrageenan-based jellies and mousse desserts are suitable for vegetarians and certain ethnic groups. To solidify and set a jelly, the conventional fruit jellies utilize pectin along with high amount of sugars. In low-calorie jelly, the pectin is substituted with mixtures of κ-carrageenan and ι-carrageenan. Furthermore, most of the cold drinks and fruit beverages are modified in cold conditions by adding sugar, aspartame, acid regulators, and flavour along with κ-carrageenan, ι-carrageenan, and λ-carrageenan for a pleasant mouth feel (Tsai 2006). A mixture of κ-carrageenan and ι-carrageenan, along with locust bean gum or pectin, is used in sorbet (a creamy option to ice cream with no fat) to provide smooth texture (Isaacs et al. 2018).

11.5.2.3 Carrageenan in Meat-Based Foods

In meat processing industry, carrageenan and brine solution are used to process hams; this process helps to bind carrageenan to the protein so that the soluble protein is retained thus imparting a decent colour, texture, and cooking yield to the ham (Blakemore and Harpell 2009). Carrageenan is also used in pre-cooked poultry processing units such as pre-cooked chicken and turkey pieces. A major concern in poultry processing industry is the loss of water occurring during pre-cooking stage. This loss of water results in lower per unit weight of product thereby reducing the yield. Moreover, it may also result in loss of texture and compromising the quality of the product. Hence to retain moisture, the meat is injected with salt brine, phosphate, and carrageenan (Foegeding and Ramsey 1987). As the meat is roasted, the carrageenan and water molecules attach within the protein and form a weaker chain association and ionic cross-linking, thus enhancing the texture and imparting tenderness. Moreover, this process ensures retention of moisture thereby promoting palatability and cooking yield (Farhan and Hani 2020). Carrageenans and hydrocolloids are also used in low fat meat products as fat substitute. Such low-fat meat is formulated with carrageenan to keep juiciness and tenderness intact (Fontes-Candia et al. 2020) (Fig. 11.5).

11.5.3 Agar

Agar is a mixed polymer primarily consisting of two components: the agarose and agaropectin. Agarose consists of straight chain polymeric units of agarobiose. Agarbiose is made up of disaccharide units of D-galactose and 3, 6-anhydro-L-galactopyranose, while the agaropectin consists of β-1,3-linked D-galactose units altered with sulphate and pyruvate moieties (Olatunji 2020). The agar is extracted from agarophytes belonging to the Rhodophyta (red algae) phylum. The Rhodophyta algae such as *Gelidium*, *Gracilaria*, *Pterocladia*, and *Gelidiella* are used for the extraction of agar. The *Gelidium* species are well-recognized for the gel strength; agars extracted from these species provide a good gelling property. However, the agar extracted from *Gracilaria* and other species require certain

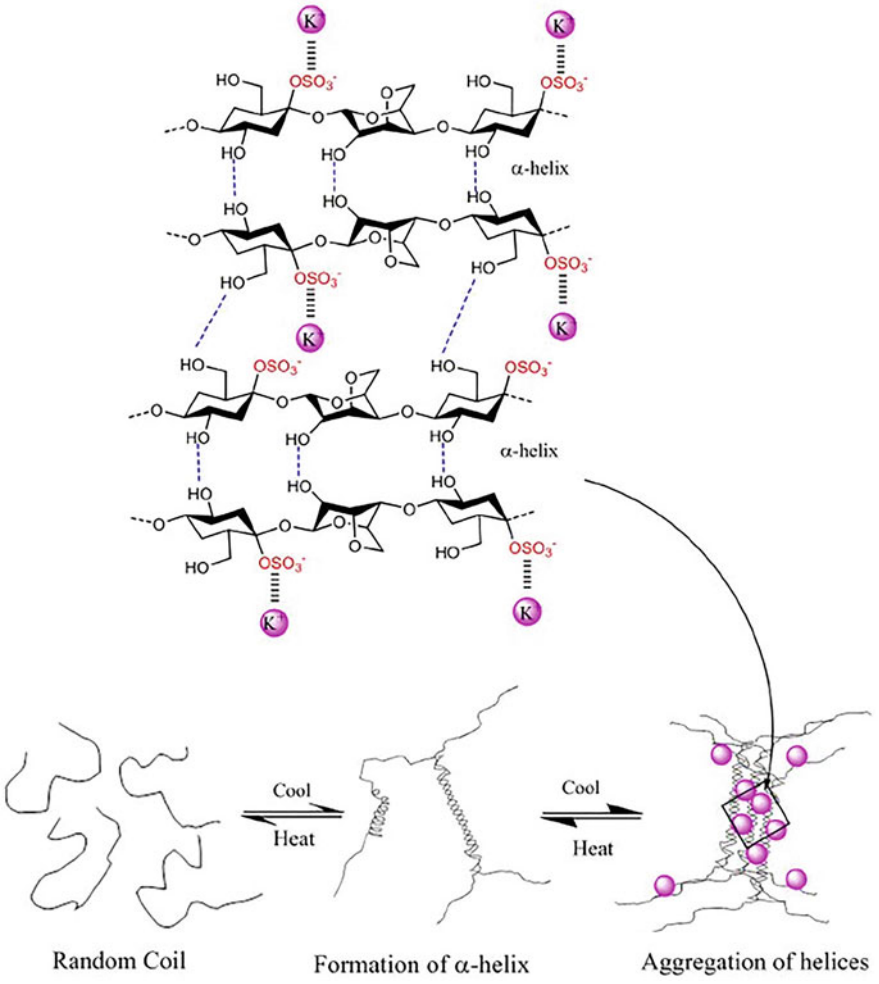

Fig. 11.5 The gelation process of κ-carrageenan in the presence of K^+ ions (Adapted from Rhein-Knudsen et al. 2015)

pretreatment and molecular modification to enhance the gel strength. Agar is a very effective gelling agent. Agar has the ability to form gels even at very low concentrations (0.2%). Moreover, these gels are thermally reversible and firm. The gelling properties are attributed by hydrogen bonding between adjacent chains of repeating units of galactose and 3,6-anhydrogalactose; hence no other external agents are required to form a gel (Chen et al. 2021). In addition, the gel structure is not disturbed or affected by the presence of any salts or proteins. The gel hysteresis characteristic, i.e. differences between melting and setting points, is significantly greater for agar as compared to other gelling agents. In general, the agar melts at 85 °C and sets at 38 °C; hence, agar solutions often require boiling temperature to

dissolve (McHugh 1987a, b). Moreover, upon cooling, the gelation of agar solution is instigated by coil-to-helix shift, supported by the accumulation of the helices to create a complex network structure, resulting in gelation.

11.5.3.1 Agar as Replacement for Gelatin

Gelatin is a combination of peptides and proteins obtained from partial hydrolysis of collagen extracted from the skin, bones, and connective tissues of animals (cattle, chicken, pigs, and fish) (Poppe 1992). Although gelatin is regarded safe ingredients in food industry, however in some vegan and vegetarian groups, consuming gelatin may cause heaviness in the stomach, bloating, heartburn, and belching. Gelatin can also cause allergic reactions (beef meat allergy) (Mullins et al. 2012; Sakaguchi et al. 1996). Hence in such cases, agar is used as a vegetarian substitute for gelatin. Moreover, agar has been categorized as GRAS (Generally Recognized As Safe) by the US FDA and by EU (E406) (Mortensen et al. 2016).

11.5.3.2 Agar in Gel-Based Food

Agar is primarily used as a gelling agent and as a stabilizing agent to control the viscosity of the product. Hence, in confectionery, agar is used to prepare jellies, jams, marshmallows, candies, or candy fillers. Agar is also used in marmalade production, as an alternative to pectin or gelatin, for providing thickening and gelling of marmalade. Moreover, the use of agar results in providing superior gelling properties and as a strengthening component in custards, soufflés, tarts, pies, cobblers, sorbets, etc. Agar-based jams are excellent alternative to pectin-based jams. In comparison to pectin-based jam, agar has the advantage to form gels, even in the presence of low sugar concentrations. The Japanese dessert Mitsumame is prepared with fruit salad with agar gel cubes. This gel cubes are coloured and imparted flavours using various salty, spicy, sweet, and tangy seasonings.

11.5.3.3 Agar as Foam Stabilizers

Foams are widely use in the food industry such as whipped cream, meringue, and mousse. Foam is generally produced by dispersing air bubbles, suspended in a continuous phase in the matrix. The foam structure provides a required texture to foods and aims to reduce mass per volume, thereby reducing the calorie intake since less product is required within a pack (Green et al. 2013). Often production of foams relies on usage of stabilizers, since air has low viscosity and low surface tension, which can result in higher chance of coalescence. Hence, agar gels are used as a stabilizer in foams (Olatunji 2020). Agar gel specks are suspended in a continuous phase, thereby avoiding coalescence and maintaining colloidal nature. Moreover, agar gel specks readily absorb the water molecules from the foam cavities thus preventing the release of water molecules. This phenomenon results in stabilizing the foam as agar specks act as a viscosity enhancer. The presence of the agar gel specks in foam also acts as a barrier preventing fluid drainage from the foam (Ellis et al. 2019). Agar therefore has an important role to play in stabilizing the microstructure of foam-based food and providing a healthier option towards high-calorie fat-based foams.

11.5.3.4 Agar as Food Packing Material

Agarose is known to exhibit thermoplastic properties, and hence agar-based films are appropriate substitutes for edible food packaging materials (Mostafavi and Zaeim 2020). One of the major constraints for use of agarose in food packaging is the fragile nature of agarose gels owing to low moisture content and poor mechanical properties. Hence, agarose is combined with other materials such as carboxymethyl cellulose (CMC). The agar-CMC in combination with silver-modified montmorillonite (Ag-MMT) nanocomposite is known to demonstrate potential antibacterial activity against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria, as a packaging material for food preservation (Makwana et al. 2020). Several other fillers also such as locust bean gum (Choi et al. 2010; Sousa and Gonçalves 2015), nanoclays (Abdul Khalil et al. 2017), cellulose nanocrystals (Oun and Rhim 2015; Reddy and Rhim 2014), grape fruit seed extracts (Kanmani and Rhim 2014), lignin (Shankar and Rhim 2017), and polylactic acid (PLA) (Rhim 2013) have been integrated with agar to retain the water and enhance antibacterial activity against food-borne pathogenic bacteria.

11.6 Conclusion

Globally, demands of polymers are too high. As per the statistics portal, reports suggest that currently the synthetic plastic turnover is more than 299 million tons and is expected to grow more by 9% per annum. Currently, biobased polymer manufacturing has just initiated and is found in its application in many sectors including the food, agricultural, and pharmaceutical industries. The highest commercial market of biopolymers is European markets. The European market covers approximately 5–10% of the biopolymer market with annual production about 50 thousand tonnes. It is also estimated that usage of biopolymers in food industry will rise significantly due its unique extrinsic and intrinsic properties. The major usage includes its application in food packaging and transporting industries. Few commercial products are biopolymer-based foils, films for prevention of food spoilage.

Biopolymers in food industries have fulfilled the ecological conditions; however the concerns still persist with its thermal, mechanical, and economics associated with its commercialization. The extended shelf life, flexibility, quality, and microbiology safety are positive aspects of the biopolymers. To replace the synthetic polymers, these qualities of biopolymers still need to be optimized for making biopolymers economically viable and more sustainable.

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Recent Aspects of Fortified Foods: An Overview on Field Testing Tools and Fortification Program Analysis Methods

12

Kiran Patruni and Gurveer Kaur

Abstract

Malnutrition is a current global challenge faced by mankind, mainly caused due to deficiency and excess of macro and micronutrients such as growth retardation, anemia, mental retardation, overweight, night blindness, reduced IQ, neural tube defects, cancer, hypertension and depression, etc. According to UNICEF, India was at the tenth position among countries with the highest number of underweight children and at the 17th position for the highest number of stunted children in the world. Fortification of staple food with macro and micronutrients is the common practice to meet the deficiency disorders and to provide complete growth. Despite decades of scientific research in this area, still complications arise for establishing connecting links from individual energy intake levels of targeted population to policy making, implementation, and evolution. Thus, the present chapter aims to cover the details about field testing kits for analysis fortificant levels of iron and vitamin followed by brief concepts about the practicing methods used for diet assessment at individual, household, and population level to decision making at analyst and policy maker levels during implementation of food fortification program. Overview of field-testing tools and fortification program analysis methods could help for development of more stringent protocol which could meet the global standards for implementation of fortification program.

Keywords

Food fortification · Filed testing · Fortification program · Methods · Tools

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12.1 Introduction

Fortified foods are tested by standard qualitative and quantitative methods to ensure the safety control and actual level or concentration of fortificant are determined for proper labeling of the product. The fortified food is tested based on specific fortificant or micronutrients that have been encapsulated into the food. Methods for analysis of fortificant in various foods are approved by Food Safety and Standards Authority of India (FSSAI), and the ability and selection of test for fortificant in fortified food depends on the technology of encapsulation and type of food. Rapidness of test for fortificant permits program manager to easily determine whether fortified food fulfills technical specifications and objectives. The development in testing methods has grown from time-consuming experiments, i.e., instrumental methods in laboratories to rapid analysis using portable devices over years according to the demand of ease accessibility and rapidness of determining the content and presence of fortificant such as folic acid, iron, iodine, vitamin A, etc.

The results that originated from these methods of analysis provide important information for indicating if:

1. Processes of fortification is working appropriately and concentration levels of fortificant are within defined requirements at the production and factory level, based on inspection and screening by Quality Control plus Assurance Department.
2. With required conditions fortified food items are available to customers.
3. At national levels consumption of micronutrient level regulations and standards are required imported fortified food items.

The requirements of testing are addressed at three levels based on production and distribution systems: qualitative field testing is needed for monitoring effective delivery of micronutrient to the consumers, and it produces the presence or absence of results; semi-quantitative tests are adequate for the manufacturers; and standard quantitative measures are required at the regulatory levels. Fortified food samples need to be evaluated by the most stringent methods, which can collect information very quickly, cost-efficient, accurate, selective, sensitive, and easy to handle. In a practical manner, the execution cost along with complexity of testing methods depends on different variables such as state of fortification compound used for fortification (i.e., iron salts vs. reduced iron), type of the matrix (i.e., wheat flour vs. salt or sugar), the technique used for microencapsulation (i.e., spray dying vs spray cooling), the availability of methods for identifying the fortificant, and the type of factor noticed for quantification (i.e., spectrophotometry versus titration volume) (Yuan et al. 2008).

The establishment of the suitable methods based on the chemical analysis and instrumentation techniques has become a major concern to the food industrial sector, research organization, academic centers, and government organization. Further, these established methods will be used to measure the nutritional information in food products and their impact to improving public health, safety by measuring

labeling, also in order to control the toxicity. Therefore, several field testing methods have been developed for analysis of fortificant according to the purposes, needs, and conditions.

12.1.1 Field Testing Analysis

12.1.1.1 Field Testing of Iron-Fortified Food

Iron is fortified in different forms which include ferrous fumarate, electrolytic, ferrous sulfate, ferric pyrophosphate, and NaFeEDTA (sodium ferric ethylene-di-amine-tetra-acetate), in variety of food such as wheat flour, sauces, therapeutic foods, etc. (Hurrell 2002).

12.1.1.1.1 Qualitative Method to Determine Iron

Determination of presence of the micronutrient becomes important before quantifying it for initial screening for making testing of fortified food more cost-effective and to save the time. Qualitative testing is used at manufacture, distribution, and retail stages for quality control. Identification and the presence of reduced or elemental iron is done based on the magnetic properties. Iron spot test (IST) is a user-friendly qualitative method used for field testing to determine added iron in fortified flours (all type of flours) and breads (Nichols et al. 2012). The IST detects all form of iron used in fortification except NaFeEDTA, and after the solution (potassium thiocyanate in hydrogen peroxide), application to iron-fortified food appearance of red spots indicates the iron presence (Johnson and Wesley 2010), and for NaFeEDTA, the IST is used using potassium thiocyanate in acidic medium. The density of spots can detect the level of the fortification, and that is the reason that the IST is recognized as semi-quantitative method at factory level. The IST is very commonly used as field-friendly test at milling units to ensure the presence of iron for quality control. Accuracy and feasibility of iron colorimetric tests depend on the technique of fortification or microencapsulation according to exposure of iron for analysis. The National Institute of Nutrition in India developed a field testing kit for the IST for salt and flours fortified with iron by spray drying on the surface, but it was not for commercial use (Yuan et al. 2008).

In order to conduct rapid qualitative analysis of iron in fortified foods, researchers have developed the field-based qualitative and semi-quantitative testing kits based on chemical approach by taking constraints in accounts that are easy to use and handle, analysis using standard charts should be rapid, and the field test must include easy exposure of iron for analysis with simple provision (thermally by melting, chemically by dissolving, or mechanically by grinding) of coating removal (Wegmüller et al. 2003). Yuan et al. (2008) developed low cost field testing kit for microencapsulated iron in double-fortified salt, and they validated the kit against standard spectrophotometric methods (AOAC) with high correlation coefficient ($r = 0.9884$). For elemental iron, the rapid field test kit is not amendable and safe due to high acid requirement for dissolution.

12.1.1.1.2 Quantification of Iron in Fortified Foods

Quantitative methods to determine iron level with accurate and precise concentration in premix and fortified food and method of quantification are selected according to cost, instrument availability, and effectiveness. The standard laboratory method used to quantify iron in fortified food is atomic absorption spectroscopy (AAS) (AACC International 2002). However, AAS is expensive and non-portable method of analysis so many researchers have conducted on development of other quantifying methods. Sample preparation involves extraction of iron for analysis depends on state of iron used in fortification. Principles behind extraction of iron are total combustion of organic compounds (total iron), digestion, and reduction (ferric), and water/acetone mixture (ferrous) and iron are determined in fortified foods by analyzing absorbance against concentrations in standard curves. AAS method is faster, but it determines the total iron rather than salt of origin. Soeiro et al. (2010) used atomic absorption spectrometry to find iron level with high accuracy and sensitivity in fortified flours. To overcome these disadvantages, researchers have developed inductively coupled plasma atomic emission spectroscopy (Poitevin 2012), spectrophotometer (De Oliveira et al. 2018; Hanson 2020), photoelectric colorimeter, visual analysis for quantifying iron in fortified cereal flours, salt, sauces, etc. The spectrophotometer determined the ferrous ion by forming color due to reaction of chromogens with iron. Niedzielski et al. (2014) determined the forms of iron in several fortified foods such as cereal-based products, desserts etc. and developed and optimized method of extraction based on colorimetric methods. A cloud point extraction (CPE) method has been developed, to determine the iron levels in fortified food items like beer (Filik and Giray 2012), wheat, and maize flours (Silva et al. 2018), based on Br-PADAP complexation followed by spectrophotometric quantification.

For rapid and less expensive quantification of iron, portable type/handheld spectrophotometers have been developed and validated against standard AACC method. Testing kit named iCheck photometer is developed by BioAnalyt for quantification of iron and validated for number of iron-fortified foods. This portable test kit contains two units, one is iCheck-iron (measuring unit) and another is iEx IRON (disposable reagent vial) in which reaction is performed. The iCheck-iron device quantifies iron based on photometric procedure and determines the concentration from extrinsic and intrinsic iron from fortification and food matrix, respectively. Laillou (2013) determined the iron in fortified fish sauce and soy sauce using iCheck-iron device and compared the results with atomic absorption spectroscopy. They found it viable for field supervising of fortified iron in soy and fish sauces after incubation times of 24 h for NaFeEDTA and 1 h for ferrous fumarate or ferrous sulfate. The iCheck-iron kit has been used for fortified premix, flours, corn soy blend, etc. for all forms of iron and validated with AAS method of iron quantification. A paper-based sensor suitable with a smart phone by using phone camera to measure intensity of color of chromogen as an alternative to photometer is developed and used for quantification of iron (FeSo4 and FeFum) in corn and wheat flours by using ferrozine (Waller et al. 2019). Hanson (2020) developed an inexpensive, reliable, and rapid method for quantifying iron (all forms) in cereal flours using

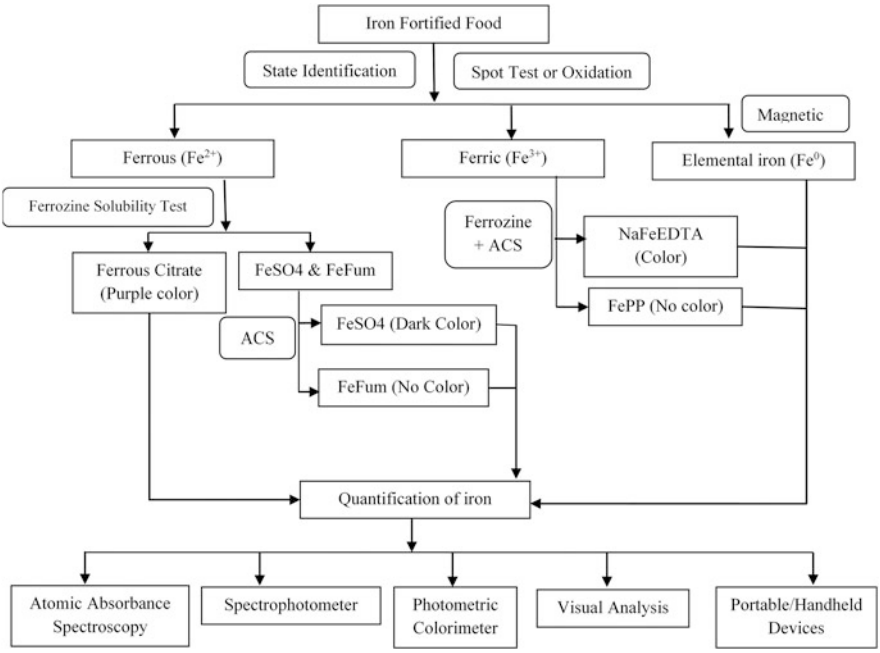

Fig. 12.1 Process flow chart of iron (Fe) analysis in fortified food samples

spectrophotometer and visual analysis (using chromogens and quantifying by standard charts) (Fig. 12.1).

12.1.1.2 Field Testing of Fat-Soluble Vitamins in Fortified Food

Vitamins A, D, and E are majorly fortified in the staple foods (flours, sugar, milk, and edible oils) as fat soluble vitamins.

12.1.1.2.1 Qualitative and Semi-Quantitative Test

The qualitative methods which are used to detect the vitamin A levels in fortified foods basically work on the principle of blue color development after colorimetric reaction with chromogen reagents (trichloroacetic acid). The intensity of color is directly proportional to concentration of vitamin A in fortified food so method can be used as semi-quantitative method, and it gives approximate concentration of retinol in fortified food. In order to determine rapid qualitative analysis, BASF has developed a mobile test kit based on photometric analysis at very low cost for fortified staple milk, sugar, and edible oils. Krawinkel et al. (2009) evaluated BASF testing kit for vitamin A in fortified vegetable edible oils.

12.1.1.2.2 Quantitative Test for Fat Soluble Vitamins in Fortified Foods

Quantification methods are used to ensure the required and adequate level of fortificant in the fortified food for quality assurance and control. The laboratory

methods for quantification of vitamin A are spectrophotometry and high-performance liquid chromatography (HPLC). Spectrophotometric method of quantifying vitamin A is based on release and extraction of micronutrient using selective solvent and then measuring absorbance of extract at 325–326 nm in UV spectrophotometer (Horwitz and Latimer 2007). This method is highly selective for fortified sugar, and effectiveness and accuracy of measurement depends on extraction of fortificant.

The HPLC method (AACC) is used to quantify retinol after saponification and dilution of samples using UV detection, and concentration is determined by comparing peak heights of sample with standard solutions (De Vries and Silvera 2002). The HPLC method is very sensitive and precise and used as standard method for vitamin A analysis to compare other developed methodologies and devices. To reduce the time of quantification and for rapid field testing with mobility of equipment, researchers have developed many portable devices based on photometer for different fortified foods. Bioanalyt has developed a portable testing kit (iCheck Chroma) for fortified oils to determine vitamin A concentration. The iCheck Chroma (two units; iCheck Chroma (measuring unit) and disposable iEx ELAN (reaction unit)) follows photometric quantification based on colorimetric reaction and gives concentration of vitamin A in less than 2 min. Rohner (2011) quantified vitamin A in fortified palm oil using iCheck Chroma device and validated it against standard HPLC method. Quantification of vitamin A in fortified soy, rapeseed, and ground oil has been compared with standard HPLC method by Renaud (2013). Based on fluorimetric analysis of vitamin A, BioAnalyt developed another portable testing kit named iCheck Fluoro for food (premix and fortified food) and biological fluids by measuring the fluorescence. The iCheck Fluoro with disposable iEx MILA unit determined the concentration of vitamin A in milk (powders and liquid), sugar, flour, etc. (Dary 2011; Sanchez-Mena et al. 2012). Dary and Imhoff-Kunsch (2012) and Engle-Stone (2014) analyzed vitamin A using iCheck Fluoro and compared the results with the HPLC method of cow milk. Vitamin A in sugar is quantified using iCheck Fluoro and validated against the HPLC method (Zambo et al. 2012; Zambo 2013). Laillou (2013) assessed the portable hand device application to measure the levels of vitamin A in fortified flour, sugar, and milk, and results were comparable with HPLC quantification. Major advantage of portable device iCheck Fluoro is removal of sample preparation or pretreatment step (either by extraction or by saponification) to quantify vitamin in fortified food. Vitamin A- and D-fortified dairy milk was analyzed using a sandwiched enzyme-linked immunosorbent assay (ELISA) kit, and vitamin A is quantified using ELISA kit (food r-biopharm product), but validation of ELISA kit is to be done in laboratories (Schweigert et al. 2011). The most common method used for carotene is the HPLC, but to reduce cost and time of quantification, proxy methods (the spectrophotometer, iCheck Carotene (developed by Bioanalyt), and near-infrared spectroscopy using both a desktop (dNIRS) and a portable (pNIRS) device) are used by Jaramillo et al. (2018) to determine total carotene content in biofortified cassava, and results showed that the spectrophotometer method is offering most prominent tools for detecting total carotene content abided by iCheck Carotene, dNIRS, and pNIRS.

Vitamin D-fortified infant and nutritional formulas and milk powders were analyzed using Liquid Chromatography–Tandem Mass Spectrometry (Gill et al. 2016). Kloosterman et al. (2017) determined the concentration of cholecalciferol (D3) and ergocalciferol (D2) in fortified food (milk, infant food, and dessert) and dietary supplement by the HPLC method using UV absorbance. Vitamin D was quantified and analysis was compared using liquid chromatography with ultraviolet and dual parallel mass spectrometry in fortified orange juice (Byrdwell et al. 2011) and other fortified foods (skimmed milk, cheddar cheese, cereal, salmon and orange juice) (Byrdwell 2009; Byrdwell et al. 2008; Phillips et al. 2008; Jakobsen and Saxholt 2009). A study was conducted on quantifying vitamin D3 in fortified food samples such as cheddar cheese, yoghurt, or ice cream in both crystalline and emulsified form using the standard HPLC method (Kazmi et al. 2007). The methods for quantification of vitamin D have shown the laboratory-scale methods with high accuracy, but development of simple, faster, and portable device for quantifying vitamin D would benefit the industry level testing.

Vitamin E (tocopherols)-fortified foods are analyzed by HPLC, gas chromatography, and micellar electro kinetic chromatography (MECK) to quantify vitamin E. According to standard method and accuracy of analysis, HPLC is mostly used. HPLC methods were used for vitamin E quantification in food including reversed phase separation followed by mass spectrometric detection (Lanina et al. 2007).

12.1.1.2.3 Field Testing of Water-Soluble Vitamin-Fortified Foods

Water-soluble vitamins (vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B9 (folic acid), vitamin B12, and vitamin C) are fortified in rice and wheat flours, fruit juices, etc. Blake (2007) has reviewed the quantification of water-soluble vitamins using microbiological assays (MAs) and spectrophotometric, biosensor, and chromatographic techniques. The extraction of water-soluble vitamins plays an important role in quantification along with technique used for analysis. Thiamin and riboflavin extracted in autoclaves using dilute sulfuric acid and niacin is extracted by alkaline digestion using calcium hydroxide, and generally the HPLC method is used to determine thiamin, riboflavin, and niacin. Food fortified with folic acid and B12 is analyzed using microbiological assays. Although different types of chromatographic techniques are established, microbiological assay (MA) is still considered a specific method used for vitamin level measurements. As MA offers superior sensitivity, it is mostly used for measurement of vitamin B12, folic acid, and biotin analyses. Additionally, MAs stand for user-friendly method for identification, differentiation, and quantity estimation by obtaining the sum values for vitamin levels consisted of folate group, without the need for extensive method development. The adjustments of the HPLC linked to ultraviolet-visible absorbance (Heudi et al. 2005) and fluorometric methods (Gujska and Kuncewicz 2005) to detect water-soluble vitamins have been presented in the literature. Lebiedzińska et al. (2008) quantified added folic acid (FA) in fortified fruit juices and cereal products using reversed-phase high-performance liquid chromatography, coupled with coulometric electrochemical detection, and coupling this separation with coulometric detection gave a suitable instrumental method for determining folic acid in fortified food

samples. It was found that coupling different techniques with the HPLC method gave rapidness and more accuracy to the quantification of vitamins in fortified foods.

Araújo et al. (2012a, b) quantified folic acid after a pressurized liquid extraction (PLE) method from fortified wheat flours using reverse-phase high-performance liquid chromatography (RP-HPLC) and found PLE method more rapid and efficient results compared to a standard solid–liquid extraction (SLE) method. Bogachuk et al. (2011) determined water-soluble vitamins (B1, B2, B6, B12, PP, B5, B9, C, B8) in fortified food products and premixes with by using micellar electro kinetic chromatography on short end of the capillary technique, and results were comparable to the standard methods. Naz et al. (2016) measured the level of three B-complex vitamins, thiamine, nicotinamide, and pyridoxine, in fortified infant food (milk products and cereals) by the HPLC method.

To overcome the disadvantages of non-mobility and complexity of the standard and the modified HPLC methods, researchers have worked for development of new, simple, sensitive, and low-cost methodologies. The enzyme-linked immunosorbent assay (ELISA) is a developed method for detection and quantification of water-soluble vitamins in fortified food (Gan and Patel 2013; Guan et al. 2015). Kong et al. (2017) developed an indirect competitive ELISA method and a lateral-flow immune-chromatographic (ICA) strip assay for the detection of vitamin B12 in food samples, and the results were comparable with the microbiological assay method, so this method was suitable for on-site detection and mass sample screening. R-Biopharm developed ELISA kits for detection and quantification of water-soluble vitamins using VitaFast® tests (based on microbiological tests for vitamins B1, B2, B3, B9, B12, and C), EASI-EXTRACT® test (immune-affinity columns permit a sample preparation and concentration of vitamins from complex matrices before the analysis by HPLC or LC-MS/MS for folic acid and vitamin B12), and RIDASCREEN® ELISA tests (quantitative analysis of single added vitamins by means of an antigen-antibody reaction using microtiter plate photometer and the RIDASOFT® Win.NET software for vitamin B9 and B12) in fortified milk, juices, cereal-based products, etc.

Vitamin C levels in food are frequently estimated using UV spectrophotometer, volumetric, and HPLC methods. In order to obtain more reliable and accurate quantification of vitamin C levels in food, HPLC techniques in combination with different identification analysis, such as UV, fluorescence, or electrochemical techniques, are in practice (Fontannaz et al. 2006). Moreover, there will be a requirement of stringent methods for analysis determination of vitamins levels in fortified food which helps in easy detection and quick identification with accurate measurements. Studies have shown that when the HPLC is coupled with mass spectrometry (MS), technique shows promising results for detection of vitamin level in food (Garrido Frenich et al. 2005; Blake 2007).

12.1.1.3 Field Testing of Other Fortificants in Fortified Foods

Iodine as a fortificant in form of iodate is widely applicable for fortification of salt. For qualitative analysis of iodine, colorimetric reaction method is standardized. Analysis is based on transformation of iodide into iodate in the presence of bromine water, which gives blue color as indication of presence of iodine in the sample.

Intensity of blue color gives the concentration or level of iodine present on the salt, but color intensity and stability can be affected by the alkalinity of the salt (Guamuch et al. 2007a, b). Titration with sodium thiosulfate quantifies the iodine content in the fortified samples. For more accuracy and rapid assessment of iodine, spectrophotometric methods and portable devices are developed and validated (Pandav et al. 2000). Spot test devices for iodine or iodine checkers have been developed based on spectrophotometric and titrimetric reactions and used to quantify iodine in salt (Dearth-Wesley et al. 2004; Nair et al. 2012; Kulkarni et al. 2013). BioAnalyt developed a paid test kit for iodine quantification named iCheck Iodine, measures the purple color reaction in the test vial, and calculates the iodine content in mg/L. Rohner et al. (2012, 2015) and Yadav et al. (2015) measured the iodine in table salt and edible salts using iCheck Iodine device, validated against titrimetric methods, and found more accuracy when compared the results with WYD iodine checker.

Inductively coupled plasma-optical emission spectroscopy has been used to analyze copper and zinc in food premixes and a simple and rapid method is developed by Perring et al. (2005) by energy dispersive X-Ray fluorescence (XRF). But there is lack of research and validation available on this technique. Poitevin (2012) determined nine nutritional elements (calcium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium, and zinc) by microwave digestion and inductively coupled plasma-optical emission spectrometryin fortified food products and validated against standard laboratory methods

12.1.2 Food Fortification Program and Suitable Methods

Food fortification by selection of suitable vehicle such as micronutrients or vitamins for a targeted individual, household, targeted groups, and community of developing or developed nations to fulfill the nutrition gap will be the major focus area of research from long time onwards (Osendarp et al. 2018; Berti 2012). In this process, several studies are conducted at ground levels by considering parameters such as individual person energy requirements (Berti 2012), ages (Fernandez-Rao et al. 2014), gender, preschool childrens (Srivastava et al. 2012), pregnant and lactating women, special micronutrient, and vitamin-deficiency disorder (Petry et al. 2020). In this process of program implementation, several observations were noticed that nutrient gap majorly meet up by giving suitable fortified food vehicle which suits the requirement of individual energy intake (Coates et al. 2012). Further, fortification program is a public health-promoting program well known for minimizing the risk of nutrition gap in global population and also helping in fulfilling micronutrient deficiency (Wimalawansa 2013). Further, the data collection of controlled fortification of nutrient components in food along with study for understanding safer intake at individual, household, and population data assessment will be majorly useful for implementing evolution of food fortification program at larger scale (Neufeld et al. 2017).

In this public health benefit program, establishment of connecting links between trainers, researchers, market analyst, planner, government organization, industrial managers, investors, and philanthropist is a major missing link. Whereas several studies have been conducted on raw data collection, dietary assessment, and validation of energy intake of staple food via sensory and efficacy studies at the individual level, family, groups, gender, age, and population levels (Pachon et al. 2015). Moreover, detailed studies on usefulness, quality acceptance, and cost of production, distribution, establishing regulatory guidelines, and marketing strategies at government and industry levels are also majorly focused areas of current researcher (Spohrer et al. 2013).

This chapter addressed briefly on well-known existing methods and their advancements which have potentially benefitted for data analysis at every stage of food fortification program reported in last decades. However, till date there is no gold set standard methods available for complete data analysis from individual level to policy makers (Coates et al. 2012). Therefore, connecting data from individual micronutrients, vitamins for targeted diseases, and their implementation, evolution of dietary data at ground level to cost estimation and market applicability is still at its infant stage of study.

In the food fortification program, the dietary data assessment is one of the key components which connecting the ground level information to market applicability (Osendarp et al. 2018; Berti 2012). The Monitoring, Assessment, and Data (MAD) group is dedicatedly working for connecting research gaps related to nutrition requirements from individual to market partners for implementation on a large scale (Dary and Imhoff-Kunsch 2012). Considering this point of view, some established methods and their related information are provided further (Table 12.1).

12.2 Conclusion and Future Scope

Levels of iron in iron-fortified food products are mainly detected by spectroscopic and colorimetric methods. Further, recent advancement of portable devices based on photometric principles will be easy to handle and detects the very low levels of different forms of iron. Similarly, in case of vitamins, despite of routine lab analysis methods using chromatography, ELISA kits, and microbiological analysis, BASF developed user-friendly testing kits using mobile devices and also worked on the principles of photometric principles. In addition, BioAnalyt has developed a portable testing kit (iCheck Chroma) for fortified oils to determine vitamin A concentration. However, there is no gold set programs specifically used for food fortification program, but methods enlisted based on primary data screening mainly are 24-h recall, Fortification Rapid Assessment Tool (FRAT), and Relative Dietary Energy Adequacy Ratio (RDEAR) which are costly and cover the individual risk factor and short time information, and data accessibility is flexible. Further, methods based on secondary data such as Household Consumption and Expenditures Surveys (HCES), Food Balance Sheets (FBS), and Fortification Assessment Coverage Toolkit (FACT) cover large set of data and long period information, and data accessibility is rigid,

Table 12.1 Food fortification program, diet assessment methods for measuring consumption of nutrients, utilization, and challenges

S. no	Method	Abbreviation	Developed by	Type of data collection	Targeted costumers	Frequency of data collection	Purpose	Limitations/ challenges	References
1	24-h recall	NA	Individual level, government organization for targeted specific need	Primary data collected by food models, photographs, or weighing or volumetric estimation techniques	Individual	Flexible	Inadequate micronutrient intakes, selecting vehicles, and appropriate fortification levels	Cost of implementation	Dary and Imhoff-Kunsch (2012), Coates et al. (2012)
2	Fortification rapid assessment tool	(FRAT).	Health bridge (formerly PathCanada), under contract to the micronutrient initiative	Primary data collection by combination of 24-h recall data and semi-quantitative food frequency questionnaires	Individual	Flexible	Identifying food vehicles for fortification and for setting appropriate and safe fortification levels	Covers small set of data only	Hess et al. (2013)
3	Household consumption and expenditures surveys, Household income and expenditure surveys, Household budget surveys, Living	(HCES), (HIES), (HBS), (LSMS)	Imhoff and colleagues suggested the practical recommendations	Secondary data	Household family members	3 to 5 years' time scale	Identify the social economical condition of family. Helps in program designing at national level	Lack in individual risk assessment	Coates et al. (2012)

(continued)

Table 12.1 (continued)

S. no	Method	Abbreviation	Developed by	Type of data collection	Targeted costumers	Frequency of data collection	Purpose	Limitations/ challenges	References
	standards measurement surveys								
4	Food balance sheets	(FBS)	Food and agriculture organization (FAO)	Secondary data	National data	Annual data	Information useful for setting trends, pattern, and levels for national diet	Information related to nutrient consumption and distribution of individual and population	Berti (2012), Fernandez-Rao et al. (2014)
5	Relative dietary energy adequacy ratio	(RDEAR)	Food and agriculture organization (FAO)	Primary data	Food distribution within households or between age and gender groups	Daily data	The diet of all members of a target population. Helps to design the food fortification program	RDEAR ratio values have shown no specific interpretation.	Osendarp et al. (2018)
6	Fortification assessment coverage toolkit	(FACT)	Global Alliance for improved nutrition (GAIN)	Primary and secondary data	Population-based (i.e., staple foods and/or condiments) and targeted (e.g., infant and young child)	Annual	Detail designing related to nutrition, health, coverages, utilization of fortification, and quality of fortified food	High cost	Friesen et al. (2017)

under control of government agencies and policy makers. Therefore, in order to connect links of established fortificants, application at ground levels needs more systematic approaches by selecting quick, cost-effective testing tools as well as development of bridging tools from individual data to policy implementation at large scale.

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Synthesis, Characterization, and Beneficial Effects of Green Antioxidant for Food Industry

13

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Abstract

Reactive oxygen species are produced due to the numerous abiotic stresses in human, animal, and plant cells. These highly reactive species damage the protein and lipid structures as well as DNA and carbohydrates present in the living cells. In current years, boundless attention has been engrossed on exploitation of natural antioxidants in daily food products. A variety of plant resources like spices, fruits, herbs, and vegetables and animal proteins from fish, egg, etc. are recognized to be natural resources of bioactive materials which have antioxidant activities. Due to the organic value and economic influence, the attention of extraction of natural antioxidants from crops spices, various foods, and food

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by-products has been increased significantly. Extensive researches have been performed to evaluate organic substances from natural sources. To develop the fresh food products, successful studies have been reported to use antioxidative additives in food and beverages. Moreover, to describe the antioxidant activities, these natural bioactive additives have positive properties on the human cells with recognized health aids.

Keywords

Antioxidants · Bioactive compounds · Reactive oxygen species (ROS) · Polyphenols · Carotenoids · Food industry · Extractio

13.1 Introduction

Antioxidants are the most abundant and essential organic compounds for living cells since they are involved to produce complex mechanisms in metabolic pathway (Matsui et al. 2011). To protect the plants from the fungal and bacteriological invasion, antioxidants produce various phytoalexins. These compounds are similar to isoflavonoids in structures (Wilson et al. 2017). The antioxidants can assist to uphold the nutrients level, taste, color, consistency, aroma contents, and components functionality owing to the low volatility and tendency of high stability. Antioxidants are found in various food supplements as well as in food additives (Griffiths et al. 2016). The levels of antioxidants are measured in body fluids and different tissues. Some kinds of antioxidants found in juices, pulps, jams, etc. contain high flavonoids and polyphenolic compounds (Franco and Martinez-Pinilla 2017). These phenolic groups have potential attribution to diminish the chronic diseases like diabetes and cardiac problems. Antioxidants can be found in the fermented and processed grain food complements. These antioxidant biofactor supports to reduce the lipid oxidation reaction with the help of their scavenging nature on the peroxyl radicals (Foyer and Noctor 2005). They hinder the cellular oxidant stresses due to the presence of free radicals by their strong antioxidant properties.

Antioxidants preserve essential nutrients in the food from cultivated area to living cell with their preservative activity (Wilson et al. 2017). Vegetables and organic product are often put away and fixed in a low oxygen climate at low temperatures in anonymity; yet in spite of this intercession, oxidation responses despite everything occur during storing (Sindhi et al. 2013). The antioxidants are significant classification of food additives, common or manufactured, intended to keep food from ruining through oxidation, therefore lessening loss of supplements, and looking after surface, colors, sensitivity, newness, usefulness, and fragrance (Samaranayaka and Li-Chan 2011; Wang et al. 1996). They additionally have a significant physiological role in between inter- and intra-cell flagging systems and metabolic procedures in plant and creature life (Nair et al. 1998).

Free radicals are at least one unpaired electron comprising reactive particles and can harm proteins, the structure of nucleic acids, starches, and lipids, prompting a

few sicknesses including early maturing, malignance, and atherosclerosis (Lobo et al. 2010). Antioxidants are very much beneficial for human health. It reduces the oxidative stress thus ultimately preventing the cellular damage (Franco and Martinez-Pinilla 2017). Epidemiological inquiries have just uncovered that higher consumption of antioxidants as food nutrients reduces the risk of several diseases (Lobo et al. 2010). Investigating natural antioxidants and its contribution in human wellbeing and sustenance is a rising field. A few organic sources, such as therapeutic vegetation, flavors, and organic products, as well as microorganisms have been assessed as wellsprings of possibly safe biotic compounds (Nair et al. 1998). These natural organic agents are used in agricultural, medical, and industrial areas (Jayachitra and Krithiga 2010).

Superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydrogen radicals ($\dot{O}H$) are known as reactive oxygen species (ROS). These species are formed during enzymatic activities. With excess production of superoxide radicals ($O_2^{\cdot-}$) to hydrogen radicals ($\dot{O}H$), various diseases can be found in the human body (Frei et al. 1988). DNA damages, periodontal disease, aging, and inflammation diseases are reported due to the involvement of reactive oxygen species (ROS) (Jain et al. 2013). These reactive oxygen species are generated due to the activity of bacteria and metal ions (Jain et al. 2008). To eliminate the adverse effect of reactive oxygen species, antioxidant treatment is advantageous to treat the inflammation diseases. Recently, processed grain food has potential to restrict the development of reactive oxygen species. With strong antioxidant properties, these processed food can inhibit cellular oxidants stress. Antioxidant biofactor contains a variation of components such as flavonoids, vitamins, tannins, α-tocopherol, catechins, etc. that have antioxidant activity (Nair et al. 1998; Wang et al. 1996). Researchers have suggested that this biofactor-rich food has substantial antioxidant properties that might be of significance to prevent degenerative diseases.

Humankind for quite a long time relies on plant biodiversity to cure different illnesses. Therapeutic potential of plant materials has already been demonstrated scientifically in the field of nutrition (Wang et al. 1996). Some diseases can be treated using green compounds isolated from plant cells. The phytochemicals extracted from different fruit cells are used for the treatment of diabetes. These non-nutritive herbal components have various properties which are used in the medical sciences recently. The color and taste in processed and fresh crops, fruits, and vegetables are varied with the presence of different phytochemical compounds (Yin et al. 2012).

13.2 Sources of Antioxidant

The bioactive ingredients with different concentrations are found from various plant-derived compounds. Leaves of the plants, roots, stems, fruits, and its edible and nonedible parts are the major source of bioactive materials (Jayachitra and Krithiga 2010). The waste materials, derivatives, and secondary products of fruit and vegetable processing industries contain rich source of antioxidants. The enormous amount of fruit waste materials is generated during processing of fruits and vegetables which

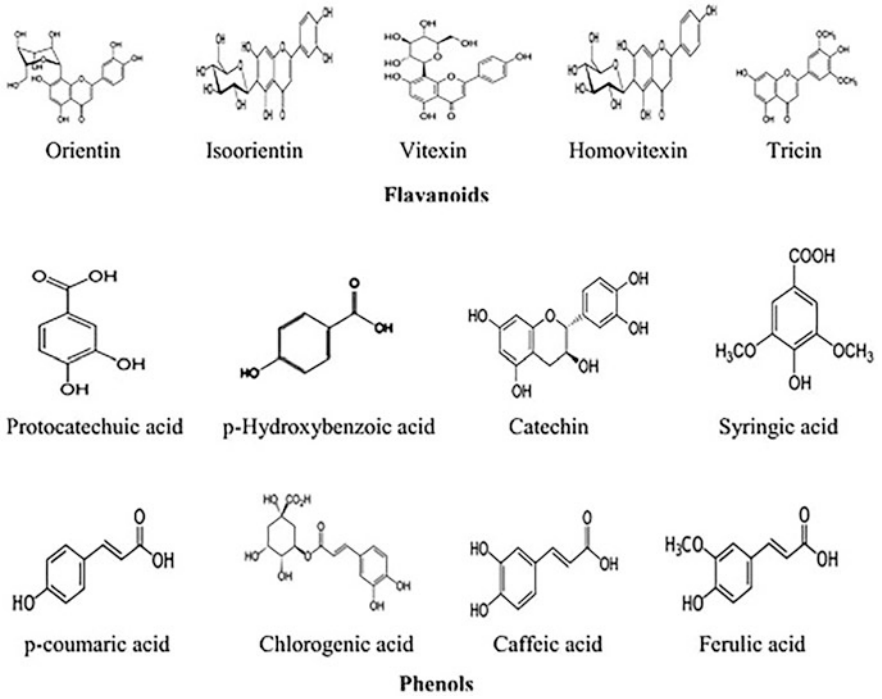

Fig. 13.1 Structural identification of various flavonoids and phenolic compounds present in various parts of bamboo such as leaves and shoots

are comprising various phytochemicals, dietary fiber, polyphenols, flavonoids, etc. It is reported that some seasonal fruits like mango, guava, banana, blue berry, walnuts, pomegranates, strawberries, and grapes are considered as important source of polyphenolic compounds (Wang et al. 1996). Huge number of bioactive compounds such as flavone, phenolic acids, carotenoids, lycopene, and vitamin C are found in orange peel, fiber, etc. Consider various vegetables like tomatoes, carrots, red beets, cabbages, and potatoes are also the major sources of phenolic compounds known as natural antioxidants. Several factors like cultivation process, condition of soil, seasonal effects, harvesting process, and extraction methodology are accountable to highlight the impact of natural bioactive compounds.

Bamboo is recently recognized as a potential source of natural antioxidants and bioactive components (Nirmala et al. 2018). Orientin, isoorientin, vitexin, homovitexin, and tricin can be isolated from various parts of bamboo. Several groups of phenolic compounds like protocatechuic acid, p-hydroxybenzoic acids, ferulic acid, caffeic acid, chlorogenic acid, catechins, and p-coumaric acid are present in the bamboo leaves and shoots (Nirmala et al. 2018; Luo et al. 2015). Fig. 13.1 describes the structural identification of various flavonoids and phenolic compounds present in various parts of bamboo such as leaves and shoots, reproduced with copyright from *Bamboo: A rich source of natural antioxidants and its applications in the food and pharmaceutical industry Trends in Food Science*

& Technology 77, 91 (2018), authored by Nirmala, C., Bisht, M. S., Bajwa, H. K., and Santosh, O.

It is mostly rich in phenolic compounds, phenolic acids, etc. Various essential oils extracted from different spices have also been confirmed to be exceptional bases of natural antioxidant compounds. Tea leaves also contain several compounds like polyphenols, tannins, catechins, flavonoids, etc. (Yin et al. 2012). Polyphenols represent the valuable ingredients in tea leaves and tea processing solid waste materials. Compared to essential oils, tea extracts comprise less flavored natural compounds which have significant effects on antioxidant activities. Anthocyanins, lycopene, carotenoids, phenolic acids, flavanols, and catechins are found from olive, pomegranate, grape, and apple pomace.

13.3 Properties of Various Bioactive Compounds

13.3.1 Polyphenols

Polyphenols are one of the utmost vital groups of subordinate metabolites of vegetation (Brandolini et al. 2013). The widely distributed phenolic compounds have organoleptic appearances in food and beverages derived from plant cells. The phenolic compounds present in the beverages are mainly two types like flavonoid groups and non-flavonoid compounds. Flavonoids have different oxygenated heterocycles and phenolic rings. The classification of these compounds is mainly involved with various bioactive materials like flavonols, anthocyanins, flavones, chalcones, etc. Anthocyanins are the most familiar flavonoids group of compounds which are present in vegetables and fruits (Heim et al. 2002), whereas non-flavonoid compounds contain phenolic acids, monocyclic acids, benzoic acids, stilbenes, hydroxycinnamic acids, etc. These bioactive materials act as anti-inflammatory agents, antioxidants, and anticancer reagents and reduced oxidative stresses (Grzesik et al. 2018).

13.3.2 Catechins

Catechins are characterized by various subgroups such as (−)-epicatechin, (−) epigallocatechin, (−)-epigallocatechin gallate, (−)- epicatechins, and (−)-epicatechin gallate. These group of catechins are found in many nutritional fruits, vegetables, black and green tea, cacao liquor, wine, chocolates, etc. The antioxidant activities of catechins are generally reported as a result of redox properties. With the help of this activity, the catechin groups act as anti-inflammatory, reducing agents, donor of hydrogen atoms, oxygen quenchers agents, etc. (Grzesik et al. 2018). Fig. 13.2 describes the chemical structural behavior of various catechins, reproduced with copyright from *Extraction of polyphenols from grape seeds and concentration by ultrafiltration Separation and Purification Technology 48, 176 (2006), authored by Nawaz, H., Shi, J., Mittal, G. S., and Kakuda, Y.*

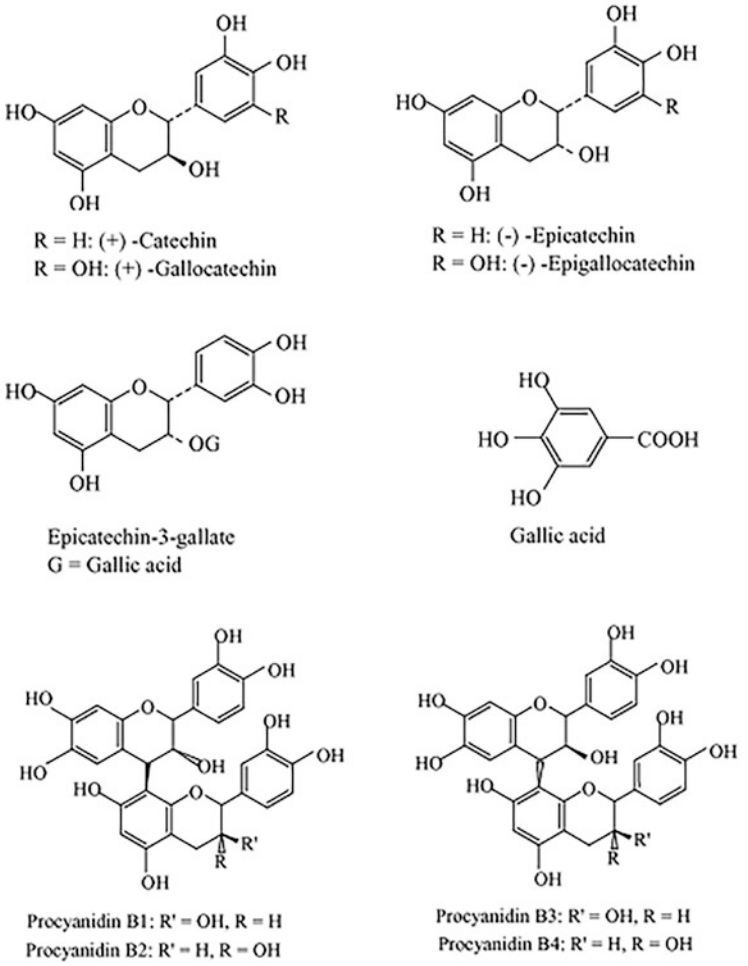

Fig. 13.2 The chemical structural behavior of various catechins (Nawaz et al. 2006)

13.3.3 Anthocyanins

Anthocyanins are the most important and largest natural bioactive compounds in nature. These phenolic groups are known as natural water-soluble pigment. Antimicrobial, pH sensitivity, and antioxidant properties are the imperative characteristics of anthocyanin compounds. Anthocyanins are extensively distributed in vegetables, lowers, fruits, and mueslis and are accountable for an extensive range of color formation (Abdel-Aal et al. 2003). Anthocyanins contain aromatics and heterocyclic groups including saccharides, glucose, galactose, xylose arabinose, etc. Additionally, various aliphatic and aromatic acids such as ferulic, gallic, caffeic, malonic, and oxalic acid are acylated through acyl groups in the anthocyanin compounds. Thus,

the degree of acylation plays important role to construct the center structure of anthocyanins. However, anthocyanin structures diverge along with the position and number of hydroxyls, methoxyl, and sugar moieties present (Yong et al. 2020).

13.3.4 Carotenoids

Isoprenoid polyene natural pigments with red, orange, and yellow color are known as carotenoids obtained from algae, bacteria, and plants. Above 800 carotenoids can be found in various plant cells (Alasalvar e al. 2001). It is also the most widespread groups of bioactive materials present in fruits, vegetables, flowers, etc. Carotenoids are exogenous pigment in nature. Carotenoid compounds are categorized into two foremost groups named as xanthophylls and carotenes. The dominant class of xanthophylls is mainly oxygenated xanthophylls. Altogether, carotenoids present in algal cells and bacteria deliver an extensive diversity of operational kinds than those originated in different parts of plant cells (Lerfall 2016). The elementary structure of carotenoid is a C_{40}-coupled polyene chain known as lycopene, which can be constructed up to eight C_5-isoprene elements (Sachindra and Mahendrakar 2005). The presence of polyene chain characterizes a chromophore which is accountable for the color characteristics. Lobster shell contains dark-blue-colored carotenoproteins which is carotenoids conjugated with proteins. Antioxidant activities of these isoprenoid polyene in existing organisms are complex (Alu'datt et al. 2017). Numerous researches have revealed that the group of carotenoids cooperate synergistically with various bioactive compounds. The synergistic effects of β-carotene with α-tocopherol have been reported widely to protect peroxidation, cell damage from free radicals, etc. (Saini and Keum 2018).

13.3.5 Phenolic Acids

Numerous researches have discussed that phenolic acid compounds are the foremost phytochemical groups present in wheat, barley, kernel, etc. (Alu'datt et al. 2017). These organic acid molecules are found mainly at the outer layer of the said plant materials. Several phenolic acids can be extracted from plant cells with different forms specifically free acids, soluble coupled, and in the form of insoluble-bound compounds (Nair et al. 1998). Free radicals are scavenged by phenolic acids by which significant rate of lipid oxidation can be reduced. Among the various phenolic acids, it has been observed that ferulic acid, 4-hydroxycinnamic acid, vanillic, sinapinic, and para-hydroxybenzoic acid are the important phenolic acid molecules which are found in barley grain (Suriano et al. 2018). Several studies have demonstrated that phenolic acids have significant antioxidant potential, ensuing in a valuable outcome to human health. These acid compounds display numerous genetic activities like free radical elimination, preventing oxidation and growth of microbes (Kelebek et al. 2015).

13.3.6 Lycopene

The common non-provitamin compound, lycopene, can be found in different vegetables and fruits such as watermelon, apricots, papayas, tomatoes, etc. (Alu'datt et al. 2017). Currently, lycopene has fascinated the attention in the field of nutrition for the potential antioxidant activity to manage several human ailments like diabetes, skin diseases, obesity, cardiovascular problems, malignances, and respirational syndromes. This bioactive material is broadly used in food processed industries, pharmaceuticals, and cosmetics preparation purposes. Lycopene delivers significant effects in upholding redox homeostasis (Zhu et al. 2020). Lycopene is a vibrant red bioactive compound which has various unsaturated acyclic groups with several linear coupled and non-conjugated binary bonds. The solubility of lycopene in water, methanol, and ethanol depends on the partition coefficient. Mostly, it is soluble in organic solvents such as chloroforms, hexane, benzene, petroleum ether, tetrahydrofuran, etc. Due to the extreme hydrophobicity in nature, the rate of absorption of lycopene in human cell is lower than the carotenoids. Lycopene is an influential antioxidant in contradiction of DNA, protein, and lipid molecule oxidation. In the presence of different carotene molecules, such as β-carotene and phytofluene, the free radical scavenging activity of lycopene can be synergistically improved (Caseiro et al. 2020).

13.4 Extraction Procedures of Bioactive Materials

13.4.1 Solvent Extraction of Antioxidants from Natural Sources

The antioxidant activity of natural extracts depends on the superiority of the original sources and the extraction methodologies during recovery process. Due to the versatility in chemical and physical properties of naturally found antioxidants, several procedures are available with various parametric conditions to extract the bioactive components from different solid matrices (Balasundram et al. 2006). Organic solvent extraction is the most conventional process for polyphenols and bioactive materials recovery. Extraction of anthocyanin, polyphenols, phenolic acids, flavonoids, and tannins has been reported using various concentrations of ethanol, methanol, acetone, hexane, dichloromethane, chloroform, butanol, etc. A combination of ethanol, acetone, and water was used to extract the pure anthocyanin from potato pomace. The extraction of flavanols and phenolic acids were reported using various organic solvents, like acetone, acetic acid, ethanol, and methanol. However, water as a green solvent was also used to extract valuable bioactive compounds from peach fruits, sweet potatoes, ripe bananas, etc. Extraction processes, operating conditions, and solvent concentrations are the major parameters to identify the maximum extraction yields of antioxidant compounds (Chemat et al. 2020). The lowermost degradation degree with maximum extraction is desirable during effective solvent extraction of bioactive materials.

13.4.2 Extraction of Bioactive Materials Using Ultrasonication

Ultrasound-assisted extraction has been applied to extract valuable organic products from various organic wastes such as fruits pomace, crop wastes, food-processed wastes, etc. (Cavalaro et al. 2019). Various phenolic compounds and anthocyanins were extracted from blueberry pomace, pomegranate peel, and mango peel using the ultrasound process (Pan et al. 2012). This process helps to disintegrate and fragmentate the plant cell walls significantly with minimum use of solvents, and less time period. The mass transfer rate increases in the presence of ultrasound effects on raw sources which results enhanced extraction of organic components (Zhang et al. 2015).

13.4.3 Integrated Pressurized Liquid Extraction of Phenolic Compounds

In order to increase rate of mass transfer between solvents and raw materials, integrated high hydrostatic pressure and pressurized liquid extraction process is applied as the efficient substitute method for extraction of bioactive materials. This process is performed to recover heat-sensitive compounds at ambient temperature and pressure at a range of 10–1000 MPa. The value-added products like flavonoids, phenolic acids, lycopene, essential oils, etc. are extracted with the integrated pressurized liquid extraction process from watercress, tomato pulps, papaya seeds, etc. (Pereira et al. 2020).

13.4.4 Microwave-Assisted Recovery of Bioactive Materials

The direct effect on the polar solvents such as methanol, water, and ethanol during extraction of organic compounds has been reported using microwave-assisted recovery process (Makris 2018). This process delivers rapid energy transmission to the solvent volume to enhance the rate of mass transfer of the organic materials (Drosou et al. 2015). Microwave extraction system permits the decrease in solvent volume and minimizes the extraction time at controlled pressure conditions. It enables the rise of the solvent temperature above the boiling point which results the increased extraction efficiency with shorter time period. To recover valuable phenolic components from fruits peels and olive tree leaves, microwave-assisted separation process has been applied successfully (Hayat et al. 2009).

13.4.5 Supercritical Fluid Extraction Process

Supercritical fluid extraction of carotenoids, phenolic components, phenolic acids, β-carotenoids, and flavonoids from various green sources has also been reported with significant recovery of these bioactive compounds. This ecofriendly process is

applied mainly in the food industry. Nontoxic solvents in the supercritical states are used for the value-added component extraction purposes. This process is performed in the absence of oxygen environment and light which decreases the compounds degradation activities (Santos et al. 2015).

13.4.6 Deep Eutectic Solvent Followed by Ultrasound-Assisted Extraction Process

A combination of organic acid salt-based deep eutectic solvent with sonolysis process has been demonstrated in recovering polyphenols from wine industry solid waste materials. The reduced toxicity, suitable operational pressure, low solvent consumption, and minimum vapor pressure production are the main reasons to choose deep eutectic solvent extraction of bioactive phenolic compounds. This newly invented extraction process has been reported with high yield value and superior bioactivities at minimum extraction time (Makris 2018).

13.4.7 Oil-Assisted Extraction of Polyphenols

Oil-assisted extraction of phenolic compounds from *Linum usitatissimum* oil plants has been carried out using petroleum ether and diethylether at 90:10, v/v ratio. The reduced lipid oxidation and extended oil stability are the significant merits of this novel oil pressing extraction process (Teh et al. 2014). The effects of nitrogen atmosphere, closed and open system, volume of solvents, press cake and oil ratio, extraction time, and number of extraction stages are the important parameters to achieve enriched phenolic compounds using oil-assisted recovery process (Fruehwirth et al. 2020).

13.4.8 Water Extraction Followed by Membrane Filtration of Polyphenols

An integrated process, involving green solvent-based extraction of phenolic compounds followed by membrane nanofiltration, has also been developed and reported. To attain a potentially high yield phenolic compounds from fruit waste materials such as pomegranate pomace, a cost-effective, ecofriendly, justifiable recovery process is essential. Extraction-membrane integrated process provides the two major operation processes such as extraction of phenolic compounds and concentration of extracted polyphenols, sequentially. Water, as a green solvent, justifies specific consideration for extraction of antioxidants from various fruits and vegetable wastes; thus, water is used as the important extraction solvent in advanced research area (Papaioannou et al. 2020). Separation of polyphenols from effluents is also the recent trend. Researches have been reported to separate phenolic compounds from tea factory waste effluents using integrated treatment process of

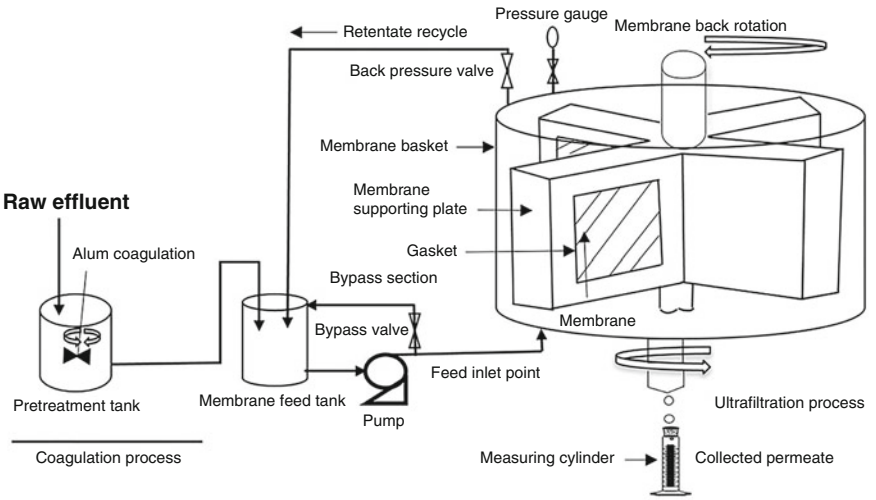

Fig. 13.3 Schematic representation of setup design of spinning basket membrane process for separation of polyphenols tea industry wastewater (Saha et al. 2019)

coagulation-spinning basket membrane ultrafiltration. Figure 13.3 describes the schematic modulation of spinning basket membrane module with coagulation process of tea factory wastewater from where polyphenols were separated, reproduced with copyright from *Treatment of tea industry wastewater using coagulation-spinning basket membrane ultrafiltration hybrid system, Journal of environmental management, 244, 180 (2019) authored by Saha, S., Boro, R., and Das, C.* Membrane filtration seems to be the most suitable process to concentrate and fractionate bioactive materials selectively from water solution and industrial streamlines mainly product streams, by-product sections, and effluents of most common food processing industries (Nawaz et al. 2006; Pereira et al. 2020).

13.5 Beneficial Applications of Antioxidants

13.5.1 Reason Behind the Choice of Antioxidants in Daily Food Habit

In this era, everyone wants to be safe and sound in terms of health by consuming hygienic food, and they are achieving this by consuming the food consisting of unsaturated fats, but in some food product, abovementioned fats are not present so in that case they incorporate the unsaturated fats by extracting from some other substance by using operations like membrane filtration, solid-liquid extraction (leaching), etc. Product quality mainly depends upon the certain parameters and approval of the consumers with respect to those parameters. In the same way, the quality of any food product is stated by the flavor, it looks, and odor. As human trend is changing and their prospective towards food is also changing shifting the category

of healthy eating foods to the foods which do not take much time to prepare. To protect the potential health of such type of products, antioxidants are used (Alu'datt et al. 2017).

13.5.2 Application of Antioxidants in Food Industries

Antioxidants are widely used in the food processing industries. To maintain the hygiene of the food products, antioxidants are used as a supplement in oils and fats and in food industries (Liaset and Espe 2008). In study, it was revealed that some of the herbs are possessing the potential antioxidants (Kulawik et al. 2013).

In order to relive the food from oxidative stress and to maintain the life of unsaturated fatty acids to keep the product fresh, antioxidants are added to the product. Oxidation reaction can be suppressed by adding more of antioxidants to the food product. As we discussed before, there are some antioxidant sources, and antioxidants can also be synthesized. These are some synthetic antioxidants, butylated hydroxytoluene [BHT], propyl gallate, butylated hydroxy anisole [BHA], and ethylene diamine tetra acetic acid (EDTA) helps to reduce the oxidation reaction and preserves the food for a long time (Carballo et al. 2019). Antioxidants associated with phenols are frequently used as reducing agent and stop the chain reaction of free radical and prevent the evolution of unpleasant smell, flavor, and order. Antioxidants also find the vast scope in the area of medical sciences and help to treat some diseases like cancer, neurodegenerative, and diabetic complications (Kamemura 2018).

Antioxidants are widely used in both the human body and in food to reduce the free radical chain reaction. Mainly in food systems, nutritional antioxidants are used to prevent the retardation of lipid peroxidation and to sustain the fragrance, odor, and aroma during the keeping of the product. They also make sure that the proper functionality of protein by decreasing the oxidation of protein (Neha et al. 2019).

In the food system, the following antioxidants can be used. Inherent antioxidants are vitamin E and ascorbic acid. Extracts from natural spices like camellia sinensis, sage, and rosemary are also used in food processing. Protein hydrolysates and proteins extracted from fish, egg, milk, and soya also exhibit the activity of antioxidants in different foods of muscle. Antioxidants are radical scavengers and assist in converting high reactive free radical species to the low reactive species (Alu'datt et al. 2017). Every human being requires the oxygen to survive, but it will become harmful when it is available in the body at higher concentrations (Neha et al. 2019).

While storing the food, fractions of peptide and hydrolysates of protein can be added as additive to reduce the reactivity of free radical species. Mentioned antioxidants can be extracted from the sources like gelation of hoki skin, yolk of the egg, potato, casein, whey, and back bone of tuna, and the same activity can be obtained by the artificial antioxidants like BHT and BHA (Xiu-Qin et al. 2009). Collaborative effects can also be seen by antioxidative tocopherols with peptide. It was studied that lipid oxidation process in salmon fillet was decreased by the

addition of hydrolysate protein available in the fish and in the muscle food antioxidants can inhibit the oxidation process. Caseinophosphopeptides, antioxidants extracted by the digestion of casein, are primarily used in tea, pastry, chocolate, juices, cereals, and mayonnaise (Holaas et al. 2008).

13.6 Conclusions

A comprehensive demonstration of the dietary and beneficial role of alimentary bioactive materials extracted from plant and animal cells is important to develop healthy diet system and to protect the main oxidation-associated ailments like heart problems, malignance, diabetes, and perceptive diseases. The current discussion equally delivers a transitory synopsis on sources of bioactive compounds which can protect human cells from oxidative stress compensations. The major character of natural nutritional antioxidants in practical food materials to manage various diseases has been delivered. The possible applications of natural antioxidants have been focused in the recent study. These natural bioactive compounds are also alternative antioxidants to replace artificial antioxidants which can be used in food processing industries to improve the market value of green materials. The quality and supplementary value in foods both are improved with adding natural antioxidants during food processing. The innovative extraction and purification technologies are justifiable in all aspect of identification and quantification of natural antioxidants. During the current situation of human lifestyle, it is essential to maintain daily food habits with proper hygienic criteria. Significant consumption of natural antioxidants protects human bodies from various critical diseases. Thus, to improve the yield of extraction of antioxidants, ecofriendly methodologies have been invented and delivered in the recent years resulting in enhancement of market value of the natural antioxidant compounds.

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Development, Prospects, and Challenges of Meat Analogs with Plant-Based Alternatives

14

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Abstract

Meat analogs are food products prepared from non-animal origin proteins and have typical meaty texture, flavor, and appearance. Meat analogs present a more sustainable method of production as compared to tradition meat production system and require fewer amounts of natural resources. It has potential to fulfil the demand of high-quality protein food in the near future. A lot of research work is going on for technological and process development of meat analogs by incorporating a wide range of ingredients with an aim to improve physicochemical, nutritive, textural, and sensory properties of meat analogs. The consumer acceptability should be conducted on large scale, and focus should be given to increase awareness about beneficial effects of consuming meat analogs to increase acceptance and popularize these products. It will also assist in formulations of new concepts and innovations in presentation and marketability of these products. Meat analogs are considered as potential alternative to real meat in the future.

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14.1 Introduction

There is increasing trends of vegetarianism due to associate health risks of consumption of processed meat products; environmental concerns as meat production at the cost of plants require more amount water, grains, land, etc.; deforestation; emission of greenhouse gases; and animal welfare and ethical concerns. Due to limited natural resources and burgeoning population, the adequate supply of high-quality protein to feed huge population will be a major challenge in the near future. The production of meat by rearing food animals is a process of getting animal protein from vegetable proteins by utilizing already strained natural resources (Kumar et al. 2017). The demand of meat for increasing population cannot meet by the total available natural resources in the near future. Thus, there is an urgent need for exploring various sustainable alternatives to animal meat possessing equivalent or higher nutritive value, organoleptic attributes equivalent to meat by applying new and innovative technologies in food sector.

The high price of meat has driven the research needs to develop cheap alternatives in the form of nonmeat proteins. As compared to animal proteins, vegetable proteins fetch lower price and are readily available in the market and may become low-cost potential alternative to meat product. Singh et al. (2008) also noted the increased popularity of low-cost vegetable proteins such as texturized soy protein (TSP). The animal welfare implication associated with conventional meat production is an important factor attributing to the fastest-growing vegan market worldwide. It could be a smart idea in the near future to sustainably feed the planet with almost 10 billion people across the globe by 2050 and ensure food safety and availability. The trend in fabrication of plant-based protein substitutes for meat proteins has markedly created major opportunity for food industry. The transition from animal-derived protein to plant-based proteins has also provided critical insights to agriculture contributing to enlighten sustainable pathways for the economy (Tziva et al. 2020). Soy protein, cereal proteins, legume protein, and oilseed protein are some of the examples for plant-based protein alternatives distributed across the market shelves as safer meat analogs (Asgar et al. 2010).

Animal protein contains all essential amino acids necessary for the maintenance and development of human body. It imparts specific functionalities and diverse attributes like taste and nutritional value to products that make it a source of quality protein (Xiong 2004). The global shortage of animal protein is imposing the emphasis on direct use of plant proteins in food products. In recent years, three of the world's most popular countries, China, India, and the USA, have shown growing interest and consumer acceptance towards plant-based products with the prospect of replacing the meat products with the same nutritional value alternatives (Bryant et al. 2019). Plant-based meat substitutes/meat analogs/faux meats/mock meats are

exhibiting resemblance in appearance, texture, and taste to certain types of meat or meat products. More healthy diets do not solely rely on animal-based foods, minimizing the health and environment risks. Therefore, the increased availability of meat substitutes by utilizing plant proteins such as textured vegetable proteins and mycoprotein is gaining popularity in the food market (Bianchi et al. 2019).

Consumer prefers fresh, natural food of high quality. This fact is reflected with more than twice market share for chilled meat alternative as compared to frozen products in the UK (Sadler 2004). Sadler (2004) listed the growing consumer concerns over food safety scares of animal products, increasing awareness about environmental concern and sustainable food production leading to meat reducers or meat avoiders, and increasing variety due to use of wide range of ingredients, improved nutritive composition, convenience, and healthy eating as major factors behind the acceptance and popularization of meat alternatives or meat substitutes.

14.2 Meat Analogs

Meat analogs also known as meat substitute, mock meat, faux meat, or imitation meat are vegetable protein food products that imitate the aesthetic attributes and/or chemical parameters of meat or meat products (Joshi et al. 2015). As per US legislation, these foods are referred to as "substitute" due to their nutritionally equivalence to meat products. Meat analogs are one of the most effective and efficient methods for promoting the use of already available broader variety of proteins for human food. Due to their cost advantages, efficient utilization of natural resources, relatively stable prices due to supply of vegetable proteins, remains stable even in case of seasonal variations, enhanced storage life, and convenient storage; this sector increasingly becomes popular in processing industry.

The replacement in food products with imitation substitutes started in the 1960s (Sadler 2004). Soy protein has traditionally been used as a popular ingredient in preparation food analogs such as fermented soybean cake and tofu. Texturized vegetable protein (TVP) was the first meat analogs processed in the middle of twentieth century, prepared by using defatted soy meal, soy protein concentrates, or wheat gluten through extrusion and originated as meat alternatives (Kinsella and Franzen 1978). The need for healthy food products having environmental sustainability implications of consumer's diet put the meat alternatives into mainstream at the beginning of twenty-first century. Plant-based mock meat from vegetables, pulses, grains, oils, legumes, and fungi witnessed a great expansion due to its nutritional and sensorial properties (fibrous texture, flavor, appearance) similar to conventional meat obtained from farm animals. Current research in this field focuses primarily on the utilization of non-traditional cheap widely available protein sources in meat analogs. Insect-based products would also aim to be the next potential source of high-quality protein for human food (Ismail et al. 2020).

14.3 Market Scenario

The substitution of meat analogs with plant-based alternatives was driven by increase in vegetarianism, consumer's ethical consciousness about animal welfare rights, and safeguarding human and planetary health. This is contributing majorly in shaping the market of meat analogs. Plant-based meat alternatives have entered the market as specifically designed products which simulate the taste and mouthfeel of animal-based products. With the change in time and needs, history of meat alternatives from 1965 CE to 2020 has been observed with various new ingredients and products in the market. Soy-based "tofu" was the first reported plant-based meat analog dating back to 965 CE in China. Other traditional references to plant-based meat alternatives include wheat protein (gluten) dating back to 1301, Yuba in 1587, and *Tempeh* in 1815 and many other combinations of cereals, nuts, and legumes since 1895 (Bohrer 2019). *Kinema* is another example of traditional plant-based (soya bean) fermented foods used as meat analogs (Joshi et al. 2015).

Quorn (brand) meat is another example of plant-based meat product. It was launched in the UK in 1985 using fermentation technology to produce filamentous mycoprotein from soil fungus *Fusarium*. The fibrous texture of mycoprotein will impart meat-like properties to the final product (Wiebe 2002). Mycoprotein contains high fibre and less fat along with high-quality protein due to availability of valuable amino acids (Asgar et al. 2010). *Quorn* products take the shape and texture of various meat products such as chicken patties, nuggets, cutlets, and ground beef (Joshi et al. 2015) including steaks, burgers, chicken breasts, sliced meats, and ready meals.

Risofu is another plant-based product used for production of rice burgers and sausages in the USA. The company "Bahama Rice Burger" developed a recipe similar to rice-based "tofu" from the Shan region of Thailand. According to the company, *Risofu* combines white, brown, and wild rice to supply a wide range of nutrients (Schmidinger 2012). Seitan, also referred to as "wheat meat" or "wheat gluten," is a popular plant-based meat analog. The wheat proteins gliadin and glutenin are separated by rinsing the wheat dough for removing starch and bran and leaving a chewy mass. This ingredient is used in production of vegetarian sausages, burgers, nuggets, schnitzel, minced meat, etc.

In Asia during the twentieth century, texturized vegetable protein (TVP) becomes the first plant-based meat analog derived from the extruded defatted soy meal, soy protein concentrates, or wheat gluten in the market (Ismail et al. 2020). From the last decade, the trend in expansion of plant-based meat analogs has been observed globally. Europe and North America have expanded the market place to a large extent beyond vegetarians to meat-eating consumers (Bohrer 2019). Food companies are offering more options to their customers by bringing different plant-based analogs on market shelves. At present, the plant-based meat analog processing has come up mainly with burger patties, mince, and sausages on market shelves. In North America, various fast food restaurant chains, supermarkets, and other food stores offer plant-based burger patties mainly manufactured by Impossible Foods Inc. and Beyond Meat Inc. Total and saturated fat content is lower, but

calories and protein are the same as beef burger patty and contain zero cholesterol. The sodium content is higher in both the products.

Beyond Meat Inc. and Impossible Foods Inc. (US-based companies) are among the top key players involved in the manufacture of plant-based beef, in partnership with multinational fast food chains like Bareburger and Burger King. For the creation of meatless meats, these start-ups used plant-based products to mimic the taste and feel of the real meat. In this context, Yaffe (2019) and Lucas (2020) reported launch of plant-based impossible beefless whopper, meatless impossible pork, and impossible sausages by Impossible Foods Inc. in association with Burger King Inc. In August 2019, KFC introduced plant-based boneless "chicken wings" and nuggets in restaurant menu in association with Beyond Meat Inc. and Light Life Inc. Kroger, the largest grocery store in the USA, under its Simple Truth Line of plant-based meat alternatives prepared pea protein-based "ground beef" and "burger patties," and this new market surge is expected to be a boom for plant-based meat market (Insights CB 2019).

Unquestionably, the popularity of plant-based meat alternatives is burgeoning predominantly in developed countries with highest demand in European plant-based meat market followed by Germany, France, the Netherlands, the UK, Italy, and Sweden. In 2019, the global market for plant-based meat alternatives was estimated around 4.8 billion USD and is expected to grow to 6.8 billion USD by 2025. The market is forecasted to grow rate of 7.1% up to 2025 and 73% by 2050 (FAO 2020). European countries (51.5%) followed by North America (26.8%), Asia Pacific (11.8%), Latin America (6.3%), and Middle East and Africa (3.6%) hold the largest share of the plant-based global meat market. In Asian countries, the acceptance of plant-based meat analogs is increasing in India (94.5%) and China (95.6%) with consumers becoming more familiar about the plant-based meat alternatives having appearance, mouthfeel, and the fibrous texture similar to conventional meat obtained from animal tissue (Bryant et al. 2019). Consequently, in the future, Asian countries could possibly be the apropos market for meat analogs. The health benefits of meat substitutes are still up for debate, even though the scope for plant-based meat market is widening swiftly.

14.4 Major Ingredients

The choice of ingredients to produce meat analogs varies depending on the technical applications of product. Meat analogs can be produced from a wide variety of vegetable proteins. Vegetable proteins still form the chief ingredients of meat analogs such as wheat, soybean, groundnut, sesame, cereal proteins, vegetables, pulses, nuts, mushroom, yeast, etc. With the technological advancement, various other ingredients with inherent merits associated with processing and nutritive value are regularly added into the list of ingredients used for preparation of meat analogs such as insects, algae, bacteria, etc. Still, soya protein is widely used for preparation of meat analogs due to low cost and easy availability. Algae, bacteria, and fungi have potential to be main ingredients in preparation of meat analogs in the near future as

their cultivation does not require land, ability to multiply very fast and adapt to various climatic conditions and good sensorial attributes along with high nutritive value. Konjac fibre, a soluble dietary fibre obtained from starchy corn or root of konjac plant, is gaining popularity in preparation of meat analog due to health benefits and good taste. Howsam (1976) enlisted the use of following ingredients during preparation of meat analogs on industrial scale:

1. Plant proteins obtained from cereal grains as wheat, rice, and corn in the form of vital or residual starch.
2. Defatted oil seeds, bean flours, cereals and their derivatives as soya protein concentrate (SPC), soya protein isolates (SPI), wheat flour, defatted soya flour, etc.
3. Animal-based precursors as meat byproducts, dried egg white, fish meal, insects, etc.

The major ingredients used in the production of plant-based meat analogs are as follows:

14.4.1 Protein Ingredients

For development of plant-based meat analogs, the selection of protein ingredient is the most important. The structure and function of product largely depends on the source of protein. The physical, chemical, and nutritional content of meat analog depends upon structural as well as functional properties of proteins (Meade et al. 2005; Damodaran and Parkin 2017; Friedmann 1996). The vegetable proteins are used individually or in combination with other ingredients during preparation of meat analogs. Presently, soy proteins with added novel ingredients, such as mycoprotein and soy leghaemoglobin (legume haemoglobin containing heme), form the major ingredients used in preparation of plant-based meat analogs. Plant-based food ingredients are highly versatile due to their meat-like properties and also offer similar nutritive value that of animal proteins (Asgar et al. 2010). Animal proteins impart sufficient proportion of all essential amino acids with acceptable digestibility needed in the human diet (Bohrer 2019). Besides the nutritional content, plant proteins are considered as inexpensive sources which can be easily altered into suitable meat substitutes such as canned meat (Featherstone 2015) and pet foods (Stein et al. 2008).

Soya is the most commonly and cheapest source of plant protein used for preparation of meat analogs due to its high nutrient content and associated functional benefits and thus proves a very promising option to be used for preparation of meat analogs. It is used as soya flour, texturized soya protein, isolated soya protein, and spray-dried soya milk. Its PDCAAS (protein digested corrected amino acid score) value is equivalent to animal protein (1.0), and the biological value of soya protein isolates is comparable to meat. Soya foods are becoming popular among the health-conscious people worldwide. In meat processing, soya proteins as TSP (texturized

soya protein) are used as emulsifier and stabilizer, improving texture and increasing water retention properties of meat products. Most soya-based meat analogs are prepared by using extrusion technology. Tofu along with mycoproteins had long-lasting satiating properties for several hours. Williamson et al. (2006) observed lower food intake after consumption of tofu and mycoproteins at lunch, and it did not result compensatory intake of food during dinner.

Textured and non-textured vegetable proteins, mushrooms, wheat gluten, egg albumen, enzymes, hydrocolloids, and vegetable starches are some important ingredients commonly used in preparation of meat analogs (Kumar et al. 2017; Asgar et al. 2010). Other novel non-genetically engineered ingredients used in commercially available burger patties are beetroot which is incorporated in burger launched by The Beyond Burger to impart desirable colour to meat analog and pea proteins to produce heat-induced gels (Bora et al. 1994). The bean protein isolates form gel at lower concentration (140 g/L) as compared to soy protein isolates (160 g/L) and pea protein isolates (180 g/L) (Fernandez-Quintela et al. 1997). Other than these, soy leghemoglobin (SLH) is used in Impossible Burger to impart a meat-like flavor profile onto plant-based meat products (Fraser et al. 2018). Upon heating, peanut protein concentrate at 7.5% formed solution and produced soft gel at 10% which turned firm gel at 12.5% (Yi et al. 2013).

Wheat gluten is another example of the most traditionally used cereal protein in the preparation of meat analogs. This is due to the viscoelastic properties of this gluten which provides fibrous attributes and maintains structural consistency of meat analogs (Kumar et al. 2012). Based on nutrition content, cereal ingredients are rich in carbohydrate content and deficient in protein content in comparison to soya but exhibit lower protein value and poor digestibility compared with other sources of proteins (Joshi et al. 2015; Joye 2019; Mota et al. 2016). Legume proteins as chickpea, pea, lentil, lupine, and mung bean exhibit a poor digestibility lower than animal proteins or processed soy protein. However, they offer complementary promising application and other unique processing attributes when structured (Kyriakopoulou et al. 2019; Huang et al. 2018).

Wheat gluten is isolated from wheat flour at the time of isolation of starch. It has cohesive and elastic properties and is widely used in preparation of meat analogs for its functional properties such as excellent binding, swelling, dough preparation, solubility, leavening properties, and nutritional qualities. Wheat gluten is a rich source of glutamine but low in lysine and threonine. It has price advantage as compared to soya and mycoprotein and the production of wheat gluten on large scale on industrial scale under controlled drying to retain the functional properties of the product. Kumar et al. (2012) prepared meat analogs by incorporating wheat gluten, and the developed products exhibited good organoleptic attributes. Addition of wheat gluten also improves the baking properties of analogs along with flavor and colour scores.

Mycoprotein is an ingredient offering a good range of protein content nutritionally comparable to animal-derived proteins. It is basically a fungus product and an eco-friendly protein alternative with a protein digestibility-corrected amino acid score (PDCAAS) of 1.00. It can be used to improve processing characteristics by

combining with other ingredients, mostly egg albumin (Joshi and Kumar 2015; Joye 2019; Mota et al. 2016; Kumar and Kumar 2011). The fibrous protein of mushroom increases sensory attributes of meat analogs especially improving chewability. The fungal hyphae are rich in high-quality proteins, dietary fibre, vitamins, mineral contents, and polyunsaturated fatty acids (PUFA). The consumption of mycoprotein has been associated with several health benefits such as cardiovascular problems, obesity, etc.

Fusarium graminearum, an edible filamentous fungi, was first used for commercial production of burger patties and sausages by using fermented mushroom, egg, and seasonings or flavourings. It is commonly available in European countries as an alternate to meat. It provides meaty flavour and improves taste of the developed product due to high concentration of sulphur-containing amino acids. Good quality of meat analogs can be prepared by using mycoproteins due to formation of flaky or fibrous texture contributed by protein filaments. These protein filaments are showing similarity with meat fibre (Kumar and Kumar 2011). Mushroom could be utilized as raw material for sausage preparation, and mushroom imparts light grey colour and specific flavor to the developed product by replacing meat at higher concentration (Uzunov and Colova 1972). Kumar and Kumar (2011) developed analog meat nuggets by adding 22.5% mushroom in place of texturized soya protein and noted that mushroom incorporation resulted in significantly increasing the organoleptic attributes of analog meat nuggets by improving flavor and overall acceptability.

The egg white or egg albumin is incorporated in meat analogs for improving nutritive value, functional properties, binding, and organoleptic properties of products as crispiness and flavor. Ovalbumin and other globular proteins present in egg contribute significantly to enhance functional properties of egg by improving viscosity and binding as during heating, globular molecules unfold, and forming molten structure. Kumar et al. (2010a, b) noted that the addition of egg albumen liquid had significant effect on the physicochemical properties and organoleptic attributes of analog meat products.

14.4.2 Carbohydrate Ingredients

Meat analogs contain carbohydrate to impart structural characteristics. Owing to their emulsifying properties, polysaccharides play an important role in improving consistency and water binding capacity by improving functional and structural attributes in meat analogs (Lin et al. 2000; Yao et al. 2004). Carbohydrate ingredients are mainly starches or flours (potato, corn, wheat, cassava, pea, and rice) or the binding ingredients or gums such as methylcellulose, acacia gum, xanthan gum, and carrageenan. They are added to modify texture and consistency. These starch form gel and imitate fat-like properties and thus are also used as low-cost fat replacers in the development of low-fat meat products. Sucrose balances the hardening impact of salt by inhibiting moisture removal and improving product shelf life by retarding bacterial growth.

Binding ingredients such as gums are added during preparation of meat analogs to improve the stability of product and reduce cooking losses by improving water holding capacity and forming stable water/oil emulsions (Kumar et al. 2017; Joshi et al. 2015). These ingredients catalyse the protein, lipid, and water of the processed products and help in forming a stable structure. On looking into the nutritional aspects of these components, it is difficult to judge their health advantages in meat analogs. In terms of offering higher dietary fibre, they can be either viewed as health promoting or as detrimental for health due to higher refined starches or sugars (Topping 2007; Viuda-Martos et al. 2010).

Methylcellulose, a dietary fibre of cellulose, is an efficient binder in meat analogs (Schuh et al. 2013). In the gastrointestinal tract, methylcellulose forms a viscous solution and has effects on glucose metabolism similar to dietary fibre (Jenkins et al. 1978; Maki et al. 2008). On the other hand, acacia gum, guar gum, xanthan gum, and other gums are used in the manufacture of meat analog (Watson 2007; Biswas et al. 2011). This has led food producers to restrict or limit the application of these compounds in processed food products (Tarte 2009; Asioli et al. 2017).

14.4.3 Lipid Ingredients

In meat analogs, vegetable oils such as canola oil, coconut oil, sunflower oil, corn oil, sesame oil, cocoa butter, etc. are used as lipid ingredients (Bohrer 2019). In modern meat analogs, the lipid content is approximately equal to traditional meat products. Fats and oils improve the organoleptic attributes of food products by improving juiciness, tenderness, mouthfeel, and flavor release (Kyriakopoulou et al. 2019). These oils do not contain cholesterol and are viewed as healthier option for improving the flavor as well as textural profile of meat analogs (Lin et al. 2000; Emin et al. 2017; Dekkers et al. 2018a, b; Pietsch et al. 2019). Nowadays, oleogels are given much attention as a substitute of saturated fats in plant-based meat analogs (Martins et al. 2019). The application of fat replacers such as oat-hull-based ingredient and β-glucan (oat soluble fibre) is reported to enhance water binding capacity and structural characteristics of reduced-fat products (Pinero et al. 2008; Summo et al. 2020).

14.4.4 Flavor Enhancer

The acceptance of meat analogs by the consumer is basically affected by flavour and taste. The sensorial stability of the product during storage is very critical. Savoury spicing, meat, savoury aromas, and their iron precursors are applied along with iron complexes for improving flavor of meat analogs (Fraser et al. 2017). The flavouring of food products is challenging during processing operations such as extrusion due to alterations in physicochemical attributes. The aromas of spices added in the product premix are affected by the structural alterations in the raw materials undergone heating. Moreover, complex chemical reactions may occur depending

on chemical structure of these compounds. To imitate aromas in meat analogs, several compounds such as nucleotides, reducing sugars, thiamine, and amino acids are applied by the food industry (Fraser et al. 2017; Moon et al. 2011).

A number of technological interventions have been investigated to obtain the "meat-like" flavor in meat analogs. Kyriakopoulou et al. (2019) defined the isolation of unique volatile compounds that occur naturally, often in association with different heat treatment, as the critical method applied to enhance the flavor and aroma characteristics of meat products. After thorough research and testing, extracted flavouring compounds such as savoury yeast extract, paprika, sugar, spices, and herbs are generally added into meat analog formulations at approximate amounts to mask off-flavours of legume proteins.

14.4.5 Colouring Agents

The appearance and colour of meat products is an important factor considered by consumer for choosing meat and meat products in terms of quality and sensory attributes before, during, and after cooking (Resurreccion 2004; Mancini and Hunt 2005). Meat analogs should therefore have similar colour characteristics to simulate meat products. In particular, colouring agents are effective additive for the plant-protein ingredients which is originally yellow brown in colour. Heat-stable colouring components such as caramel colour, turmin, cumin, malt or annatto, and carotene are currently being used (Rolan et al. 2008; Vrljic et al. 2015). At high temperatures, thermally unstable colours degrade, so heat-labile colourants and sugar reduction are used in a combination to provide shape and texture of both raw and cooked meat (Rolan et al. 2008). To secure thermal stability of colour pigments, other ingredients such as ascorbic acid, apple extract, and citrus fruit extracts are used in combination (Mattice and Marangoni 2020). Herbafood Inc. produced vegan burger patties and meat analogs by utilizing apple extract (HERBAROM) for imparting natural red colour of raw patties which turns brown upon cooking and multifunctional apple and citrus fibres (HERBACEL AQ Plus) for providing textural and structural resemblance to meat. These novel ingredients are very suitable in the development of novel plant-based meat alternatives. These ingredients also safeguard the quality of meat analogs by acting as antimicrobial and preservative agents. Betanine and beet root extracts are the suggested colours for meat analogs (Hamilton and Ewing 2000; Rolan et al. 2008; Kyed and Rusconi 2009). Other colouring agents like lycopene, annatto, and leghemoglobin are also used to compensate for red colour of meat, and to mimic the colour of chicken meat, titanium dioxide is commonly used (Fraser et al. 2018; Oreopoulou and Tzia 2007; Beyond Meat 2020). Reducing sugars also added to get brown colour of the product due to their interaction with the amine protein (Maillard type reaction). Dextrose, xylose, maltose, mannose, lactose, galactose, and arabinose are also explored for this purpose, as stated in several patents (Hamilton and Ewing 2000; Rolan et al. 2008).

During cooking, the protein (myoglobin) imparts characteristic cherry red colour of meat by undergoing a series of chemical changes. It transforms from

nitrosylmyoglobin into nitrosylhemocrome, changing pink colour from red at 65 °C temperature (Reig et al. 2008). A similar concept has been reported in various cases for meat analogs (Mancini and Hunt 2005). In modern meat analogs, the choice of ingredients to impart characteristic colour differs from product to product (Bohrer 2019). To simulate the naturally occurring colour attributes of meat products, the Beyond Burger utilized beet juice extract, and the MorningStar Farms added tomato paste to get similar colour in plant-based meat analogs. The use of sarcoplasmic proteins is also gaining popularity to get desired colour attributes resembling meat products. Another meat analogs, the Gardein meatless meat balls, incorporated a reduced iron compound to get a similar colour. It has chemical and structural similarity with haemoglobin and myoglobin (Bohrer 2019; Fraser et al. 2018; Robinson 2019). Still, more research activity is needed to find out heat-stable colourants of clean label.

14.4.6 Other Ingredients

Traditionally, meat is a rich source of several beneficial compounds such as vitamin E, zinc, sodium ascorbate, vitamin B1, vitamin B2, vitamin B3, vitamin B6, and cobalamin. During preparation of meat analogs, these compounds could be easily incorporated to balance the nutritional composition of meat analogs (Damayanti et al. 2018). Besides providing health benefits, they play important role in enhancing quality and storage stability of meat analog.

14.5 Types of Meat Analogs

Broadly, meat analogs or alternatives can be categorized under three main categories classified as follows:

1. Plant based (soy, wheat, pea, rice, etc.)
2. Fermentation based (mycoproteins)
3. Insect based

14.5.1 Plant-Based Meat Analogs

Consumer's attention towards plant-based mock meat manufactured from vegetables, pulses, grains, oils, and legumes has witnessed a great expansion due to its nutritional and sensorial properties (fibrous texture, flavor, appearance) similar to conventional meat obtained from farm animals (Joshi et al. 2015). Moreover, the consumer's shift towards plant-based meat products is mainly due to ethical consciousness, environmental concern, and animal welfare rights concerns contributing majorly in increasing interest in meat analogs (Hartmann and Siegrist 2017; Siegrist and Hartmann 2019). Among all the proteins, soy proteins (soya protein isolates,

20.3%; soya protein concentrates, 33.4%; and textured vegetable proteins, 9.6%) are the most explored one, with high-functional and balanced amino acid profile followed by wheat proteins (46.8%) acting as binders and extenders by providing fibrous meat-like texture and reducing cooking losses during processing. Alternatively, pea proteins (pea protein isolates, 12.2%, and pea protein concentrates, 28.4%) could be a great substitute for soy proteins because of their hypoallergenic nature and better functional properties in extending the nutritional and textural properties of meat analogs (Schreuders et al. 2019). Similarly, rice proteins (7.2%) and vegetable proteins (4.7%) are also gaining attention for formulating plant-based meat alternatives (Avebe 2020). Soy meat proteins are instantly digestible with protein digestibility of 63.4% and nitrogen balance of 1.31 g N/day from soy protein concentrate and 66.1% and 1.16 g N/day from soya protein isolates relative to beef, having protein digestibility of 73.2% and nitrogen balance of 0.42 g N/day (Cheftel et al. 1992; Kumar et al. 2017).

Different structuring techniques like thermo-extrusion, simple shear flow, spinning, and cross-linking are current production processes for preparation of meat-like fibrous structure from plant and mycoproteins (Dekkers et al. 2018a, b). During intense processing of meat analogs, red pigments, vitamins, and minerals are supplemented to impart meat-like appearance and nutritional profile similar to meat. Plant-based mock meat has been promoted as "better for health" and "better for planet" foods because of essential amino acids, lower saturated fat, and cholesterol free profile (Guo et al. 2020). The major ingredients in fibrous meat analogs are plant proteins (20–50%), vegetable lipids (0–5%), polysaccharides (2–30%), and other ingredients. Functional properties of plant proteins such as emulsification, solubility, foaming, viscosity, gelling, and binding ability play a crucial role in forming structure, colour, texture, and flavor of meat analogs (Chiang et al. 2019) (Figs. 14.1 and 14.2).

14.5.2 Fermentation-Based Meat Analogs

Mycoproteins are the proteinaceous foods containing essential amino acids, carbohydrates, and vitamins. Mycoproteins obtained from the fermentation of fungal biomass have the advantage of forming elongated fibres associated with lowering blood cholesterol and sugar levels in human body by reducing the level of low-density lipoproteins and enhancing the formation of high-density lipoproteins (Denny et al. 2008; Finnigan et al. 2010). Mycoproteins are considered better meat substitutes because of their low cholesterol profile, better nutritional composition, and strong effects on satiety. Important fungi like *Penicillium roqueforti* and *Penicillium camemberti* are important component of ripened blue cheese and mould-ripened soft cheeses due to its benefits of improving the texture, flavor, nutritive value, and shelf-life of the cheese (Nout and Aidoo 2011). In East Asian countries, red yeast rice, a traditional folk medicine, is manufactured by utilizing *Monascus purpureus* for treating hypercholesterolemia, limb weakness, circulation problems, indigestion, and diarrhoea (Moore and Chiu 2001). Since 2002, Food and Drug

Fig. 14.1 Double screw extruder (Source: Product Development Laboratory, Department of Livestock Products Technology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India)

Administration (FDA) admitted mycoproteins as generally recognized as safe foods (Denny et al. 2008). The filamentous microalgae proteins having meat-like chewable characteristic extracted from *Spirulina* is a recent advancement in domain of mycoprotein-based meat analogs (Percival 2019). The high-fibre and protein content of fungal biomass is having additional health benefits, because high-fibre diet is usually associated with reduced risk of certain cancers and obesity, making fungal biomass, a promising meat substitute (Börjesson and Meddings 2019). Similarly, mushrooms have potential to meet our daily requirements of protein, minerals, and

Fig. 14.2 Double screw extruder (Source: Product Development Laboratory, Department of Livestock Products Technology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India)

vitamins. The higher dietary fibre and protein in hyphae imparts chewability to the products (Naylor et al. 2001).

Filamentous edible fungus *Fusarium graminearum* was the first commercial meat substitutes used in the preparation of burger patties and sausages because of their meaty flavor attributed to sulphur content amino acids and glutamic acid in fungi. Recently, in Quorn®, Marlow Foods Ltd., UK, commercialized mycoproteins are sold as Quorn in the supermarkets of 19 countries (Marlow Foods Ltd. 2019). In the future, the worldwide market for mycoprotein-based meat analogs is likely to see powerful growth, owing to its quality of imitating the taste and texture of animal

meat of high quality. Fungal proteins with fibrous and flaky texture align the protein filaments perfectly with more acceptable flavor, relative to beany flavor of textured soy proteins. The fungal biomass production method is expensive with market price analogs to animal meat (Ritchie et al. 2018). In the coming future, the maximum market demand for mycoprotein products as meat alternatives and meat extenders will be in East and South Asian countries. Presently, mycoproteins are widely acceptable in European countries; however, cost reduction and limited regulations governing *mycotoxins contamination of food* are an ongoing global concern.

14.5.3 Insects-Based Meat Analogs

Edible insects have been explored as alternative protein source owing to their high nutritive value, environmental sustainability, and affordability (Azzollini et al. 2019; Smetana et al. 2019; Bohrer 2019). Insects also known as mini-livestocks are gaining importance to be used as alternate supply of animal protein. Oonincx et al. (2010) noted 50 times higher emission of GHG and 10 times higher ammonia production during livestock farming as compared to insects farming. Insects diverted a significant portion of energy towards growth rather than spending it on maintaining body temperature. Authors noted that GHG emission significantly higher for pigs (methane (2–4 g/kg mass gain), nitrous oxide (106–3457 mg/kg mass gain), carbon dioxide equivalent (80–1130 g/kg mass gain), and ammonia (1140–1920 mg/day/kg mass gain)) as compared to *Tenebrio molitor* (methane (0.1 g/kg mass gain), nitrous oxide (25.5 mg/kg mass gain), carbon dioxide equivalent (7.58 g/kg mass gain), and ammonia (1–2 mg/day/kg mass gain)), *Locusta migratoria* (methane (0–0.1 g/kg mass gain), nitrous oxide (59.5 mg/kg mass gain), carbon dioxide equivalent (17.72 g/kg mass gain), and ammonia (26–46 mg/day/kg mass gain)), and *Acheta domesticus* (methane (0–0.09 g/kg mass gain), nitrous oxide (5.3 mg/kg mass gain), carbon dioxide equivalent (1.57 g/kg mass gain), and ammonia (142 mg/day/kg mass gain)).

Edible insects are balanced source of essential amino acids, proteins, lipids, and micronutrients such as iron, copper, and zinc. International Platform of Insects for Food and Feed (IPIFF) organization having 28 members from 14 different countries promoted insect industry as the best edible proteins in human diet. The organization is comprised of more than 300 edible insect-producing companies with mass scale production (Derrien and Boccuni 2018). Presently, edible insect-based companies are engaged in production of intensively studied edible insects with yellow mealworm (*Tenebrio molitor) having* 40% production, accompanied by lesser mealworm (*Alphitobius diaperinus*) having 15% production, house cricket (*Acheta domesticus*), and banded cricket (*Gryllodes sigillatus*) having 15% production each (Derrien and Boccuni 2018). Proteins derived from edible insects have shown good interfacial properties, emulsion stability, and gelling behavior (Yi et al. 2013). As per the reports of Kim et al. (2016) and Azzollini et al. (2019), edible proteins from yellow mealworm performed well in preparation of sausage emulsion and extruded snacks up to 10% incorporation. Similarly, Smetana et al. (2018) reported improved meat-

like texture of extrusion fibrous soy-based meat analogs added up to 30% lesser mealworm proteins.

Edible insects are best alternative proteins for substituting meat obtained from animals (Yi et al. 2013). In recent years, the market demand for edible insect protein products has increased drastically in Europe and North America. Although insect-based burger patty and mealworm balls have already been placed in various supermarket chains in Switzerland and Germany. The ensure food safety and nutritional quality of edible insect proteins still remain a major challenge in the food industry (EFSA 2015).

To get the desired impact on large scale and to make it a reliable source of animal protein and nutrition, insect farming should be adopted on industrial scale as harvesting in the wild condition has limitations and could not be a reliable and regular source. However, the large-scale insect farming causes issues regarding insect welfare during farming, species-specific requirements, proper sanitation, harvesting, and killing. At insect farm, proper biosecurity measures should be taken; otherwise spread of various bacterial, viral, and fungal diseases could collapse of whole farm.

14.6 Nutritive Value and Food Safety Concerns

It is very easy to control the nutritive value of meat analogs by careful selections of ingredients used during their preparation. These products with desired nutritive value and organoleptic attributes can be easily prepared as compared to traditional meat products. Although meat analogs mimics the attributes of meat, still there is differences in structure, composition, and sensory qualities of meat analogs and traditional meat. Redman (2010) reported higher acceptability of meat as compared to meat analogs prepared with 40% protein content. The author prepared meat analogs having cross sections similar to skin of meat. Kumar and Kumar (2011) prepared analog meat nuggets by adding textured soya protein, mushroom, wheat gluten, egg albumen, spices, and flavouring ingredients and compared this product to chicken meat nuggets and reported meat analogs having comparable sensory attributes to meat nuggets but better lipid profile. The structural and textural features such as hardness, chewiness, and cohesiveness of meat analogs were reported lower than chicken meat nuggets. Talabi et al. (1986) compared the microbiological value of meat analogs with meat and reported that meat analogs had better microbiological profile than meat products. This could be due to differences in processing and preparation methods of these two-type products. Havlik et al. (2010) studied the possibility of consuming meat analogs with aim to reduce purine intake. The authors reported significantly ($p < 0.05$) higher purine content in meat analogs prepared by addition of wheat and egg white. Lousuebsakul-Matthews et al. (2014) studied the effect of consumption of meat analog and legume intake on prevalence of hip fracture among Caucasian men and women and reported less incidences of hip fractures in populations with higher intake of meat analogs. Van Nielen et al.

(2014) reported that replacement of meaty diet with meat alternatives resulted in lower incidences of metabolic syndrome and led to better blood lipid profile.

14.7 Major Challenges

There are several challenges related to direct application of vegetable proteins to mimic the meat products. The formulations and nutrient content are the major challenges during production of meat alternatives. The key goal behind the use of any alternative to meat products is to enhance functionality, flavor, appearance and colour, and storage stability (Barbut 2017). However, plant-based ingredient reduces the formulation cost and improves nutritive value as well as the consumer image of the product. However, there are several challenges associated with several components like specific taste of meat, appearance, colour, mouthfeel, and nutrition value of the product.

The characteristic off-flavor is produced in plant-based meat analogs due to oxidation between the components, and masking these aromas is a possibility; however, it is very difficult to imitate the exact meat flavor. More than 1000 water and fat-derived compounds are responsible for the characteristic taste of meat (Claeys et al. 2004 Mottram 1998). The flavour will be a very important parameter for success but a challenge for food technologists. The strong off-flavours emanating from soy-derived products are major drawback in their popularization and acceptability. Grassy and beany flavour due to lipoxygenases enzyme and astringent flavour due to saponins and isoflavones (antinutritional factors) restrict utilization of soy protein as meat analogs (Joshi et al. 2015).

Various proteins exhibiting allergic reactions have been found in legumes (Singh and Bhalla 2008; Riascos et al. 2010). The presence of phytic acid in leguminous seeds decreases the bioavailability of essential minerals by binding with salts. This results in loss of mineral and micronutrient, which causes human deficiencies. Gluten is the main cereals protein, and some individuals has chronic intolerance to gluten proteins known as celiac disease (CD) due to damage of absorptive epithelium of the small intestine (Sadler 2004). These constrains have certainly restricted the use of these plant proteins for preparation of meat analogs (Joshi et al. 2015). However, a number of methods can be successfully used to eliminate or inactivate these antinutritional factors in order to allow the possible use of ample plant protein sources available for the production of alternative meat analogs at lower prices (Arntfield 2009).

In addition to these challenges, the perceiving of the meat analog in the mouth is very critical. In order to achieve juiciness in product, the approaches are limited to the use of additional product hydration and the incorporation of fat or extracts such as beetroot juice, which often offer the look of a "bleeding" product, similar to meat. To improve the sensorial characteristics of meat analogs, new ways to integrate water, fat, and flavourings are strictly required. The presence of fewer amino acids and trace elements in plant-protein ingredients makes it much more challenging to produce a food that should have the nutritional value of meat.

14.8 Consumer Acceptance

Consumer acceptance plays vital role in development and marketing of a product. The wide acceptability of products by consumers is very critical for success of the products. This consumer acceptability is still dominated by its organoleptic attributes such as flavour, texture, juiciness, binding, appearance and colour, etc. Kumar et al. (2019) observed that consumers prefer traditional meat over meat analogs despite the later having several inherent advantages ranging from environmental concerns, ethical issue to nutritive value. Thus, the main focus lies on the production of meat analogs having sensory attributes and cooking method similar to meat products by wholly replacing meat.

Under the multidisciplinary Dutch research programme of PROFETAS (Protein Food, Environment Technology and Society), the more sustainable alternative was studied for food production and consumption. The study focused on development of NPFs (Novel Food Proteins) with an aim to reduce meat consumption. Elzerman et al. (2011) evaluated the acceptance and appropriateness of meat alternatives in meal by replacing meat and noted that appropriateness was significantly affected by colour of meat alternatives-meal combination rather than flavor and texture. To increase their acceptability, authors suggested serving of meat substitutes with various meal combinations rather than serving these alone. Hoek et al. (2013) studied sustainability aspects of meat analogs and noted that initial preference of meat alternatives was far lesser than meat and at the same time caused frequent boredom. On continuous exposure for a long time resulted in increased liking. Hartmann and Siegrist (2017) reported that consumers are still not well informed or aware about the detrimental effect of environment upon meat consumption and the unwillingness of consumers to shift dietary preference from meat to other suitable alternatives.

Gomez-Luciano et al. (2019) studied the consumer behavior and willingness to purchase meat alternatives, viz. plat proteins, in vitro meat, and edible insect-based proteins. The wide availability of plant proteins alternative to meat was noted as important factor affecting consumer's acceptance and willingness to purchase these products, and this acceptance towards meat alternatives was more prominent among consumers from high-income countries as compared to consumers from low-income countries. Authors also reported that the attributes of these alternative proteins are most important factors affecting consumer willingness to purchase these products rather than environmental concerns, convenience, or preference for healthy meal or apprehension while buying new food (neophobia).

14.9 Recent Advances and Future Trends

The growing concern for health of natural resources as well as humans has led to a shift towards sustainable food production system with higher plant-based foods and fewer animal-based foods (Kumar et al. 2021). These products are mostly popular in Western countries. However, due to growing interest of consumers towards these

products, Asian countries are also becoming a potential market for plant-based meat alternatives (Ismail et al. 2020). Recent research in plant-based proteins as alternative ingredients for meat products includes advanced extractions and processing of proteins from traditional and non-traditional sources. However, promising physical structuring techniques are helpful, but require further improvement in creating meat-like textures (Jones 2016).

This trend of plant-based meat has potency to overcome the limitation of conventional utilization of protein from plant sources, especially from legumes and cereals. Research and development have shown that it is easier to achieve meaty texture by utilizing plant-based products, such as soy and legumes with suitable technological interventions such as extrusion, shearing, spinning, etc. Advances in the meat-analog processing enabled the application of substitute ingredients such as lupin, legumes, and oilseed crops. Together with increasing meat-like structure, the interest in more locally grown protein crops has favoured the combination of different protein ingredients for the production of new products with improved attributes. Research and development work on the development of novel meat analogs is underway, and these advances are expected to provide market participants with several opportunities in the future (Kyriakopoulou et al. 2019). In spite of the notable number of new product releases, the sector has not yet reached maturity (Fig. 14.3).

As per the estimates of Tubb and Seba (2019), the estimated cost of plant-based mock meat will be five times cheaper relative to existing animal proteins. Gordon et al. (2019) reported a substantial boom in plant-based global meat market with annual growth rate of 85 billion USD by 2030. Since 2015, worldwide market launch of plant-based meat free products in form of burger patties, sausages, and nuggets is more than six thousand, four hundred eighty-five (Asgar et al. 2010; Curtain and Grafenauer 2019; Fresan et al. 2019; Caporgno et al. 2020). Beyond, Meat Inc. (US) and Impossible Foods Inc. (US) are the top two players in global plant-based meat market followed by Garden Protein International (US), Nestle (Switzerland) Sunfed (New Zealand), Naturlii Foods (Denmark) and Sainsbury's Morningstar Farms (US), Quorn Foods (UK), Maple Leaf Foods (Canada), Boulder Brands (US), Vegetarian Butcher (Netherlands), Tofurky (US), Gold and Green Foods (US), and VBites (UK).

14.10 Conclusion

Meat analogs prepared with plant proteins provide a superior alternative to meat for health conscious at affordable price. Use of a wide variety of ingredients during preparation of meat analogs resulted in availability of varieties and convenience. Systematic investigation of some potential obstacles like safety and nutritional aspects, allergenic plant proteins, consumers acceptance, deteriorative chemical alterations in heat-sensitive compounds, microbiological contamination, regulatory challenges, financing, and technological hurdles need to be addressed adequately.

Fig. 14.3 Vegan burger patties with apple extract (Source: herbafood.de)

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Part IV

Novel Technologies in Food Industry

Comparative Study on Bio/Micro and Nanoencapsulation Technologies Applications in the Food Industry

15

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Abstract

Bio/micro and nanoencapsulation technologies have developed as a potential strategy for delivering and preserving bioactive nutrients. Micro- and nanoencapsulation technologies might enhance the flavor, texture, consistency, and taste of the food products. The antimicrobial ability of the encapsulated

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materials provides stability and usable ability in food packaging materials, thereby enhancing the shelf life of the food products as well as safer for human utilization. The rate release of the compounds might be controlled by using encapsulation, thereby maintaining the flavor and sweetness of the food product. This book chapter focuses on the encapsulation of bioactive compounds and its importance in food science, synthesis of bio, micro, and nano-encapsulation techniques, carrier agents used in micro and nano-encapsulation, technological aspects, and safety aspect.

Keywords

Encapsulation · Food · Nutrients · Bio-active compound · Nanotechnology

15.1 Introduction

Presently, the global population continuously increased, along with the growing interest in natural food ingredients to reduce the risks of various diseases; the emergence of new food products is unavoidable. Since many people do not always have access to many bioactive nutrients in a typical diet, they should introduce them as an alternative to their daily diet (Oh 2016). Bioactive nutrients are sensitive compounds to temperature, oxygen, light, pH, enzymatic activity, and ionic strength during processing and storage, or under gastrointestinal conditions (Joye et al. 2014). Thus, they must protect against the inevitable detrimental environmental effects of food processing and storage. Additionally, their bioavailability was significantly impeded by the limited diffusion of these compounds passing through the intestinal mucosa and the low intestinal epithelial permeability. In this regard, bio/micro and nanoencapsulation technologies have developed in this way as a potential strategy for delivering and preserving bioactive nutrients (Soleimanpour et al. 2020; Ray et al. 2016).

Micro- and nano-encapsulation technologies might enhance the flavor, texture, consistency, and taste of the food products. On the other hand, the antimicrobial ability of the encapsulated materials provides stability and usable ability in food packaging materials, thereby enhancing the shelf life of the food products as well as safer for human utilization. Moreover, encapsulation controlled the timely and targeted release of bioactive molecules (probiotics). The rate release of the compounds might be controlled by using encapsulation, thereby maintaining the flavor and sweetness of the food product. In this chapter, following a concise overview of the importance of bio/micro and nanoencapsulation, diverse encapsulation technologies appropriate to bioactive nutrients will be presented. Afterward, applied carrier agents in micro and nano-encapsulation technologies will be discussed. Finally, the technological challenges and food safety aspects in encapsulation and future outlooks on the encapsulation of bioactive food ingredients will be presented.

15.2 Encapsulation of Bioactive Compounds and Its Importance in Food Science

Encapsulation generally is a process in which active ingredients are embedded within another component to form capsule-shaped particles with different diameters of micro and nanometer scales. Although the nanocapsules with less than 1 mm in diameter are generally accepted as nanocapsules, in the pharmaceutical and cosmetic fields, the size lower than 100 nm is considered as nanocapsules (Sadeghi et al. 2017; Jafari 2017). The active ingredient loaded in the micro and nanocapsules is often known as the encapsulant agent, core, payload, coated material, internal phase, or fill, although its wall is often referred to as the encapsulating agent, shell, coating, membrane, external phase, carrier material, or matrix (Jafari et al. 2008; Deng et al. 2020). Based on the distribution of encapsulant in the encapsulating agent, the encapsulating micro and nanoparticles can be mainly categorized into two basic categories of reservoir type (or capsule) and matrix type (micro/nanosphere) (Soukoulis and Bohn 2018). The structure of reservoir type is defined by a shell of an encapsulating agent with an interior hollow space which is filled with the active ingredient. The encapsulant in this type can be one or multiple cores which can be coated with single or multiple walls (Jafari et al. 2008). While in the matrix type, the active ingredients are uniformly distributed in the spherically formed matrix of the carrier material; therefore, the encapsulant can be located in the core or surface of it. Regarding which encapsulation techniques are applied, these types of encapsulating micro and nanoparticles can obtain.

The most important reason for encapsulation is the protection of active ingredients against environmental agents such as exposure to oxygen, water, enzymes, light, and heat so that the shelf life of active ingredients is also improved (Manojlović et al. 2010; Zuidam and Shimoni 2010). Encapsulation can be applied to mask unpleasant taste, smell, and color, trying to prevent interference with the quality of the product. Controlled release of active ingredients is another reason that can be achieved by the encapsulation process. Encapsulant release can be triggered in the response of the various agents including changes in temperature, ## the water content in microenvironment through dissolution; mechanical forces (chewing, mastication); shear, pressure, pH, and in presence of the special enzyme (Gaonkar et al. 2014). The controlled release has the potential to be tuned to deliver the active ingredients in a specific stage of consumption or target position in the consumers' body. Furthermore, the delayed-release or sustained-release can also be achieved by the encapsulation process. Also, during encapsulation processing, the physicochemical properties of active ingredients can be modified as their liquid forms can be converted into flowable solid powder. Considering the encapsulation of active ingredients minimizes their unwanted interaction with other food components, it also improves their bioavailability (Ray et al. 2016). Finally, the simplicity of managing the encapsulation processing and the reduction of the product cost are other reasons for the encapsulation of food ingredients. The most common bioactive ingredients which can be encapsulated include vitamins, flavors, and essential oils, essential fatty acids, antioxidants, antimicrobial agents, natural food colorants,

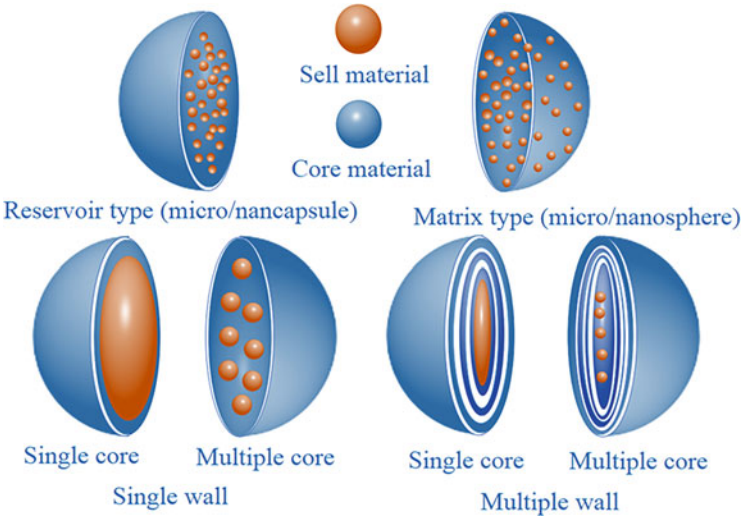

Fig. 15.1 Schematic illustration of the different types of encapsulating micro and nanoparticles' morphology

enzymes, and phenolic compounds. In the following, the synthesis method used for bio/micro and nano-encapsulation is discussed. However, choosing an appropriate method, encapsulating material, and formulation are some important factors that should be considered to produce micro and nano-encapsulate with the required properties and functional performance (Fig. 15.1).

15.3 Bio-Encapsulation, Micro-Encapsulation, and Nano-Encapsulation Techniques (Top-Down Approach and Bottom-Up Approaches)

This section provides a brief description of the most common encapsulation techniques in the food industry. Designing an effective encapsulation technique along with appropriate elements is a critical point to develop an encapsulation system with the desired application. The biological functionality, physicochemical properties of the delivery system, and the total cost are important factors that should be considered in the selection of the encapsulation technique. Therefore, the criteria required must be well-defined before selecting an encapsulation technique for a successful encapsulated system. The important criteria for selecting the encapsulation techniques based on intended performance in the final applications are included: physicochemical properties (such as stability, solubility, color, flavor, test, particle size distribution, charge density, physical state, particles' permeability, and resistance to unpleasant conditions), rheological properties (the flowability, shear resistance, compression resistance of the formulated structure), and biological functionality (the release profile of bioactive encapsulant from the delivery system)

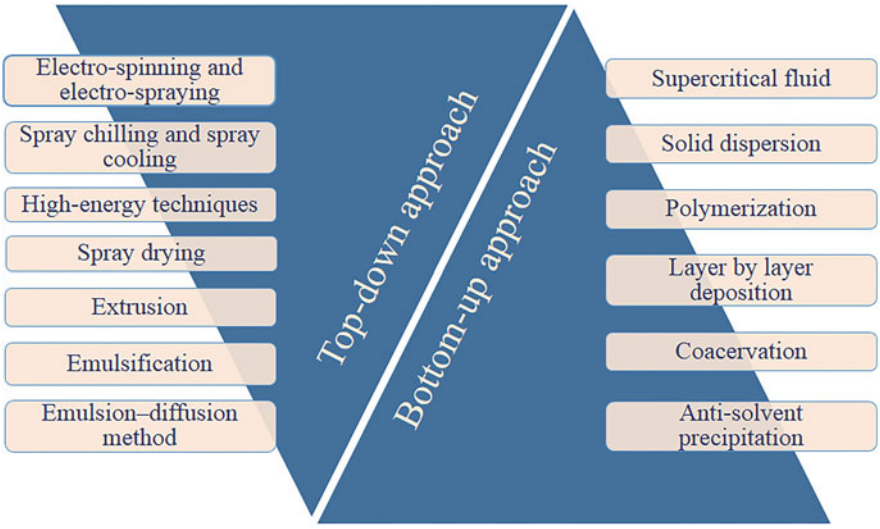

Fig. 15.2 Top-down and bottom-up approaches in micro and nano-encapsulation techniques

(Jafari 2017; Park and Lu 2015). Generally, the micro and nano-encapsulation techniques are classified into two main strategies including top-down and bottom-up approaches. In top-down strategies, encapsulation systems are fabricated using high-energy instrumental processes leading to size reduction to micro and nano-scale such as electro-spinning and electro-spraying, high-pressure homogenization, microfluidization, and sonication. In contrast, in bottom-up strategies, encapsulation systems are formed from small elements by low-energy-based processes such as self-assembly, coacervation, inclusion complexation, supercritical fluid, anti-solvent precipitation, polymerization, and layer-by-layer deposition (Jafari 2019). In the following, the most common micro and nano-encapsulation techniques are reviewed in more detail. However, more examples of their use for specific applications in the food industry can be found in the references (Fig. 15.2 and Table 15.1).

15.3.1 Anti-Solvent Precipitation

Anti-solvent precipitation or solvent displacement is a technique based on the spontaneous emulsification of an internal organic phase into an external aqueous phase. The organic phase is a miscible solvent in the external phase, containing the dissolved encapsulating agent and bioactive ingredient. The external phase in precise concentration acts as anti-solvent and makes the precipitation of encapsulating agent combining with the bioactive ingredient from the organic solution, and diffusion of the internal phase in the external aqueous medium and solvent displacement happens (Shishir et al. 2018). Supercritical carbon dioxide (SC-CO_2) is also used as an anti-solvent, and this technique is known as supercritical anti-solvent precipitation (SAS)

Table 15.1 Common encapsulation techniques

Technique	Bioactive compounds	Materials of carriers	Size (μm)	Outcomes	Ref.
Anti-solvent precipitation	Curcumin, vitamin B9, Essential oil (eugenol and thymol), β-carotene	Chickpea protein, zein, soy lecithin, sodium caseinate, carboxymethyl chitosan (CMCS), and tea polyphenols (TP)	>0.1 μm	• Sustained-release profile • High encapsulation efficiency • Higher thermal and light degradation stability • Higher stability in simulated gastrointestinal conditions	Ariyarathna and Karunaratne (2016), Ariyarathna and Karunaratne (2015), Chen et al. (2015), Wang et al. (2018)
Coacervation	Ceylon cinnamon extracts, capsaicin	Gelatin, maltodextrin, and tannins	>0.1 μm	• Higher stability during storage • Higher thermal stability	Brito de Souza et al. (2020), Wang et al. (2008)
Electrospraying	Aspalathin, (−)-epigallocatechin gallate (EGCG)	Chitosan, lecithin, poly (lactide-co-glycolide), and Eudragit S100®	>0.1 μm	• Controlled release profile • Prolong the antiviral activity of EGCG against murine norovirus via gradual bioactive release	Human et al. (2019), Gómez-Mascaraque et al. (2016)
Electrospinning	Gallic acid, phenolic-enriched extracts	Hydroxypropyl methylcellulose (HPMC)/ polyethylene oxide (PEO), zein	>0.2 μm	• Lower oxidation during storage • Improving the thermostability of the extracts • Controlled release profile	Aydogdu et al. (2019), Moreno et al. (2019)

Emulsification	Curcumin, vitamin D3, and saffron petals	Lignin, basil seed gum (BSG), whey protein concentrate (WPC), and tween 80	>0.1 μm	• Stabilizing the formulation and controlled the release profile of encapsulated curcumin • Improving the potential of bioavailability	Bertolo et al. (2019), Gahruie et al. (2020)
Extrusion	Ascorbic acid, quercetin	Glassy low-dextrose equivalent maltodextrin matrix, carnauba wax, shellac, and zein	>1 μm	• Controlled the steady dissolution rate of ascorbic acid • Good taste-masking	Chang et al. (2019), Khor et al. (2017)
Freeze drying	Anthocyanin-rich blackberry, anthocyanins from black glutinous rice (BGR)	Maltodextrin	>10 μm	• High anthocyanin content and low moisture content, hygroscopicity, and solubility • Increasing the microencapsulation efficiency, increasing the total anthocyanin content and antioxidant activity	Yamashita et al. (2017) Laokuldilok and Kanha (2017)
High-energy techniques	Eicosapentaenoic acid and docosahexaenoic acid, vitamin E, lupulon, and xanthohumol	Monolayered (lecithin + maltodextrin) and multilayered (lecithin + chitosan-maltodextrin), corn oil, lecithin	>0.3 μm> 0.167 μm	• Improving the microencapsulation yield, microencapsulation efficiency, and oxidation stability • Modulating the vitamin content, stability, and bioaccessibility • Improving the stability and encapsulation at nanoscale sizes	Solomando et al. (2020), Lv et al. (2018), Khatib et al. (2019)

(continued)

Table 15.1 (continued)

Technique	Bioactive compounds	Materials of carriers	Size (μm)	Outcomes	Ref.
Layer by layer deposition	Limonene, 5-aminosalicylic acid and glycomacropeptide	Soy protein isolate (SPI) fibrils and high methoxyl pectin (HMP), chitosan, and alginate	>1 μm	• The higher stability of limonene in microcapsules • Modulating the release profile	Ansarifar et al. (2017), Rivera et al. (2015)
Polymerization	Lymphocytic choriomeningitis virus glycoprotein (LCMV33–41)	Poly(alkyl cyanoacrylate)	>0.1 μm	• Increasing the entrapment efficiency of peptides within poly(alkyl cyanoacrylate) nanoparticles	Liang et al. (2008)
Precipitation of pressurized emulsions or hydrogels	β-Carotene	n-octenyl succinate (OSA) starch,	>0.3 μm	• High retention of antioxidant stability	de Paz et al. (2012)
Solid dispersion	Flavonoids (flavanone glycosides, naringin and hesperidin, and their aglycones, naringenin, and hesperetin)	Polyvinylpyrrolidone (PVP) and poly(ethylene glycol) (PEG)	>2 μm	• Enhancing the dissolution of flavonoid aglycones and hesperetin	Kanaze et al. (2006)
Spray chilling and spray cooling	Vitamin B12	Vegetable fat and soy lecithin	>10 μm	• Controlled release profile • Improving the stability of vitamin B12	Chalella Mazzocato et al. (2019)

Spray drying	Propolis extracts bioactive compounds, folic acid	Maltodextrin DE 13, vinal gum, and gum arabic, whey protein concentrate, and commercial resistant starch	>1 μm	• Increasing the physical stability by gum adding as well as a higher degree of encapsulation efficiency and higher antioxidant activity • Improving the encapsulation efficiency and storage stability of folic acid	Busch et al. (2017), Pérez-Masiá et al. (2015)
Supercritical fluid	Hydroxytyrosol-rich olive oil	Eudraguard®	>1 μm	• High encapsulation degree	Tirado et al. (2021)

(Visentin et al. 2012). Anti-solvent precipitation is a simple and low-cost technique. Due to this process does not require any particular equipment, it can be easily scaled-up. Both reservoir type (capsule) and matrix type (micro/nanosphere) can be obtained by anti-solvent precipitation (Ezhilarasi et al. 2013). Nano-encapsulating system with defined size distribution can be also attained by this method using rapid mixing devices and ultrasound. As these devices are applied, the mixing rates increase, the nucleation rates enhance, and agglomerations decrease; therefore, the uniform particles are produced (Thorat and Dalvi 2012). This method has been also used for various bioactive ingredients such as antioxidants (Dai et al. 2017), vitamins (David and Livney 2016), essential oil components (Chen et al. 2015), and other bioactive compounds.

15.3.2 Coacervation

The coacervation technique is defined as deposition of the colloid particles of a simple or a mixture of macromolecules (aspolyelectrolytes) from a solution phase that is separated into two-phase through a liquid-liquid phase separation mechanism. One phase is poor in the concentration of macromolecules, while the other phase is rich in the macromolecule droplets that formed the coacervate phase encapsulating the active ingredient. Dependent on the number of polyelectrolyte types used in this technique, it can be named a simple coacervation (one polyelectrolyte type) or a complex coacervation (two or more polyelectrolytes types). The adjusting pH and ionic strength of solution phase, physicochemical properties of biopolymers as electrolytes (such as nature, charge, concentration, molar mass, their ratio, and their interaction), and cross-linking agents are some of the important factors that should be considered in this technique to form coacervates with the desired shape, morphology, size, and thermal stability (Anandharamakrishnan 2014). Coacervation is one of the most widely used encapsulation methods in the food industry due to the high loading present of active ingredients (up to 99%) and their controlled release through mechanical stress, pH, temperature, or sustained release (Gouin 2004). By this technique, capsule type (or reservoir type) in nanometer size (usually more than 100 nm) can be obtained. Protein and polysaccharide such as gelatin, chitosan, alginate, and gum acacia as wall materials and polyanion tripolyphosphate (TPP) and glutaraldehyde as the coacervation cross-linking agent are mostly used in this technique (Ezhilarasi et al. 2013; Gan and Wang 2007).

15.3.3 Electrospinning and Electrospraying

Nowadays, electrospinning and electrospraying have fascinated great attention in the pharmaceutical and food industries due to their simplicity and low-cost processing (Wen et al. 2017a). These techniques can be used for the production of nano- and microsized particles as well as fibers; however, more investigations are required to the efficient operation of these techniques in the encapsulation of bioactive

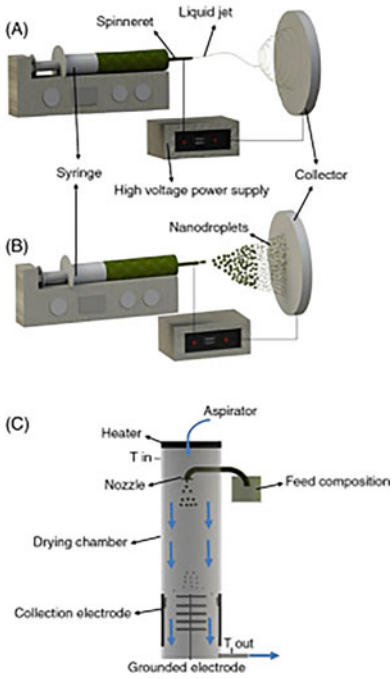

Fig. 15.3 Equipment-based nano-encapsulation processes: (**a**) Electrospinning; (**b**) Electrospraying, and (**c**) Nano-spray dryer. The image was taken with the permission (Faridi Esfanjani and Jafari 2016). © 2016 Elsevier B.V. All rights reserved

compounds in the food industry (Wen et al. 2017b). These techniques have a similar process, as a uniform electrohydrodynamic force is used to eject the solution of prepared polymer formulation (melt, or more commonly a solution or dispersion) at a constant rate for production of desired particles or fibers. Indeed, a high-voltage supply is applied to make a fine mist of polymer solution via a surplus electric charge. The accelerated solutions or mist is collected on the collector part (Esfanjani and Jafari 2016). However, these techniques are very similar to each other's, but the polymer concentrations are different. The electrospinning is used a higher polymer concentration for the production of nanofibers, whereas electrospraying yields particles using lower polymer concentration. These systems allow manufacturing micro and nanoparticles without thermal processing and changing in temperature which results in their fascinating application in the encapsulation of bioactive compounds (Tabarestani and Jafari 2019). However, protein-based bioactive compounds need surfactant or plasticizer for nanofiber production in the electrospinning process, whereas there is no need for any surfactant or plasticizer during the electrospraying process (Tarhini et al. 2017). Recently, novel electrospinning- and electrospraying-based methods such as emulsion electrospinning or electrospraying and coaxial electrospinning or electrospraying for co-encapsulation of different compounds and ##change the release profile of encapsulated bioactive compounds (Bruni et al. 2020; Yao et al. 2017; Matsuura and Maruyama 2017) (Fig. 15.3).

15.3.4 Emulsification

Emulsion-based systems are composed of colloidal droplets of an immiscible phase (inner phase) dispersed in a continuous phase (external phase). There are two main simple emulsion systems including oil-in-water (O/W) and water-in-oil emulsion (W/O). Dependent on the type of emulsion systems, this technique can be a suitable method for encapsulating both lipophilic and hydrophilic active ingredients in the food industry. Active ingredients should be dissolved in the inner phase of the emulsion system. This phase is then dispersed in the continuous phase using the mechanical energy of devices such as homogenizers, sonicators, and microfluidizers (high-energy approaches) or the inner chemical energy of the system such as phase inversion methods and spontaneous emulsification (low-energy approaches) (Barbosa-Cánovas et al. 2005). Multiple emulsions composed of water-in-oil-in-water (W/O/W) consisted of small colloidal droplets of water emulsified in larger droplets of oil that are re-dispersed in a continuous phase of water. Multiple emulsions can be used for the encapsulation of both hydrophilic active ingredients (dissolved in the inner water phase) and lipophilic active ingredients (dissolved in the oil phase). In comparison with simple emulsions (W/O/W), multiple emulsions can enhance the encapsulation and protection properties and also improve the release profile of active ingredients and the storage stability of products (Barbosa-Cánovas et al. 2005; Cho et al. 2003).

15.3.5 Emulsion–Diffusion Method

The emulsion–diffusion method is another kind of emulsion-based system in which the inner phase is miscible in the continuous phase (Quintanar-Guerrero et al. 1998). The inner phase (organic solvent containing active ingredients and encapsulating materials) and external phase (usually water phase) are separately prepared. Emulsification of the inner phase causes it to diffuse to the external phase and subsequently results in the generation of spheres encapsulating active ingredients. In this method, mechanical energy is usually applied to produce the emulsion (high-energy approach) (Fathi et al. 2014). The emulsion–diffusion method often leads to the formation of capsules with nanometer size. The size of nanocapsules is dependent on applied mechanical energy (such as the shear rate), temperature, the chemical compositions and volume of inner phase and emulsifier, and the concentration of encapsulating materials (Moinard-Chécot et al. 2008). This technique provides an efficient procedure for encapsulating both hydrophobic and hydrophilic active food ingredients (Zambrano-Zaragoza et al. 2011; Surassmo et al. 2010); however, it should be considered that any residual organic solvents remaining in the final product may have toxic effects.

15.3.6 Extrusion

Extrusion is a favorable industrial-scale method in the encapsulation process of hydrophilic or hydrophobic bioactive compounds. It refers to the injection of a solution into another solution media to make gelation with the purpose of encapsulated bioactive large porous particles productions (Bamidele and Emmambux 2020). However, the particles can protect the encapsulated bioactive compounds during storage conditions; their large and porous structures have led to their limited use for a large number of bioactive agents with the purpose of high stability and controlled release profile (Jia et al. 2016). Hence, new approaches including melt extrusion, co-extrusion, centrifugal extrusion, and rotating disk or multi-nozzle system have been applied to improve the extrusion technique (Rodríguez et al. 2016).

15.3.7 Freeze-Drying

The freeze-drying or lyophilization method is one of the most favorable procedures used for drying thermo-sensitive ingredients (Sanchez et al. 2013). This technique is also considered as a stabilizing method that improves the physicochemical stability of the particles encapsulating bioactive ingredients and their storage conditions. The stages of the freeze-drying process include freezing of encapsulation materials and active ingredients dissolved in water at low temperatures (between −90 and − 40 °C), drying via sublimation at low pressure, and the secondary drying of the unfrozen water via increasing the temperature or decreasing the pressure. However, the main drawbacks of this method are high-energy consumption, the long duration of the process, and producing particles with an open porous structure that results in exposure of active ingredients to the atmosphere (Paul Singh and Heldman 2009). However, freeze-drying is generally applied for the water elimination from encapsulation particles that are produced by other encapsulation techniques.

15.3.8 High-Energy Techniques

High-energy methods use powerful destructive force to achieve a distributed amount of dispersed oil-based droplets or particles. The system consists of two process droplet disturbances and a droplet coalescence, the balance of which results in the formation of smaller particles. High applied energies can induce the production of spherical shape particles which can be caused by the generation of higher disruptive forces compared to restoring forces. The particle size distribution is depended on the design of devices (pressure, valves, and impingements), the number of passes or cycles, environmental conditions, and physicochemical properties of samples. High-energy intensities and processes for a long time can lead to the production of smaller particles due to decreasing the interfacial tension which results in increasing the

emulsifier adsorption rate and fall of the disperse-to-continuous phase viscosity ratio (ηD/ηC) (Jafari 2017; Anandharamakrishnan 2014).

15.3.8.1 High-Pressure Homogenizer

High-pressure homogenization is a frequently used technique to yield small-sized particles in the food industry (Saricaoglu et al. 2019; Ruiz-Montañez et al. 2017). In this technique, a pre-prepared mixture of aqueous and oil-based phases is passed from a narrow slit which resulted in producing small droplets via the produced high shear stress (Jurić et al. 2019). The high-pressure passing process of the mixture from the multiple passes can be led to the broken down of large particles to smaller ones via intensive disruptive forces performing on the large particles (Levy et al. 2020).

15.3.8.2 Microfluidization

Microfluidization is a high-energy-based procedure that uses defined geometric microchannels to transfer the liquid and separate it into two streams that impinge on each other, producing an area of high turbulence and shear that can cause droplet disturbance (Muñoz et al. 2019a, b). The key benefits of this method are high reproduction, the development of various sizes of particles, and the lack of harmful organic solvents. Besides the process, it can be repeated several times to achieve the preferred particle size. On the other hand, high-pressure and longer emulsification periods may contribute to the re-coalescence of the emulsion droplets referred to as over-processing (Mahdi Jafari et al. 2006).

15.3.8.3 Sonication

Sonication is a common technique for generating nanometer-sized particles on a laboratory scale. Applied high-intensity sound waves induce cavitation bubbles, which result in extreme troublesome forces and break down the oil and water phases into small droplets (Koshani and Jafari 2019). To date, the sound waves have been electrically or mechanically generated by batch or continuous processing, but the superior effects of the batch process are encountered in the feasibility of the continuous process facilities (Kentish et al. 2008).

15.3.9 Layer-by-Layer Deposition

Layer-by-layer (LbL) deposition technique, a solution-based assembly approach, is widely used for the generation of multilayer nanoencapsulation particles (Shishir et al. 2018). In this technique, polyelectrolytes with opposite charge deposit alternatively around a charge template to form a multilayer system. Inorganic nanoparticles (such as gold nanoparticles) and polymeric nanoparticles (such as polystyrene nanoparticles) are common colloidal templates that are used for the LbL procedure. After the development of the encapsulation system with a needed number of layers, the used template is eliminated, and a nanocapsule with an internal (or interior) hollow cavity is produced. The two main approaches for loading active ingredients

within multilayer nanocapsules include active ingredient adsorption on the surface of the template before deposition of the multilayers on the template and active ingredient diffusion into the internal (or interior) hollow cavity of the encapsulation system after generation of multilayers nanocapsules (Pinheiro et al. 2015). Compared to a single-layer encapsulation system, multilayer nanocapsules have higher protection to active ingredients against environmental agents such as gastrointestinal tract stress. Also, sophisticated equipment is not required, and this method is a low-cost and easily adaptable technique (Pavlitschek et al. 2013; Ye et al. 2005).

15.3.10 Polymerization

Polymerization through mini-emulsion approaches is another emulsion-based technique to produce encapsulation systems. Mini-emulsions are composed of submicron dispersion oil droplets (50–500 nm) in the aqueous phase. In this procedure, amphiphilic oligomers are used as a surfactant that is assembled in the interface of oil and water. When monomers and a water-soluble initiator (such as potassium persulfate) are added to the system, the amphiphilic oligomers are activated and start to polymerize. As the polymer chains gradually expand, the core is formed, and the encapsulation system can be produced (Quintanilla-Carvajal et al. 2010). The structure and hydrophobicity of oligomers are important factors that affected the nanocapsules morphology (Hu et al. 2007).

15.3.11 Precipitation of Pressurized Emulsions or Hydrogels

Precipitation of pressurized emulsions or hydrogels is a technique for the preparation of water-insoluble active ingredient formulations (Horn and Rieger 2001). In this instrumental method, it is first needed to preparation of three solutions including (1) aqueous solution of encapsulating materials and emulsifier, (2) solution of active ingredients in a hydrophilic solvent (such as acetone or short-chain alcohols), and (3) an appropriate organic solvent separately. Then the hot, pressurized encapsulant solution, the organic solvent, and the cold encapsulating materials solution are pumped to the mixers and put into contact that causes a thermal shock resulting in the nanocapsules formation instantly before the precipitation happens, as shown in Fig. 15.4 (Fathi et al. 2014). After that, the solvent of the system is removed using a backward pressure valve. In this process, recommended operating temperatures around 200 °C and pressures above 50 bar are needed to preserve the liquid phase of the hydrophilic solution. The concentration of emulsifier, the applied pressure, and the ratio of the organic solvent to the water have a significant effect on the properties of encapsulation particles. The higher applied pressure leads to the generation of the particles with a smaller size, while a significant rise in particle size occurs at the high organic solvent/water ratios, and increase in the emulsifier concentration can improve the encapsulation efficiency of particles (de Paz et al. 2012, 2014). As high operating temperatures are applied, thermally stable polymers

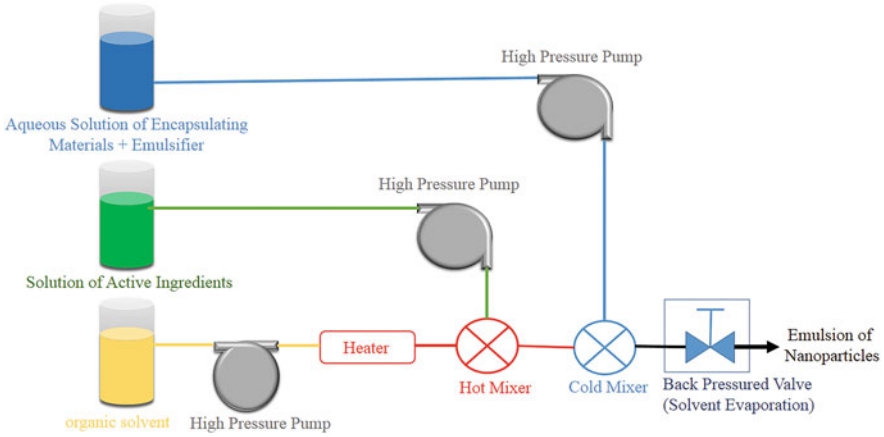

Fig. 15.4 Schematic illustration of precipitation of pressurized emulsions process

(such as cyclodextrins) should be used for the fabrication of nanocapsules through this method (Fig. 15.4).

15.3.12 Solid Dispersion

Solid dispersion is a favorable procedure to improve the dissolution rate of poorly water-soluble compounds through enhancing the wettability, diminishing the size of particles, and producing amorphous particles which results in increasing their bioavailability. Solid dispersion refers to the dispersion of poorly water-soluble compounds into polymeric or lipid-based media to form a solid product via melting and solvent evaporation methods (Zhang et al. 2017). The dispersed solids can be in molecularly, amorphous, and crystalline forms which result in the classification of solid dispersions into solid solution, eutectics, glass suspension, and glass solution (Shishir et al. 2018). In addition to bioactive compounds and the carrier media, the addition of surfactants can lead to higher stabilization of the formulation via decreasing the recrystallization and improving the solubility of encapsulated compounds (Zhang et al. 2017).

15.3.13 Spray Chilling and Spray Cooling

Spray chilling and spray cooling techniques are based on the dispersion of the bioactive agents into a molten media (fat or wax) and its atomization through heated nozzles into a case with low temperatures (spray-chilling) or case at room temperature (spray-cooling). The produced particles tend to melt at 32–42 °C, so the investigation of fat crystallization has significant importance in produced particles by the spray chilling or cooling procedure. Indeed, these processes are appropriate

methods for encapsulation of lipid-based bioactive compounds especially lipid-soluble vitamins (Katouzian and Jafari 2016; Wegmüller et al. 2006).

15.3.14 Spray Drying

Spray drying is an old and frequently used technique in the food industry for the encapsulation of bioactive compounds. The homogenized matrix of a bioactive compound and the carrier is atomized into the drying case with a hot air supply, resulting in encapsulated powders with diverse particle sizes. The resulted particles have shown excellent solubility, protection, and a controlled release profile for encapsulated bioactive compounds (Ray et al. 2016; Anandharamakrishnan 2014).

15.3.15 Supercritical Fluid

A supercritical fluid is a substance (carbon dioxide, propane, water, and nitrogen) with liquid and gas-phase properties that are due to the presence of the substance above its critical point (temperature and pressure) (Klettenhammer et al. 2020). The technique refers to the solubilization of the bioactive compound and the carrier in a supercritical fluid and its atomization and spraying which result in evaporating the applied fluid and precipitate the particles. This encapsulation technique is an excellent method for the encapsulation of thermosensitive bioactive compounds (Garba and Ismail 2020).

15.4 Carrier Agents Used in Micro and Nano-Encapsulation

One of the critical priorities in the encapsulation of bioactive compounds is the choosing of an appropriate encapsulating agent. Because encapsulating agents have to be approved as "generally recognized as safe" (GRAS) materials, the number of encapsulating agents is quite restricted (Robin and Sankhla 2013). In other words, encapsulating agents must be compliant with bioactive agents and should also be a good emulsifier and low viscous at high concentrations and have good dissolving and network-forming characteristics (Shishir et al. 2018). Additionally, they should be able to preserve the bioactive compounds at different conditions without any instability as well as tolerance## the acidic and enzymatic barriers to improving the bioavailability of bioactive compounds (de Souza Simões et al. 2017). As the cost is an important factor in the industry, the encapsulating agents should be available at a reasonable price. Besides, the physicochemical and rheological behaviors of encapsulating agents and bioactive compounds should be considered (Shishir et al. 2018) (Table 15.2).

Table 15.2 Materials used for encapsulation of bioactive compounds

Type of encapsulating agents	Encapsulating agents	Bioactive agents	Ref.
Polysaccharide-based materials	Starches (dextrins, maltodextrins, and cyclodextrins), celluloses (carboxymethyl cellulose (CMC), methylcellulose, cellulose ethers, hydroxypropyl celluloses, and cellulose acetate, cellulose nanocrystals (CNCs), and nanofibrillar cellulose (NFC)), pectins, chitosan, alginate, and gums	Vitamin A, ascorbic acid, saffron, curcumin, bifidobacterium lactic B107, and lactobacillus acidophilus	Ribeiro et al. (2020), Chang et al. (2019), Rajabi et al. (2019), Sabet et al. (2020), Albertini et al. (2010), Shishir et al. (2018)
Proteins	Whey proteins, caseins, gelatins, soy proteins, cereal proteins (zein, wheat protein, barley protein, potato protein, and Amaranth (Amaranthus hypochondriacus) proteins), and pulse proteins	Caffeine, vitamin B 12, quercetin, orange essential oil, astaxanthin, β-carotene, and folic acid	Gunasekaran et al. (2007), Zhang et al. (2015), Li et al. (2019), Tavassoli-Kafrani et al. (2018), Edelman et al. (2019), Yang et al. (2014), Aceituno-Medina et al. (2015), Shishir et al. (2018)
Lipid-based materials	Lipids (fatty acids, plant sterols, phospholipids, hydrogenated fat, glycerides, sorbitan esters, and hard fat), and waxes (beeswax, paraffin wax, microcrystalline wax, and carnauba wax)	Ascorbic acid derivatives, α-amylase	Khalid et al. (2014), Haghighat-Kharazi et al. (2018), Shishir et al. (2018)
Polymers	Polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyvinylpyrrolidone (PVP)	Aloe vera skin extract (AVE), vanillin, and aloe vera	Solaberrieta et al. (2020), Kayaci and Uyar (2012), Torres-Giner et al. (2017), Shishir et al. (2018)

15.5 Pros and Cons of Encapsulation Techniques in the Food Industry

As described above, bioactive compounds can be loaded within micro or nanoparticles to protect themselves from the inevitable environmental effects of food processing and storage. Generally, encapsulation is performed to maximize the shelf life of the active agents, but it can mask the unpleasant odor, taste, and color of the bioactive agents while preventing interference with the efficiency of the product. Encapsulation can be used to control the release profile of food ingredients using different stimuli, including pH, temperature, shear or pressure, and enzymes, and also to enhance the absorption of bioactive agents (Gaonkar et al. 2014). In general, the encapsulation technique has several benefits in the food industry, which are classified as follows (Zuidam and Nedovic 2010; Ray et al. 2016):

1. Protecting the bioactive compounds from environmental and storage factors to enhance nutritional shelf life.
2. Improving the bioavailability of bioactive compounds.
3. Controlling the profile release of encapsulated bioactive compounds.
4. Improving the handling of liquid bioactive agents by converting into fine powders.
5. Separating of bioactive agents within a mixture that may be reacted with another one.

15.6 Bio, Micro-Encapsulation Versus Nano-Encapsulation

The size of the encapsulation particles is a key factor in encapsulation technology which depends on the production methods. Generally, capsules with diameters ranging from 1 to 5000 μm are expressed as microcapsules (King 1995) and colloidal-size capsules with diameters ranging from 10 to 1000 nm are considered as nanocapsules (Konan et al. 2002). As the size of the encapsulation particles is reduced to the nanoscale, their surface-to-volume ratio is dramatically increased which provides several advantages over traditional microencapsulation. Compared with microcapsules, nanocapsules enhance the bioavailability, improve stability and shelf life, and provide an opportunity for triggered controlled release of bioactive ingredients (Shefer and Shefer 2008). Nanoencapsulation particles have more accessible active sites on their surface that can be used for future modification for targeted delivery. Due to their enhanced adhesiveness, nanocapsules can also have a longer retention time in the mucosal covering epithelium of gastrointestinal trace (Chen et al. 2006). Furthermore, increasing surface area improves the nanocapsules' interaction with targeted agents such as tissue cell receptors and cell membrane that result in more penetration, intracellular uptake, and higher efficiency (Esfanjani et al. 2018). However, encapsulation of active ingredients loaded in nanocapsules is a more complex process compared with producing microcapsules, and more innovative and sophisticated technologies are required for the nanocapsule preparation

(Rafiee et al. 2019). Furthermore, the evaluation and characterization of the nanoencapsulation particles involve the use of specialized, advanced instruments (Livney 2015).

15.7 Technological Challenges and Food Safety Aspects in Encapsulation

Currently, various techniques have been proposed for the encapsulation of bioactive ingredients, but only some of them such as spray-drying and freeze-drying are commonly used in the food industry (de Souza Simões et al. 2017; Đorđević et al. 2016). It is needed to improve the conventional technique for industrial application to enhance their output and quality, the economic cost of the product, handling complications, and safety issues. Each encapsulating method has its advantages and limitations which should be considered for their industrial applications. To overcome the challenging problems in encapsulating techniques and fabrication of the encapsulation particles with the desired functionality, several key factors are suggested in the following: as the encapsulation efficiency and stability are dependent on the wall materials, it is necessary to find out the suitable and safe materials and evaluate their efficiency for each encapsulation methods. To improve encapsulation efficiency and further product stability and desired release profile, the important factors such as technical parameters and formulations should be optimum. Various encapsulation techniques should be investigated and compared and find out the suitable one for the known bioactive ingredients. The appropriate encapsulation technique is commonly determined based on the application and intended physicochemical properties of the final product, release profile, economic aspects, energy consumption, toxicity, and safety issue. In most cases, applying a combination of several encapsulation techniques is required to achieve the best result.

15.7.1 Safety Aspects

In addition to the various advantages of nanoencapsulation systems, safety, environmental, ethical, and regulatory issues have been raised concerning the effects of nanoparticles on people and the environment in recent years. Due to their small size, nanoparticles can pass through the various biological membranes and remain in the different tissues; therefore, they can interfere in the biological reactions resulting in toxic effects (Jafari et al. 2017). Besides, nanoparticles can be extensively absorbed by small intestinal cells, and their accumulation may cause inflammatory bowel disease (Jafari et al. 2017; Roblegg et al. 2012). The surface charge of nanocapsules may also cause cytotoxicity, and it is reported that the nanoparticles with a positive charge are more cytotoxic effect than neutral or negative ones (El Badawy et al. 2011). These studies showed that the increased uptake of nanoparticles in different cell tissue, and their increased surface area with high reactivity, along with other unique physicochemical properties can lead to inflammation responses and

laceration (Baek et al. 2012; Zhang et al. 2007). Furthermore, due to their high surface area, nanocapsules containing food ingredients can compete in uptake with normal food in the intraepithelial region (Fröhlich and Roblegg 2012; Tetley 2007). The surface modification and different kinds of coating approaches can modify the surface chemistry of nanocapsules to decrease their toxic effects in living systems. Nevertheless, complementary studies are needed to provide more knowledge on nanocapsules safety for long-term use in the food industry (Ezhilarasi et al. 2013; He and Hwang 2016; Katouzian et al. 2017). On the other hand, the wall material and other compounds used in the encapsulation process (such as organic solvents, emulsifiers, and different formulation additives) can have toxic effects. Generally, natural biopolymers are regarded as safe and biodegradable compounds, so they can be considered as the first option for encapsulation bioactive ingredients. Nevertheless, these natural compounds may also possess adverse effects on cell survival.

15.8 Conclusions and Future Trends

Encapsulation is an efficient method for preserving the bioactive agents against unfavorable environmental and industrial conditions, as well as undesired flavor and aromas. Compared with the micro-encapsulation process, the nano-encapsulation process can lead to higher bioavailability, more stability, and shelf life and provide an opportunity for triggered controlled release of bioactive ingredients. Various techniques like anti-solvent precipitation, coacervation, electrospinning, and electrospraying are used for bio/micro and nano-encapsulation of bioactive compounds. The physicochemical properties of bioactive compounds, desired size of particles, release profile, and cost of production are significant factors in the selection of the carrier and encapsulation process. To date, new techniques like electrospinning and electrospraying have attracted much attention in the encapsulation of bioactive compounds due to their high encapsulation efficiency, excellent release profile, and enhanced thermal, light, and oxidative stability. Even so, their commercial use is limited by poor throughput. The prospect of studies should be focused on optimizing encapsulation devices as well as the quality, toxicity, and safety of encapsulated formulations. In a nutshell, there is a hopeful viewpoint for applying encapsulation and related technologies in the food industry due to increasing the number of food companies that introduced micro and nano-encapsulation in their nanofood commodities.

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Recent Advances and Use of Tools for Functional Foods and Nutraceuticals

16

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Abstract

Nutraceuticals and functional foods are known to have multiple health benefits from maintaining the proper well-being of the host to prevention and treatment of diseases. The therapeutic areas targeted by nutraceuticals are metabolic diseases, cardiovascular diseases, diabetes, obesity, GIT health, immune system modulation as well as prevention of various chronic diseases such as cancer. The development in the technologies made it possible to consume these bioactive compounds with their maximum bioactivity and functionality. There are several traditional as well as molecular biology techniques that are incorporated in the food and nutritional research to enhance the health benefits obtained from bioactive compounds present in the food. This chapter highlights the introduction and role of nutraceuticals in various diseases and also elaborates the recent research and techniques involved in the nutraceutical research.

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16.1 Introduction

Nutraceuticals and functional foods have gained considerable interest in the recent years due to their nutritional qualities, natural origin and therapeutic effects in many diseases. Modern age lifestyle disorders such as cancer, diabetes, obesity, osteoporosis, hypertension, allergies, etc. are becoming a challenge for the whole world (Chintale Ashwini et al. 2013). Conventional allopathic medicines are available but come with several side effects (Niggemann and Grüber 2003). Also, it has been proved that diet and exercise play a significant role in the maintenance of a good health as well as have the preventive and therapeutic effects against diseased conditions. The term nutraceutical was first coined by Stephen de Felice in 1979 (DeFelice 1992). It can be considered as the merger of two terms, nutrition (which provides health benefits and nutrients) and pharmaceutical (treatment of diseases), to make nutraceuticals and is defined as the 'food or certain compounds present in foods that impart health and medical benefits to the consumer as well as function in the prevention and treatment of targeted diseases' (Chintale Ashwini et al. 2013). The concept of functional foods is not new; people have been using herbs and many medicinal plants like tulsi (*Ocimum tenuiflorum*), neem (*Azadirachta indica*), ashwagandha (*Withania somnifera*), shatavari (*Asparagus racemosus*), pippali (*Piper longum*), etc. for the treatment of many infections and diseases since ancient times. Certain plants and food items like vegetable, fruits, spices and cereals that contain various nutrients, compounds and phytochemicals which provide additional health benefit alongside the basic nutritional needs are termed as functional foods. Functional foods have the provision to include both micronutrients like vitamins and minerals and macronutrients like proteins, fats (omega fatty acids) and carbohydrates. When these nutrients work in the prevention and cure of a particular ailment rather than just fulfilling deficiency conditions, they are termed as nutraceuticals (Sharma et al. 2017). The spectrum of nutraceuticals is very broad including functional foods, herbals, dietary supplements, genetically engineered foods, fermented products, probiotics, etc. All of these are known to have the action against specific targeted diseases. They cover most of the therapeutic areas such as digestion, cold and cough, sleeping disorders, anti-arthritic and prevention of certain cancers, high cholesterol, hypertension, diabetes, etc. Nutraceutical research is now co-supported by the emerging techniques in biological research to develop health-benefitting products which alter the expression and constitution of genes or metabolic pathways in a positive direction (Dahiya 2013). There are many techniques and methods used to enhance the bioavailability and bioactivity of functional foods such as fermentation and probiotic fortification. The use of starter culture for the fermentation of food products to deliver desired result is also one of the techniques. To increase the functionality of nutraceuticals, the delivery systems of the nutraceuticals

are also improved by the use of nanotechnology and various carrier vehicles such as emulsions, biopolymers, etc. Lastly, the interrelation of molecular biology with nutritional research has led to the understanding of effect of food on gene expression. The omics technology is involved from the last 20 years and has given a major breakthrough.

16.2 History and Development of Nutraceuticals

The concept of using food, especially herbs for treating many ailments, is a couple centuries old. The idea comes from the statement quoted by Hippocrates, the father of modern medicine, 'let food be thy medicine and medicine be thy food', almost around 2500 years ago. He clearly established the relation of food and its importance in the treatment of various ailments. There are several examples of using traditional herbs as a medicine globally. Ginseng is one traditional drug used for treating cancers and for chemotherapy for over 2000 years in China (Sharma et al. 2017). The medicinal importance of several herbs such as coriander, cumin, turmeric, fennel garlic, curry and dried mint found in the pyramids was identified by Egyptians. Triphala is one of the most preferred tonics in Ayurveda. It is a mixture of three herbs, namely, *Terminalia chebula* (Combretaceae), *Terminalia bellirica* (Combretaceae) and *Emblica officinalis* (Phyllanthaceae). It benefits almost all of the organs in the body particularly the skin, liver and digestive system. Turmeric from South Asia is a well-known anti-bacterial herb and is also a potent inhibitor of HIV (Chanda et al. 2019).

16.3 Concept of Nutraceuticals

As defined earlier, nutraceuticals are food working similar to medicines, although there are more approaches to it. Pharmaceuticals usually undergo clinical trials for the verification of the effects and are patented. However, in nutrition, foods having beneficial properties are not patented as well as there are no verification methods for treating diseases. Nutraceuticals are generally perceived as the preventive measures against any diseased condition. Nowadays, the major side effects of traditional medications have driven people towards more natural and safe treatment. These foods contain a combination of various compounds responsible for their action. These are called phytochemicals, which have a wide range of therapeutic effects against a number of diseases. These phytochemicals can act as (1) substrates for various biochemical reactions, (2) cofactor/inhibitor for enzymatic reactions, (3) scavengers to minimize the effect of toxic compounds, (4) enhancer of the absorption of various essential nutrients and (5) compressor of inflammatory reactions in the body (Dahiya 2013). Hence, the interrelation of food with health is well established now, but the main goal is to apply the benefits of food as therapeutic compounds.

16.4 Classes of Nutraceuticals

There are a wide variety of food items consumed all over the world to slow down symptoms and prevent chronic diseases other than the medications. The broadly range nutraceuticals are classified on different basis. Based on their production, they are classified into traditional and non-traditional nutraceuticals.

16.4.1 Traditional Nutraceuticals

These are the foods or compounds sourced from natural foods and are consumed without making any changes to its form. They are simply natural with new information about their potential health benefits (Chintale et al. 2013). They include all sorts of carbohydrates, proteins, amino acids, vitamins, minerals and phytochemicals present naturally in the food as well all the herbs, probiotic and prebiotics. There are many amino acids and vitamins obtained from the diet, which are involved in various metabolic pathways such as folic acid and vitamin B12. Lycopene is one compound known to have anti-cancer effects by reducing oxidative stress (Atessahin et al. 2005). All the fruits, vegetables, grains, meat and fermented products consumed in the natural form are classified as traditional nutraceuticals (Gupta et al. 2010). They broadly include nutrients/functional foods, herbals, probiotics and prebiotics and nutraceutical enzymes.

16.4.2 Non-traditional Nutraceuticals

As the name suggests, nutraceuticals which are modified artificially to basically enhance the nutritional quality are termed as non-traditional nutraceuticals. They include genetically engineered crops and fortified foods viz. oil fortified with vitamin A, wheat flour fortified with iron and folic acid; and milk fortified with vitamin D. Genetically engineered crops result in producing crops with high nutritional content or with specific properties. One example is golden rice. They are also called recombinant nutraceuticals (Chanda et al. 2019).

Nutraceuticals are also classified into different classes such as functional foods/nutrients, herbals, dietary supplements and probiotics.

16.4.3 Functional Foods

They are the nutrients which are already present in the food and have well-established functions, such as vitamins, amino acids, fats and proteins. They are called functional foods as they also have the ability to prevent certain diseases to some extent. Omega-3 fatty acids reduce the bad cholesterol in blood and lower the risk of CVD. Oats, bran, psyllium and lignins are beneficial for heart disease and colon cancer (Khan et al. 2014).

16.4.4 Herbals

Many herbs have been used traditionally as therapeutic foods. These may be consumed directly as food or in the form of extracts.

16.4.5 Dietary Supplements

These are the dietary compounds taken orally and contain concentrate of specific nutrients. They are usually meant for treating deficiencies, but some have special functions such as sports nutrition, weight-loss supplements, etc. They are available in the form of tablets, liquid, powders and extracts.

16.4.6 Probiotics

The concept of probiotics is studied most in the recent times as they have the potential to act as therapeutic agents for most of the ailments and thereby helps in improving overall health. These are the live microorganisms, usually lactic acid-producing bacteria, which are ingested orally to maintain the balance of gut microbiota. The main functions are to protect from pathogenic infection and enhancement of the immune system. Apart from these, a wide range of other benefits are being discovered. Probiotics are also being modified to project them for a specific function, acting as a medicine for a disease. This concept is called designer probiotics and is very new in the nutraceutical research.

16.5 Benefits of Nutraceuticals

Several researches over time have shown how beneficial can nutraceuticals be. The pros of nutraceuticals cover all the aspects from health benefits to becoming consumer-friendly. Their popularization in the past decade is a fruit of numerous researches being conducted continuously around the world, trying to untangle their role in the treatment and prevention of risk of several illnesses.

Being a part of our usual diets, nutraceutical does not have any side effects when consumed with the purpose of treating or preventing an ailment. The fact that proper intake of all the nutrients in a balanced diet from childhood can prevent the risk of chronic diseases such as cancer and CVD (cardiovascular diseases) is well known. One of the best examples of nutraceutical is dietary fibre as it prevents the risk of colon cancer, lowers blood cholesterol, improves gut health and prevents the infections in GIT (gastrointestinal tract) such as ulcers and IBD (inflammatory bowel disease). The pros of nutraceuticals also account their ease of availability, and economically, they are much affordable if taken in natural forms. Also, the probiotics are useful for maintaining proper gut health and microbial ecosystem. With time, many nutraceutical compounds present in various foods have been

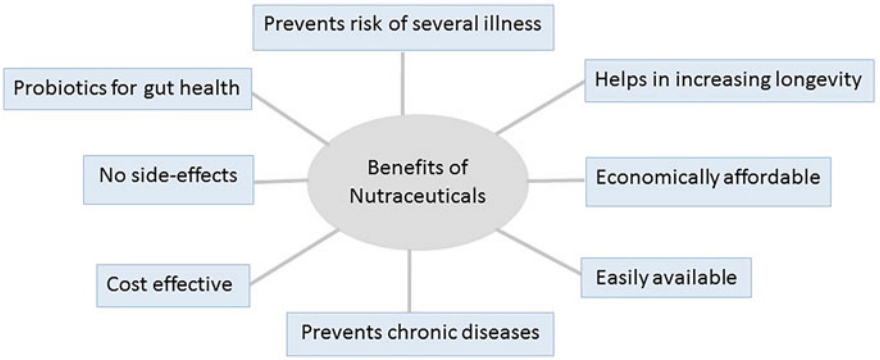

Fig. 16.1 Representation of certain benefits of nutraceuticals

proved to prevent and treat different diseases. The benefits of nutraceuticals have been shown in Fig. 16.1.

16.6 Enhancement of the Bioactivity, Bioavailability, Functionality and Health Benefits of Functional Foods and Nutraceuticals

The growing demand for nutraceuticals and their popularity among individuals have put the burden on nutrition science to find ways for improving the bioactivity, bioavailability and functionality of these functional foods and nutraceuticals. An interdisciplinary approach for this purpose is being carried out to unveil the easier and better strategies for the advancement of techniques and methods that will make this target more approachable. These strategies have been discussed in the upcoming sub-sections.

16.6.1 Enhancement of Bioactivity

Identifying and characterizing the potential of a food to see if the food can be classified as a nutraceutical is easier. The challenges appear when the bioactivity of the nutraceutical compound present in these medicinal foods is low. Demand to search for techniques/strategies to increase or enhance the bioactivity then comes into limelight. The majorly used popular strategies for this purpose are fermentation and probiotic fortification.

16.6.1.1 Fermentation

Fermentation is a metabolic process in which carbohydrates are oxidized to liberate energy. It is one of the oldest techniques, initially adopted for the preservation of foods, but it was also known to provide special aromas, texture and flavours to the

food. Today, however, the technique is being used to increase the bioactivity of already present nutraceutical compounds such as phenols, phytochemicals, antioxidants, etc. Fermentation of whole grains and cereals is widely explored as they contain numerous phytochemicals, but low bioavailability is a major issue. Lactic acid fermentation is preferably used because it is comparatively inexpensive and overall nutritional and organoleptic qualities are improved. The activity of LAB (Lactic Acid Bacteria) during cereal fermentation is well documented (Rollan et al. 2019; Petrova and Petrov 2020).

It was also shown that "routine", which is a flavanol glycoside found in buckwheat and quinoa was metabolized into quercetin by *Enterococcus avium* strain. Quercetin is a flavanol which has many documented health benefits (Shin et al. 2015). Fermentation also initiates the structural breakdown of the cell wall through microbial activity which eventually releases bioactive compounds (Adebo and Medina 2020). This usually occurs in grain fermentation. Enzymes such as proteases, amylases and xylanases synthesized from the microorganisms also play a role in the efficient bioactivity of phenolics in grain fermentation. The phenolics present are esterified to the cell wall matrix in certain cereals like bran; but they are not readily available. Fermentation is an effective strategy to release the bound phenolics and increase the bioavailability (Adebo and Medina 2020). Lactic acid fermentation is the best known method adopted to improve the functionality, nutritional value, taste and safety of food products. In a study including fermentation of whole grain sorghum with *Lactobacillus* strain, the increase in concentration of catechin, gallic acid and quercetin was documented (Adebo and Medina 2020). Another example is the fermented product *koji*, made by the fermentation of millet, and overincrease in the total phenolic compounds (TPC) is observed in the final product due to the mobilization of phenolic compounds to their free state from bound forms by the action of enzymes produced during fermentation (Salar et al. 2016).

16.6.1.2 Probiotic Fortification

The probiotics are a popular functional food consisting of live microorganisms whose ingestion in a certain amount is tagged along with specific health benefits. Several studies have shown the effectiveness of probiotic bacteria for the prevention and treatment of diseases like obesity, type 2 diabetes, non-alcoholic fatty liver disease, insulin resistance syndrome and several types of cancer (Markowiak and Śliżewska 2017). The commonly known probiotic microbes belong to the following genera: *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and *Enterococcus*. Moreover, strains of Gram-positive bacteria of genus *Bacillus* and some yeast strains belonging to the genus *Saccharomyces* are also popular probiotic (Simon 2005). These are the native strains of probiotics and have potential therapeutic effects. This fact gave birth to the need of using probiotic microbes as a functional food. For this reason, the food fortification using probiotic in the name of 'probiotic fortification' as a strategy begun. *Lactobacillus reuteri* CRL 1098 has the ability for the production of compounds with vitamin B12 activity. The fortification of food products like soy beverages using this strain has been beneficial in preventing disease caused by the deficiency of water-soluble vitamin B12 (Molina et al.

2012). Another example of probiotic fortification using naturally occurring probiotic microorganism includes the addition of a native strain of *Lactobacillus plantarum* 15HN to yoghurt that has shown to increase the folate concentration in yoghurt manyfolds that makes it a suitable alternative for synthetic folate in cases of folate deficiency without any side effects (Khalili et al. 2019).

The co-inoculum of yeast and *L. fermentum* PBCC11.5 was used in bread production which was measured to have a twofold increase of final vitamin B2 content as compared to the product made with wild-type strain *L. fermentum* PBCC11 (Russo et al. 2014). Another example of using probiotics as a starter culture can be included in soy isoflavones, most of which are bound to carbohydrates in the soy. These glucosides are not broken down in the GIT, so a probiotic supplement rich in β-glucosidase can be used for the fermentation that can initiate the release of isoflavones from their bound form (Laino et al. 2014). LAB, i.e. lactic acid bacteria, and other vitamin-producing microorganisms can be used for food fortification. LAB has strain-specific properties, and some can be designed for specific functions to be used as starter culture in fermented cereals (Rollan et al. 2019). They are the natural alternatives and can have lower production costs. The production of riboflavin and folate was reported by the certain strains of LAB isolated from the amaranth and quinoa sourdough (Carrizo et al. 2017).

Certain challenges using the native strains of probiotics could be achieved; but several functional characteristics were not met by these native strains. This led to the need of developing designer probiotics. Many microorganisms normally involved in food fermentation can be designed for a specific functional characteristic with the help of RDT (Recombinant DNA Technology) and can be added as the starter culture in the food (Steidler et al. 2003). These would ensure the growth of specific microorganisms during the fermentation which when ingested by the host would perform its function. The role of these specially designed probiotic microorganisms can be very broad from targeting a specific disease to production of vitamins to improve the deficiency condition in the body.

16.6.2 Enhancement of Bioavailability

The bioactive impact and health-promoting effects of orally ingested nutraceuticals depend on their bioavailability which involves crossing the epithelial barrier, resistance to the digestive enzymes and stomach acids and stability in the circulation and to reach their target tissue or organ in active form. The bioavailability of these compounds also depends on the diet. The main challenge of using nutraceuticals as health-promoting compounds is their bioavailability after ingestion. To deal with these efficacy issues, novel delivery methods are drawing more attention from researchers.

16.6.2.1 Polymer Coatings

Nutraceuticals need to be protected from the harsh environment of the GIT in order to function properly. For example, 60% probiotic bacteria find it challenging to

firstly survive in the GIT, and anthocyanidins' stability depends on the pH of the GIT. Hence, polymers can be designed as the carrier vehicles for them (Lee 2017). They are definitely biodegradable that can be degraded in the body by biological processes. Some natural polymers include proteins (collagen, gelatin, zein) and polysaccharides, and synthetic polymers can be based on ester, anhydride and amide bonds.

16.6.2.2 Microencapsulation

Microencapsulation is the enveloping of the bioactive compounds into a coating, which might be present in the form of solid particles, liquid droplets or gases. There are many requirements that an encapsulation system needs to fulfil. They should protect the bioactive compounds from degradation and keep them activated and stable (De Vos et al. 2010). They also mask the unfavourable taste, if present, and increase the solubility and absorption of the compound. The shelf life and stability under storage are also increased (Manzanares et al. 2019). The size is of typically a few microns in diameter and referred to as microcapsules. The coating used is always biodegradable as mentioned in the previous section, and they can be either protein based, polysaccharide based or lipid based. The final aim of microencapsulation of the bioactive compounds is their proper digestion and absorption from the intestine into the circulation so that they can perform their designated function (Ye et al. 2018). The microencapsulation can efficiently deliver bioactive compounds in foods such as probiotics, minerals, vitamins, phytosterols, lutein, fatty acids, lycopene and antioxidants (Champagne and Fustier 2007).

16.6.2.3 Emulsions

Emulsion is a very useful tool for the delivery of the bioactive compounds. It consists of a dispersed phase (small volume) emulsified into a continuous phase, which generally has a large volume (Lee 2017). The kinetic stability is achieved by controlling many factors such as viscosity, surface charge and droplet size and also adding some thickening agent (Lu et al. 2019). The structure of the emulsion such as the droplet size can affect the kinetics of releasing the nutraceutical compound. So, the basic working of the incorporation of functional foods' ingredients is that they can be incorporated into the dispersed droplet which is again covered by the continuous phase cutting the contact from the external environment (Mao et al. 2015). They are considered to be a great option for delivering bioactive compounds as they provide protection from degradation as well as control the release. One of the advantages is that these emulsions can be modified according to the compound by modifying the structures in water phase, oil phase and interphase. Classification is based on the phase distribution of oil and water. A system consisting of water droplets submerged into oil is called oil-in-water (O/W) emulsion, and the system with water droplets immersed into oil is termed as water-in-oil (W/O) emulsion (McClements 2010). The emulsion can be efficient for the delivery of bioactive lipids like ω-3 fatty acids, carotenoids and phytosterols (McClements et al. 2007). Also, the HIPEs, i.e. high-internal phase emulsions, can be used for enhancing bioavailability and protection of beta-carotene (Tan et al. 2017).

16.6.2.4 Nanotechnology and Nanoemulsions

Nanotechnology is the newest technique used to deliver nutraceuticals to increase their bioavailability. Nanotechnology deals with substances with the capability to measure, image, manipulate, transform and control at the dimensions of 1–100 nm. It has a great potential in improving the efficacy of the bioactive compounds as it increases the absorption and solubility, facilitates controlled release, protects them from degradation and has the most effective function in targeted delivery. Nanotechnology provides tools and techniques to incorporate biological and chemical surface ligands onto the nanoparticles. These ligands recognize the target cells, and with the controlled release mechanism, they increase the efficacy and functionality of the bioactive compound by delivering to the target cell.

Many natural compounds have the nano-sized particles or assembled into nanoparticles after biological changes. For example, milk protein beta-lactoglobulin is about 3.6 nm in length. Nanotubes from the hydrolysed milk protein α-lactalbumin are self-assembled and are potential carrier for nanoencapsulated nutraceutical compounds (Momin et al. 2013). Other than these natural carriers, there are many nanodevices that have been made such as micelles (5–100 nm in diameter), liposome solid lipid nanoparticle, nanoencapsulation with biopolymers, nanoemulsions, etc. (Katata seru et al. 2019).

16.6.2.5 Nanoemulsions

Nanoemulsions is a nano-sized formulation of an emulsion, which means two immiscible liquids mixed together into a single phase, but the size range is from 20 to 200 nm (Sharma et al. 2017). Resveratrol is the antioxidant compound found in grapes and blueberries but has a poor bioavailability, so researchers encapsulated it in a nanoemulsion form by spontaneous emulsification method to overcome the bioavailability issue (Sharma et al. 2017). Fat-soluble vitamins, A, D and E are encapsulated in O/W emulsion. In an experiment, there was more inhibition reported to *E. coli* by essential garlic oil emulsion than just by simply garlic oil on a petri plate (Katata-seru et al. 2019).

16.6.2.6 Liposomes

Liposomes are structurally spherical in nature with a diameter of 20 nm and above. This technique incorporates biological and chemical surface ligands onto the nanoparticles (majorly bilayer of phospholipids) (Singh 2016). The interior core of liposomes is aqueous in nature. Nanoliposomes are nanometric version of a liposome and possess similar properties to a liposome in terms of structure and thermodynamics. Reduced size of nanoliposomes provides increased bioavailability of encapsulated compounds due to more surface area contact (Singh et al. 2012). The bioactive compounds having hydrophobic nature can be packed inside the lipid bilayers of liposomes and can be released gradually, either through diffusion or an instantaneous process of membrane disruption. The stimulation for membrane destruction can be done by changing the temperature or pH (Thomsom et al. 2009). Liposomes can be used as a delivery system for several bioactive compounds

including antioxidants, proteins, peptides, vitamins, minerals, fatty acids, etc. (Singh 2016).

16.6.2.7 Microemulsion

Microemulsions are assembly of water, oil and amphiphilic molecules into structurally droplet form. They are thermodynamically very stable. They posses transparency, low-viscosity and show isotropic dispersion. The food-grade surfactants include phospholipids and diacyl glycerides (Flanagan and Singh 2006). Microemulsions can be used with the aim of increasing solubilization and as a delivery system for bioactive compounds. As a co-surfactant, ethanol can be utilized for solubilizing the long-chain triglycerides (Flanagan et al. 2006). For packaging, the lipophilic compounds like lutein and lycopene into aqueous systems O/W microemulsions can be an effective delivery system (Garti et al. 2003). Microemulsions have many applications in several fields of research and application for industries like pharmaceutical sectors, cosmetics, etc. In the food industry, several surfactants are allowed, and several are not suggested making it a less preferable delivery system for microemulsions (Singh 2016). But using the microemulsions for the solubilization of the long-chain triglycerides still remains an important benefit of this technique. Also, the breakdown of microemulsions with increased water concentration adds to its drawbacks restricting its use in food delivery system. Microemulsions can still be useful for oil-soluble bioactive compounds like α-tocopherol when formed using lesser EMG, i.e. ethoxylated mono- and diglycerides, and POE, i.e. polyoxyethylene oleyl ether concentrations (Flanagan et al. 2006).

16.6.2.8 Biopolymeric Nanoparticles

These are biopolymer (proteins and polysaccharides)-based nanoparticles effectively used as a drug delivery system (Sundar et al. 2010). They have a nanoporous structure system which provides the properties for the better development of tissue engineering, diagnosis and targeted drug delivery systems. This technology has a very crucial role in many research studies of medicine and biology and is specially used as a crucial site-specific delivery system with increased efficiency and very lower amount of toxicity (Ramchandran and Shanmughavel 2010). Preparation methods for the protein-based nanoparticles include the following steps: emulsification, desolation, coacervation, nanoprecipitation, liquid-liquid dispersion and electro-hydrodynamic atomization (Jimenez-Cruz et al. 2015). The delivery system can be beneficial for the delivery of polyphenol-based combination (Zhang et al. 2020).

16.6.3 Enhancement of Health-Promoting Effects of Functional Foods

The functional foods show the absence of any side effect when consumed with the aim of attaining health benefits. They not only prevent the occurrence of a disease

but also provide relief in the already existing illness. The strategies for enhancing these health-promoting effects of nutraceuticals are dependent on their proper bioaccessibility and bioavailability. Using the identified or potential functional foods as a part of regular diet, creating food supplements, extracting and isolating the bioactive compounds from their sources and producing their drugs or pharmaceutical supplements or using several delivery systems like microencapsulation, nanoemulsions with increased functionality are several strategies that can be used to achieve this target.

16.6.3.1 Oral Delivery Techniques

The intake of nutraceuticals comes with a lot of benefits as well as the challenges. Oral delivery methods are the most effective way to deliver nutraceutical compounds with their proper bioaccessibility and bioavailability. There are many hurdles to overcome such as the solubility and permeability of the compound from a solid oral dosage, especially for fatty acids and phytochemicals. Another challenge is the bioaccessibility, which means that the body should be able to absorb the compound from its delivery matrix. Proper degradation and metabolism of the bioactive compounds is also the main role of an oral delivery system after the solubilization is complete (Gleeson et al. 2016). There are many systems of delivery vehicles mentioned in the above sections, which are used for oral consumption. Some of the examples are lipid- and surfactant-based systems (nanoemulsions, liposomes, solid-lipid nanoparticles), biopolymer-based systems, intestinal permeation enhancers, etc.

16.6.3.2 Probiotic Foods

These are the fermented foods but a little more specific where the fermentation is done by selective bacterial strains which are good for overall health. The probiotics can deliver specific desired results with the incorporation of genetic engineering. They can enhance the concentration of a specific nutrient in the case of nutritional deficiency or can target any specific disease. Other than that, probiotic incorporation in the diet always tends to increase the functional benefits of a particular food; there are plenty of examples available in literature where probiotics act in the prevention of certain diseases such as metabolic disorder, GIT infections, gastroenteritis, immune reactions, etc. Several probiotic foods are available in the market, and some of these are even a part of traditional diet practices. Both dairy and non-dairy probiotic foods have been developed and identified to make the probiotic foods available for the people with lactose intolerance, a metabolic disorder with inability to digest lactose/milk sugar. Curd or yoghurt is a dairy-based traditional food with tons of probiotic microorganisms and accountable health benefits like improvement in the condition of diarrhoea. Usual process for making any probiotic food includes a common step of fermentation. The fermentation is dependent on the addition of probiotic microorganisms like *Lactobacillus* sp. and *Bifidobacterium* as a starter culture. But the use of these probiotic bacterial species is not limited to the dairy products. Non-dairy-based food products produced using these probiotic microbes from fruits, vegetables, cereals and sausages are also available as

functional probiotic foods (Karovicova et al. 2002). The non-dairy-based traditional probiotic foods include non-alcoholic beverages produced from the cereals such as Boza (a cold beverage found in Turkey, Romania, Albania) and Mahewu (a sour beverage available in Africa) (Prado et al. 2008). The health benefits provided by these functional foods have also laid the foundation of the production of such probiotic potential carrying new foods. Examples include soy-based probiotic foods as frozen desserts (Heenan et al. 2004) and milk drink (Donkor et al. 2007). This new soy-based products have tremendous health benefits like they have shown to reduce the levels of carbohydrates that are responsible for the production of the gas in the intestine, result in elevated isoflavone levels (Champagne et al. 2009) and have a significant effect on lowering LDL, i.e. low-density lipoprotein (Larkin et al. 2007).

Delivery of probiotics is also a challenge because practically the survival of the probiotic bacteria is conditioned at various steps inside the body sometimes due to digestive tract enzymes or low pH in the small intestine (Lee 2017). In order to protect these bacteria from harsh conditions, the encapsulation of the probiotic bacteria can be done by emulsions, extrusion or spray-drying method. Probiotic therapy, specifically using the designer probiotics, is the alternative approach to target diseases in the most specific way possible. Many genetic alterations and even metabolic disorders can be prevented by probiotic therapy such as type 1 diabetes, cancer, etc. (Sleator 2015).

16.6.3.3 Prebiotic

The probiotic will be needing a food or nutrition source inside the host. Use of prebiotics which is a non-digestible fibrous compound helps in the better absorption and functionality of these prebiotic compounds. They promote the growth of more probiotic bacteria in the gut by aiding as a nutrition source. They are fermented in the colon and make the pH acidic which results in more absorption of the minerals such as calcium and iron. Fibrous foods and foods containing carbohydrates like oat meal, courgettes, broccoli and carrots are rich in prebiotics. The prebiotics include XOS (xylooligosaccharide), FOS (fructooligosaccharide), GOS (galactooligosaccharide), inulin, pectin and several others. These can also be added to foods to increase the health-benefitting potential of that food. Several prebiotic foods are available in the market, viz. the addition of prebiotics to infant formulas like inulin has shown to mimic the bifidogenic effect of human milk (Fan et al. 2016).

16.6.3.4 Synbiotics

Adding two supplements to the diet or maintaining check on the required amount of each with respect to the other seems troublesome. Therefore, new functional food based on ‘synbiotics’ came into existence. The synbiotic is a combination of both probiotic and prebiotic in one supplement. These are often consumed as co-encapsulated forms. *Bifidobacterium* or *Lactobacillus* genus bacteria as probiotic with fructooligosaccharides as a prebiotic seems to be frequently used in synbiotic products (Markowiak and Śliżewska 2017). Like probiotics, the encapsulation can be done for both probiotics and prebiotics in combined form, i.e. co-encapsulation.

This can be achieved by techniques such as electro-hydrodynamic atomization. The co-encapsulation has several benefits like increased survival of probiotic in acidic conditions by encapsulating in a polysaccharide matrix and improved bacterial survival during storage period, viz. co-encapsulation of starch granules along with the *B. lactis* has increased the bacterial survival (Zaeim et al. 2019).

16.7 Technological Trends for Understanding and Improving the Functionality of Nutraceuticals

The improved and better functionality of nutraceuticals remains the major concern of food technologists due to the ever-growing need and demand for these health-promoting food products. Advancements of novel technologies in the field of biotechnology, bioinformatics/computational biology and nutrition sciences have provided an interdisciplinary approach for this purpose. The techniques mentioned below have efficiently improved the functionality of these medicinal foods or nutraceuticals along with the inhibition of associated harmful pathogens.

16.7.1 Omics Technology

While traditional applications of food research were focused on providing and completing nutritional requirements; however, latest research focuses more on using food for improving health as well as for therapeutic effects. To fully understand and accomplish that goal, it is very important to understand the total mechanism and metabolism of bioactive compounds inside the body. The omics study is the new breakthrough involving and connecting genomics (gene analysis), metabolomics (metabolite profiling), transcriptomics (gene expression study) and proteomics (protein expression study) to figure out nutrigenomics in relation to the nutraceutical compounds. Nutrigenomics examine as to how diet influences the gene transcription, protein expression and metabolism (Kussmann et al. 2006). The study of these complex interactions requires the development of advanced analytical approaches combined with bioinformatics. The analytical approach may include new techniques such as NMR, GC-MS, etc. which are constantly being used in the assessment of metabolites in the bodily fluids. These techniques promote the idea of identifying a healthy phenotype which should then be promoted by healthy nutrition (Kussmann et al. 2006). With time, several discoveries and understandings have been established. One such is the understanding of the enteric nervous system, which is a collection of hundreds of neurons functioning independently in CNS and controlling the gut mobility, blood circulation, immune reactions and functions. The genes and their underlying mechanism can lead to defining functional gut disorders on the basis of biological markers rather than symptoms (Mayer and Collins 2002). It is now possible to understand the role of genes in obesity and how diet plays a major role in the activity and functioning of those genes due to the present-day genomics and proteomics technology. Kaput (2004) reported that there

are roughly 100 genes known to be involved in obesity and 20 are known to be affected by diet. The examination of dietary fat intake, food restriction and protein intake on gene expression is done by transcriptomics involving microarrays (Iqbal et al. 2002).

16.7.2 Proteomics or Protein Engineering

Proteomics or protein engineering deals with three basic things, protein expression, protein structure and protein function. This basically bridges the gap between gene and phenotypic or metabolic result. One of the best advantages of proteomics in therapy is the early caught of any changes in the protein expression from normal or metabolite changes and eventually detection of early deviations from normal. The assessment of proteins is comparatively easier than the genes (Kussmann and Affolter 2006). A major application of protein engineering regarding nutraceutical is producing the gluten using microorganisms, i.e. expressing the wheat gluten by heterologous expression (Kapoor et al. 2017). The expression system used for this purpose can be *E. coli*, yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*) or insect cells (cultured). A promoter for gene expression at high level is another need for this purpose along with stability of plasmid and use of codon. Majorly used expression system for gluten production is *E. coli* as it possess certain advantages like high yield and availability of fusion tags (Tamas and Shewry 2006). The wheat gluten so produced is a protein with certain medicinal properties like ability to lower the risk of proximal colon cancer (Um et al. 2020).

16.7.3 Genetic Engineering

Genetic engineering or genetic modification is one of the most researched and practically adopted technologies to increase the efficacy of various bioactive compounds within their source. Genetic modification is applied to crops, microorganism or even multicellular organisms for various specific functions. The main objective of developing GM crops or biofortified crops was to treat the nutritional deficiencies present in populations (Glass and Fanzo 2017). One of the best examples is golden rice in India which was a little yellowish rice having more concentration of beta-carotene than the wild crop.

Nowadays, genetic engineering has vast applications as genetically modified microorganisms are used for various purposes in clinical nutritional research. Many genetic tools are developed; development of cloning vectors is done based on the identification and isolation of plasmids from the particular strain. Using molecular biology techniques, these vectors containing the desired genes are incorporated into the organism. One such example is the use of LAB (*L. lactis*) strain for the treatment of IBD. IBD is the inflammatory disorder of the gut. *L. lactis* is a well-known probiotic strain with already known health benefits, but here the bacterial strain was modified to produce antioxidants and anti-inflammatory

cytokines (IL10). These compounds reduce inflammatory reactions and control the immune response in the body (De Moreno de LeBlanc et al. 2015). These are also termed as designer probiotics.

16.7.4 Gene Editing

Improvements and advancements in the field of synthetic biology have successfully manipulated the genome of beneficial microbiota for better functional relationship (Yadav and Shukla 2020). Genome editing is the major technique in this regard as it gave rise to certain microorganisms with nutraceutical value. Application of gene editing to manipulate and regulate a wide range of cells and organisms has created a new era of advancement. Specific tools that can be used for gene editing include CRISPR (clustered regularly interspaced short palindromic repeats)-Cas, i.e. CRISPR-associated systems like Cas9; TALENs, i.e. transcription activator-like effector nucleases (Gaj et al. 2013); and ZFNs, i.e. zinc finger nucleases (Urnov et al. 2010). The discovery of programmable DNA nucleases laid the foundation of the whole concept of gene editing. These mentioned sets of tools help in cleaving or binding the gene or DNA sequences at a targeted locus for desired manipulation. Nowadays, the most reliable and advanced tool for gene editing is CRISPR-Cas9 system due to its certain advantages over other tools. It efficiently recovers the low mutation frequency rate in the oligonucleotides, genome can be modified at several loci using this tool, and this system also has a role in the adaptive evolution of phages. A bulk of genetic studies involving genetic engineering or gene editing are based on CRISPR which is generally an mRNA sequence from the genome of bacteria (including the bacteria used as a food source) (Stout et al. 2017). Gene editing with the application of CRISPR-Cas9 tool can aid to isolate or modulate the strains of a mixed culture and even produce edited bacterial genomes having relevance as bacterial workhorses in the food industry (Stout et al. 2017). This tool utilizes the RNA-guided nuclease in association with Cas9 protein and gRNA. The homology of CRISPR to single-stranded genetic material of bacteriophage facilitates their binding during replication, while Cas protein will act as an endonuclease degrading the genetic material of the bacteriophage. Certain CRISPR families' presence has been identified in microorganisms with probiotic potential like *Lactobacillus* and *Bifidobacterium*. Therefore, endogenous CRISPR-Cas system, i.e. types I and II, can be used for the engineering or gene editing of probiotics. The type II and V systems are only used for heterologous editing in species or strains of microorganisms which do not have CRISPR-Cas9 system relationship (Yadav and Shukla 2020). This tool facilitates the integration of multicopy gene in a single transformation by the introduction of a series of landing pad system from synthetic DNA as used in yeasts (Bourgeois et al. 2018). It can also be used as a novel set of technology to reach the target of reducing the harms caused by detrimental bacteria, thereby reducing the forever concern of the food industry. The application of CRISPR-Cas9 technology can be observed in lactic acid-producing bacteria (LAB), which are widely used as a starter culture and health-providing probiotic

bacteria, because LAB have a comparatively frequent or more occurrence of CRISPR-Cas systems (Briner et al. 2015). Traditionally, if we look for natural dodging using this technique, the primary starter culture of yoghurt which consists of *Streptococcus thermophilus* comes into notice as the adaptive immunity to these starter culture bacteria against the bacteriophage also involves the role of CRISPR-Cas tool (Barrangou et al. 2007). The occurrence of CRISPR-Cas in edible/diet-associated microorganisms can be beneficial for identifying and distinguishing closely related strain providing protection against pathogenic phages; and for isolation or modulation of specific strains within a mixed culture. Example of successful gene editing CRISPR-Cas9 tool includes developing the ability to produce enhanced amount of exopolysaccharides in *Streptococcus thermophilus.* The exopolysaccharide is critical for the texture of dairy products and, thus, has a significance in the dairy industry. This enhanced production of exopolysaccharide can be achieved as a result of nucleotide sequence alteration for epsC in *Streptococcus thermophilus*. Another application includes the development of increased ability of bile salt hydrolysis in *Lactobacillus gasseri* by replacing the bshA promoter sequence (Hidalgo-Cantabrana et al. 2017).

16.8 Conclusions

Nutraceuticals and functional foods are the most promising research areas to understand the relationship of food with the body and then, based on this understanding, develop benefitting products and systems for improving health and preventing diseases. Various nutrients present in our foods such as vitamins, minerals, antioxidants and herbals are consumed by human race for centuries. The lifestyle changes in the past 50 years have led us to revisit the beneficial and therapeutic aspects of our foods. There are many functional foods and nutraceuticals established now such as turmeric, cinnamon, fruits and vegetables, omega fatty acids, vitamins, etc. These compounds actually have the ability to prevent the occurrence of various diseases such as cancer, diabetes and obesity and might have a treatment for many diseases. The challenge faced by nutraceutical consumption is its efficient delivery into the body so that it can be properly solubilized and absorbed for its targeted effect. There are various techniques used for improving the delivery systems of nutraceutical compounds such as nanotechnology, microencapsulation, emulsion system and biopolymers. To improve the bioactivity of various nutrients, fermentation and specific starter culture are also used. Other than that, probiotics and prebiotics improve the bioactivity of nutrients if delivered properly, and genetic engineering is used for the development of different nutrient-rich crops to treat nutrient deficiencies and designer probiotics to target a specific function or disease prevention/treatment. Advancements in the field of nutritional biotechnology also improved the functionality of nutraceuticals and functional foods.

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Application of Non-Conventional Methods in Food for Obtaining Bioactive Components

17

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Abstract

During the present era of paradigm shift in the chemical processing technology, from the use of high-temperature high-pressure involved processes to mild and eco-friendly sustainable processes, non-conventional processing methods are coming in the forefront of implementation. Reducing the dependence on fossil fuel-dependent classical processes, the newer processes with extreme novelty in process integration and innovation are becoming fit for the present time. Fermentation-based bio-processing and advanced separation-based bio-refining are drifting the near future towards the production of high-scale bioactive compound which could be used in all purposes of human life. Succinic acid is one such bioactive material which is found in different nutritious foods. Though it is

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extensively used as a bioactive ingredient to enhance the food quality, smell and taste, but, world widely, more than 90% of succinic acid is produced by classical chemical technological pathway, which is costly, polluting and difficult to operate. That is why, novel routes are extremely sought which will satisfy the operational flexibility, production economics and market demand. An exhaustive review has been presented in this chapter to exhibit the movement of production technologies towards the newer direction of novel strategies aiming towards the manufacturing of succinic acid. Bio-refining through membrane-integrated fermentation technology appears to be the brightest among all. To resist the overgrowing crisis of food, such novel and non-conventional routes are necessary to be critically explored by the global researchers.

Keywords

Bioactive material · Novel strategy · Succinic acid · Fermentation · Membrane · Bio-refining · Bio-processing

17.1 Introduction

Biologically active compounds are micronutrients and extranutritional ingredients that are widely found in different foods, fruits, vegetables and eatables from different plants and animal flesh. The primal influence of these compounds is to promote good health to a living person's body. Nowadays, quite a lot of research is being carried out on polyphenolic materials which are potentially antioxidants and used to be present in green plants, cereals, olive oil, vegetables, fruits, tea and red wine. Succinic acid (SA) is also such kind of a bioactive component which is widely found in broccoli, beet, fresh meat, cheese, etc. and used widely as a safe acidity regulator, preservative, stabilizer, taste improver and supplementary component in various food and beverage processing industries. SA is reckoned to be one of the top 12 highest-priority carbohydrate-derived chemical building blocks and value-added bio-products as referred by the U.S. Department of Energy and the BREW project (Saxena et al. 2017). In fact, microbial production of succinic acid with its co-products is able to substitute different petrochemical products as substantial green intermediates to produce a variety of chemical products (Law et al. 2019). The high cost and limited stock dependence of conventional fuels, upsurge in carbon footprints, with higher concentration towards the utilization of local raw feedstocks, are the governing factors of such efforts. Moreover, the worldwide growing demand of different chemicals (e.g. polyethylene plastics) is triggering the scientific efforts towards the manufacturing of green chemicals (bioplastic). Currently, succinic acid possesses a global market of about 1.6 MT/annum which is increasing by around 10% a year (Jansen and van Gulik 2014; Ahn et al. 2016). Poly(butylene succinate) is a group of eco-friendly polyesteric chemicals which could be produced from succinic acid and are famous for their admirable thermo-mechanical perseverance,

chemical stability and excellent biodegradability. Thus, it has drawn much attention of the current researchers' community due to its applicability in a wide spectrum of fields. Though bio-reactions with associated downstream purification technology were studied for the production of bio-succinic acid, more endeavours should be taken to design cost-effective sustainable technologies. But, in fact, the bio-transformation route suffers due to the high cost of substrates which in turn elevates the production cost even than that of petroleum stock-dependent pathways (Luthfi et al. 2017; Foulet et al. 2019). Indeed, there is dearth in scale-up confidence to foster industrial scale of production of bio-succinic acid, employing cheap renewable waste carbon feedstock like sugarcane bagasse constituting of lignocellulose material composed of 50% cellulose, 25% hemicellulose and 25% lignin. It could be referred as a high-volume key agro-waste generated in India, produced at a rate of about 250 kg per ton sugarcane, with approximately 250 million tons of sugarcane being harvested every year in India.

While transforming biowastes to succinic acid, the main challenges are the pretreatment of feed biowaste and downstream separation of succinic acid from the bio-reaction mixture. The pivotal factor disturbing the utilization of biomass in bio-reaction is the discharge of carbohydrates (by pretreatment) from the recalcitrant poly-carbohydrate chains in lignocellulose. Deep eutectic solvent is a type of novel eco-friendly solvent having mostly identical physical and chemical characteristics like ionic liquids (Dion et al. 2020). This is also advantageous for its ease of preparation and for showing high biodegradability and extreme compatibility, with exhibition of great efficiency in the pretreatment of lignocellulose. On the other hand, nanofiltration technology could be used as a promising separation tool for the purification as well as recovery of succinic acid. After fermentation, succinic acid is one of the most abundant materials in microbial ectoplasm, and during overproduction than usual, it comes out in the fermentation-mixture volume. A cost-effective downstream separation and purification turns imminent for the manufacture of highly pure succinic acid, and to resolve this issue, membrane coupling steps in as a promising alternative. Modular design of this technology enhances flexible operation for any scale of production as per the market demands (Sosa et al. 2016; Law and Mohammad 2018). Membranes with high selectivity are able to ensure high separation and rejection to impurities and to receive pure and clean permeate. Such wisely selected membranes coupled with classical fermentation units can simultaneously produce and purify the desired product in a single process unit with compact design, where the capital cost could be significantly reduced. No involvement of any phase changing phenomena ensuring reduced energy and cost consumption can satisfy the requirements of process intensification.

17.2 Demands and Challenges for Succinic Acid Production

A 4-carbon dicarboxylic acid or Succinic acid (SA) is recognized conventionally as amber acid in Europe for decades which used to be employed for healing process (Saxena et al. 2017). At present, succinic acid is accepted as the building block of

numerous vital compounds used in food, chemical and pharmaceutical conglomerates (Law et al. 2019). It contains huge prospective in the production of polybutylene succinate (PBS), surfactant and detergent, flavour and fragrance, and herbicide and fungicide along with food preservatives (Nam et al. 2012). In the near future, succinic acid can be an imperative forerunner for producing bio-based chemicals like recyclable plastic (e.g. PBS), polyester polyols, plasticisers and polyurethanes (a substitute of petroleum-based adipic acid) and 1,4-butanediol. A wide variety trade functions (about 56%), medical (16%), food (13%), and others (14%) take part in the process of a vast growth prospective and thrust of SA market (Jansen and van Gulik 2014; Ahn et al. 2016). Worldwide requirement of SA is about at 1.8 mil tonnes per year in 2017 which is assumed to increase to roughly to 2.5 mil tonnes by the end of 2020, displaying a growth rate of 39% between 2014 and 2020, and it is forecasted to boost by 30% in the near future, but only 30,000 to 50,000 tonnes is produced globally per year where only 10,000 tonnes is produced through renewable feedstock. According to current survey, the MRP of SA varies between US $6000 and $7000 per tonne in country by country. The rising price of the conservative feedstock and guidelines on conservative chemicals (petrochemical-based resource) are the major obstacles for the growth of the SA market. Nevertheless, the negative ecological effect of the conservative SA is predicted to improve the demand for bio-based SA internationally. Still only a minute proportion of the total demand is at present met up from those eco-friendly fermentative expertises which still doesn't get prerequisites for cheap pretreatment method of biowaste and effective downstream separation and purification. The key difficulty while evolving any sustainable technique is to achieve cost reduction while guaranteeing a green atmosphere. For downstream purification of SA, membrane technology could stand as a potential candidate where membranes are fabricated and tailored to ensure soaring level of selectivity that confirms higher purity throughout downstream processing.

17.3 Different Conventional Methods for Succinic Acid Production

17.3.1 Conventional Chemical Process

Conventionally, SA is produced by chemical production route, by oxidizing N-butane OR benzene. Thus, maleic anhydride is produced which, through hydrolytic reaction, generates maleic acid by contravention of molecular bonding. By further hydrogen addition reaction, maleic acid is lastly transformed into SA through the saturation of double bonds between carbon atoms (Zeikus et al. 1999). But, in fact, rigorously long, difficult and expensive downstream separation and purification stages are required to be integrated to manufacture commercial-grade SA. Such processing stages are costly, dependent on classical fuels and complex to handle which reckons non-sustainability for a long-term basis (Isar et al. 2006).

17.3.2 Biological Fermentation

Sugar-rich biomass like sugarcane bagasse (SB), beetroot bagasse (BB), wheats, bread wastes and several lignocellulosic resources could be employed as raw material feedstocks. Through the application of commercially available high concentrated costly enzymes or strong acid hydrolysis, the complex structures of lignin or cellulose could be cracked to transform into simple structured fermentation worth sugars. But, the same outcome could be achieved along with simple amino acids by the application of suitable species of fungi (Leung et al. 2012). Though the enzyme hydrolytic pathway is advantageous for its intrinsic features of non-inhibiting reactions with non-hazardous waste generation and low temperature and pressure operations, the high cost of the enzymes and extreme precautions during operation are the only bars against exhaustive implementation (Agbor et al. 2011). Filament structured fungi, e.g. *A. niger* or *R. oryzae*, stand as the most favourite species for solid state fermentation technology. While working with feedstock of fruit and vegetable waste, those species are able to produce more than 25 g/L simplest sugar. Following microbial fermentation with *A. succinogenes*, SA was generated with a yield of over 95% and a productivity of over 1.3 g/Lh (Dessie et al. 2018).

According to reports, producing SA using fermentation results in increased toxicity as the time passes. Chrysanthi et al. (2019) incorporated a method of fed-batch *Basfia succiniciproducens* SA fermentations using membrane electrolysis. Their process is to directly contact the broth with a hydroxide ion and hydrogen producing cathodes, thereby enabling the in situ extracting of succinate to hydrogen ions and oxygen making anode compartment where the volume is relatively low. There, acidification and precipitation of the succinate take place. The benefit of this procedure lies in producing the base for maintaining pH, while very less amount of external base is required. Recycling of the bacterial cells takes place from the cathode compartment followed by its exposure to H2 and creation of biological reducing power. The execution of fermentation was done by means of glucoses, xyloses and ultrafiltered spent sulphite liquor (SSL), while acidic sulphite pulping of *Eucalyptus globulus* wood generates the side stream. Since the membrane was impermeable to cells, colour and solids, the result represented a combination of succinate extraction, clarifications, acidifications and concentrations—all of these in just one unit operation. Ratio of SA with by-product augmented, supporting creation of SA over lactics, formics and acetic acids, with almost 15% additional full sugar to SA conversion production. The OH^- yield because of H2O reduction showed inferior NaOH handling (up to 33% less) for sustaining the pH for duration of fermentation. The utmost output also augmented by 30% to a rate of 0.41 g/L h with electrochemical system, with 1.65 kW hr. per kg SA extracted for SSL and 2.4 and 1.9 kW h/kg SA extracted for glucose and xylose, respectively. The authors, bearing in mind the aforesaid advantages, suggested that their system could be used as a step forward in technology to achieve sustainability in commercial SA manufacture from rudimentary industrial side streams.

17.3.3 Enzymatic Process

This approach enzymatic entrapment, which is cost-effective and provides excellent process consistency, might be used to repurpose leftover biological feed or enzymatic bio-reaction boosters. Moreover, this technology provides high benefit in hassle-free downstream along with improvement of the mechanical stability and thermo-chemical resistivity of the biocatalysts (Galaction et al. 2011). A variety of enzymatic entrapments could be developed using a polyelectrolyte, calcium alginates, polyvinyl alcohols, carrageenans, collagens, chitosans, glycidyl ester copolymers, nonwoven fabric, polyurethane foam, sponges, pearlite and activated carbons (Pal et al. 2016; Kumar et al. 2019). Repetitive batch-fermentation employing C. glutamicums restrained in polyurethane fillers on hydrolysate of cassava bagasse produced about 35 g/L simplest carbohydrate, from which SA was produced with a yield of 71% and output rate of 0.42 g/Lh (Shi et al. 2014; Dessie et al. 2018). Different metabolic pathway for the synthesis of succinic acid is summarized in Fig. 17.1.

17.4 Different Non-Conventional Methods for Succinic Acid Production and Purification

17.4.1 Electro-Membrane-Based Hybrid System

Handojo et al. (2019) studied the revival of organic acid (OA) from electro-membrane routes. The fundamental goal of their research was to find low-cost OA manufacturing and recovery strategies. The authors investigated electromembrane (EM) methods, namely electrodialysis, electrometathesis, electro-ion substitution, electro-electrodialysis, electrodialysis with bipolar membrane, and electrodeionization which are primarily the most potentially viable technologies to recover OA. Electromembrane routes employ the concept of electromigration of ions using ion-exchange (IO) membranes which allows the recovery of different types of organic acids yielding higher percentage with minimal energy utilization. Moreover, the recovered acid can be maximized by integrating it with fermentation process.

Adeel et al. (2019) investigated the technoeconomic feasibility of a SA bio-refinery which also produced acetic acid and dimethyl ether. Carbon negative technologies which produce chemicals are the need of the hour as they can lessen the disastrous consequences of climate change. The carbon-negative technology used in the bio-refineries that produce SA. However, they leave a huge by-product of hemicellulose and gypsum in the waste. The authors investigated the technoeconomic feasibility of the same while the by-product which is hemicelluloses (HM) is changed to acetic acid (AA) and dimethyl ether (DME), avoiding gypsum creation. The feedstock being SA for recyclable plastic, the products namely acetic acid (AA) substitute fossil derived resource, while DME can be used as energy storing entity. The authors derived a technology principally underlying in a pioneering incorporation of marketable technologies counting water-splitting bipolar

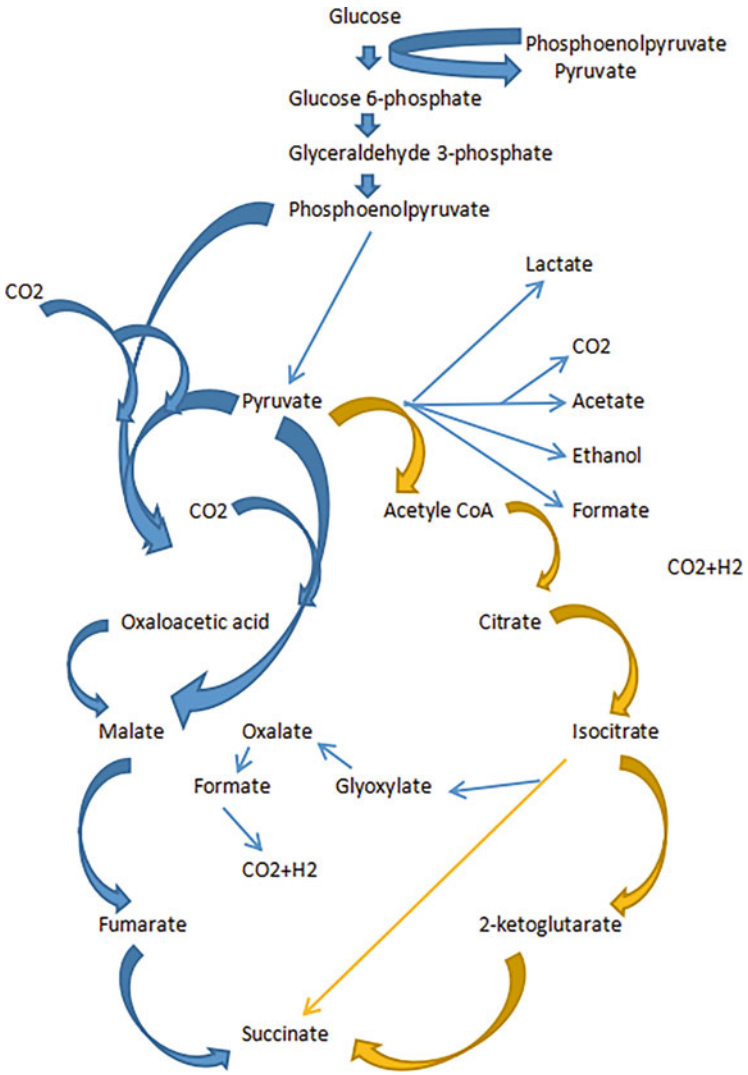

Fig. 17.1 Different metabolic pathway for succinic acid synthesis (Nhuan et al. 2017)

membrane electrodialysis for acid purification. The moulded many-product bio-refinery (Multi Case) yearly use 650,000 tonnes pulp logs, 135,000 tonnes CH3OH, 1,700,000 tonnes H2O, 42,000 tonnes carbon dioxide and 89,000 KW energy to make 220,000 tonnes SA, 115000 tonnes AA and 900 tonnes DME. All the scrounging electrical energy and heat duty is looked after inside the bio-refinery. Their findings showed a "CAPEX" of AUD $635,000,000, "OPEX" of $180,000,000 and a SA Minimum Selling Price of $990per tonne. Sensitivity and

uncertainty analyses of the Multi Case bio-refinery model show it is also flexible with cost variations.

17.4.2 Membrane-Based Hybrid System

Jeng et al. (2019) used forward osmosis-assisted crystallization process for recovering SA starting from fermentation broths using FO-assisted crystallization route. The authors used osmotically driven forward osmosis (FO) before crystallization process while performing downstream revival of bio-based SA. They used activated carbon to preheat fermentation broth which contained SA. This was followed by addition of powdered activated carbon (PAC) which demonstrated efficiency for glucose, formic acid (FA), and colour exclusion keeping the concentrations of SA constant. This was followed by Forward Osmosis to concentrate the untreated and treated fermentation broths. The process displayed a notable improvement of concentration factor (CF) next to 3.9 times for the treated broth, therefore ensuing a final SA conc. of 111.26 grams per litre. Distinctively, superior flux loss and inferior CF was noticed for unused broth, chiefly because of unfavourable result of high membrane fouls and cake layer development. SA crystals were after that effectively recuperated as of forward osmosis concentrated broth in concluding crystallization stage. The clarity and production amount of SA crystals were 90.52% and 67.09%, in that order for treated broth. The authors' effort established the progress of practicable FO-crystallization route for recovering bio-based SA.

Alexandri et al. (2019) used spent sulphite liquor as feedstock for downstream severance and refinement of SA from fermentation. They used spent sulphite liquor (SSL) formed as side-stream as of sulphite pulping of Eucalyptus globulus hardwood for separating lignosulphonate via nanofiltration in the leftover stream and SA manufacture by means of fermentation of the permeate course via *Actinobacillus succinogenes* or *Basfia succiniciproducens*. The possible addition of the procedure in conservative pulp mills in the direction of the advance of a new bio-refinery is reliant on the competent downstream partition of SA crystals at soaring yield and clarity. Their investigation focusses on the assessment of five downstream division processes, that is to say Ca precipitation, direct crystallization by means of acidification or cation-exchange resin, salting-out plus reactive extraction, for the refinement of SA as of basic fermentation broths. Reactive drawing out by means of trioctylamine in 1-hexanol and direct crystallization along with cation-exchange resins showed the way to SA recovery yielding about 73% and 79%, respectively. The authors also performed 1H-NMR analysis which indicated that those downstream division procedures lead to SA crystal purities of ca 98.5% for reactive removal and more than 99% for the direct crystallization technique together with cation-exchange resins with no obvious AA contents when recrystallization was in use. They also established that SA created by means of fermentation by side stream from pulp and paper mills possibly will be alienated at high clarity and yield from

basic fermentation broth depicting its exploitation for poly(butylene succinate) manufacture as feasible.

Li et al. (2010a, b) sidelined a single step method for recovering SA from fermentation broth through crystallization. SA is a precious 4-carbon platform chemical with broad functions in numerous areas. The authors investigated a single step revival of the preferred produce SA crystal via fermentation broth. Using fed-batch fermentation by Actinobacillus succinogenes BE-1, the concluding concentration of SA, formic acid, LA and AA in the bio-reactor reached 97.80 g/L, 23.50 g/L, 5.10 g/L and 17.40 g/L, in that order. The following acids contain dissimilar extents of dissociated and associated forms at diverse pHs, while the solubilities of these kinds of acids is dissimilar considerably. While the pH of the fermentation broth was controlled to be lesser than 2.0, crystallization of SA possibly will be done at 4 °C without difficulty, whereas chief acid by-products like LA, AA and FA could be easily mixed and made into a solution. Using this single step method, SA production was 70%, while its purity came to be 90%. Compared to that, for conventional Ca precipitation coupled ion-exchange adsorption, the yield and purity were 52% and 92%, respectively. Crystallization might be looked upon as being the last purification stage along with being the primary recovery stage for downstream partition route.

Antczak et al. (2019) employed bipolar membrane electrodialysis (EDBM) to separate and concentrate SA commencing from post-fermentation broth. They investigated how to separate and concentrate SA from 3-component representation solution and definite post-fermentation broth subsequent to pretreatment method in a ten-chamber large-scale EDBM system. Effect of current density and original concentration of succinate in diluate compartment was assessed by them bearing in mind parameters like closing SA concentration, current effectiveness and energy consumption per kg SA manufactured. In accordance with the outcome, the max conc. of the acid in concentrate compartment along with max current effectiveness being 75.4% after 180 min of method was attained while the current density along with original conc. of SA salt in the diluate compartment was 120 amperes per square metres and 200 g/L in that order. Their outcomes suggested that it's promising to employ EDBM route as a stride for separating and concentrating SA from definite post-fermentation broth subsequent to biotechnological alteration of glycerol. Furthermore, the relevance of EDBM procedure permits the conc. of SA and additionally conversion of salt to acidic form.

Orjuela et al. (2011) tried to recuperate SA using a novel procedure from fermentation broth by means of acidification and esterification in C_2H_5OH. Acid salt commencing from fermentation were positioned in C_2H_5OH together with a minor stoichiometric surplus of H_2SO_4. Concurrent acidification and esterification occurred, through inorganic sulphate salt created precipitating from C_2H_5OH solution. The succinate was then recovered like a solution of free SA, monoethyl-succinate, along with diethyl-succinate in C_2H_5OH, a mixture appropriate for additional esterification by the use of reactive distillation. They recovered succinate species almost equal to 95% for model succinate salt solution along with definite fermentation broth mixtures.

Luthfi et al. (2017) investigated SA recovery starting from aqueous solution and fermentation broth by means of polyimide nanofiltration membrane. They formulated a novel (PI) P84 NF membrane to prevail over separation shortcomings of bio-based SA recover process. They made PI membranes at various polymer conc. and thickness of the membrane. They investigated the intrinsic characteristics of those membranes by means of salt rejection method, Field Emission Scanning Electron Microscope, a porometer, Atomic Force Microscopy and Zeta Potential of the surface. The performance of the membranes for SA recovery rate was then studied commencing from an optimized fermentation broth along with an actual broth, comprising succinates, formates, and acetates. The outcome of a variety of environments of dissimilar feed conc., pressure, speed of stirring and diverse conc. Ratios of di-valent to mono-valent ion was inspected by the authors. The researchers created a 20 wt% PI membrane with an average pore size diameter of 0.23 nm and sodium sulphate rejection of 80.0 percent using the outcome. Their fabricated membrane displayed much higher succinate rejection of 89–96% with a simulated broth, while the elimination efficiency enhanced with rising pressures and lowering feed conc. In the meantime, succinate selectivity enhanced by 20.0–51.0% with increased speed of stirring and the ratio di-valent:mono-valent ionic solute conc. With the real broth, 92.0% succinate rejection was attained, that was at par with the rejection performance of the industrial membranes like NF1 membrane.

Prochaska et al. (2019) used two step method (membrane separation and reactive extraction) to remove SA from fermentation broth. The process is suggested to be ecologically feasible from model solutions along with the definite post-fermentation broth derived after bio-conversion of unprocessed glycerol (produced in a huge quantity as by-product for the duration of the manufacture of bio-diesel). An incorporated scheme to recognize this procedure was planned comprising UF, ED-BM and three-step reactive extraction (RE) with the help of industrial solvating extractants. The experimental set has been shown in Fig. 17.2. The authors used pre-clarification route using UF which removed elevated molecular pollutants found in feed solution, like bio-masses, protein and also bacteria cells. Noteworthy drop in permeate flux throughout the process was noticed because of foul of the UF membrane. Nevertheless, the fouled layer can be efficiently detached by using hydraulics along with chemical cleanings. Secondly, appliance of ED-BM route in the scheme permitted exclusion of acidification of broth which more often than not makes a substantial quantity of wastes. SA was detached in a stage reactive removal with over 90.0 percent efficacy in liquid streams following ED-BM. Extractions are able to sustain membranic methods to split well COOH groups as of the post-fermentation broth, yet, not sufficiently selective in splitting SA from further acid present in broths. The lone method to attain selective extraction SA over CH_3COOH, $C_3H_6O_3$ and $C_3H_8O_3$ is by reducing pH value to 2.0 and employ Cyanex 923 for using it as extractant.

A two-phase aqueous system for extracting (SA) produced by Actinobacillus succinogenes with fermentation broth was studied by Gao et al. (2016). A variety of hydrophilic solvents along with inorganic salts was applied to make a two-phase SA aqueous scheme, among which the two-phase C_3H_6O/NH_2SO_4 aqueous scheme

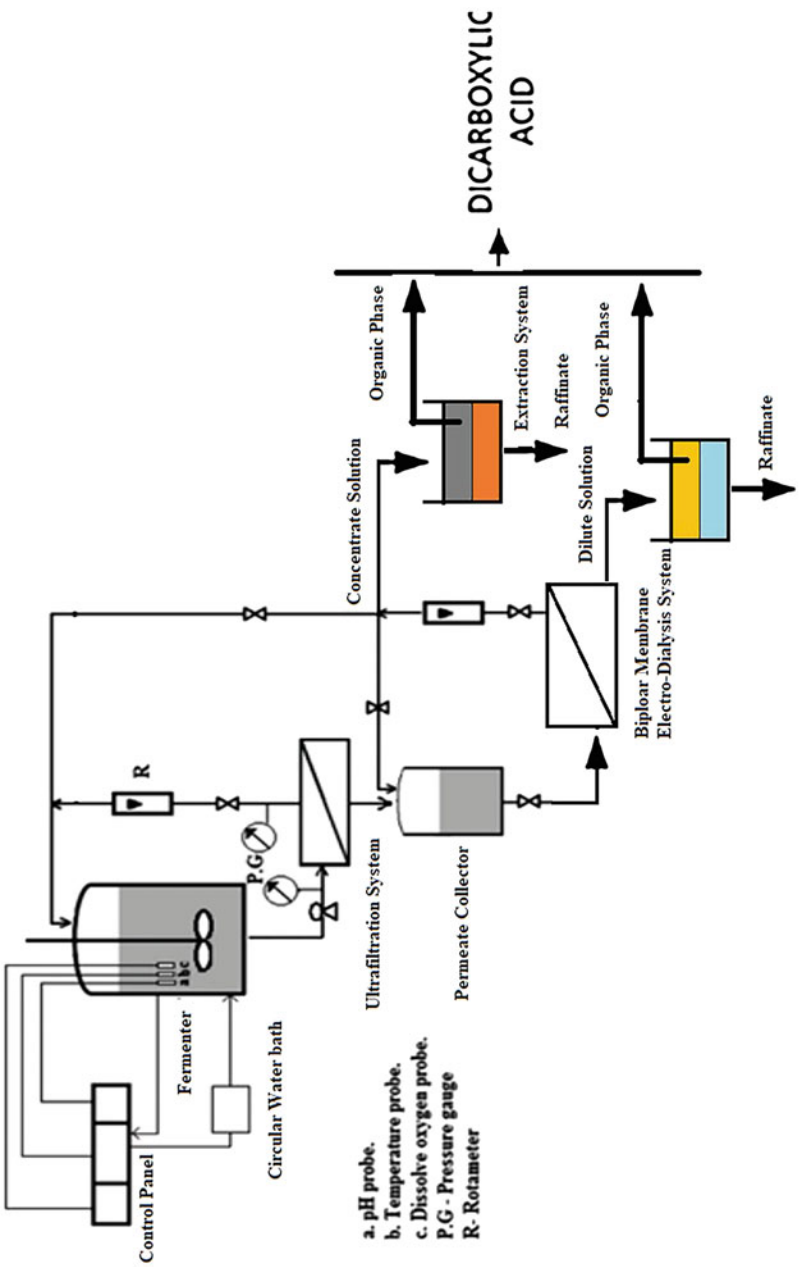

Fig. 17.2 Membrane-based extraction system used for succinic acid from fermentation broth

has been extensively examined, and also included phase drawing tests, phase composition results and pH in dividers, the elimination of cell along with protein from the fermentation broth, and the restoration of NH2SO4. Under the prepared environment, with a pH of 2.0 fermentation and a 2-part arrangement containing 35% acetone (wt/wt) and 15% (wt/wt) ammonium sulphate, the SA concentration in fermentation broth was −94.40% with the elimination of 93.60%, 98.1%, and 78.50% glucose, cell, and protein, in that order. South Africa's entire production was 77.30% and SA's efficiency was 98.70%. After adding CH3COOH, 95.90% of the NH2SO4 can be found in saline stage, indicating a small amount of waste disposal in South Africa through a two-phase water system.

A two-phase aqueous system for the extraction of succinic acid (SA) produced by *Actinobacillus succinogenes* from fermentation broth was studied by Pu et al. (2014). The alcohol content of sulphite has been tested in the ongoing culture of SA production using *Actinobacillus succinogenes* and *Basfia succiniciproducens*. Continuous concentrations were initially performed at a constant mixing rate (0.04 h^{-1}) with various commercial xylose variants (23–55 g/L) or continuous concentrations of xylose (40 g/L) and dilution levels 0.02–0.25 h^{-1}) indicating that the mixing levels of 0.02–0.15 h^{-1} resulted in satisfactory production of fatty acids in both species. In continuous cultures using nanofiltrated dissolved sulphite alcohol, the highest yield was obtained at a mixing rate of 0.02 h^{-1} (0.48 g/g of *A. succinogenes* and 0.55 g/g of *B. succiniciproducens*), while the highest yields were found in 0.04 h^{-1} culture values of *A. succinogenes* (0.67 g/Lh) and 0.1 h^{-1} *B. succiniciproducens* cultural (1.6 g/Lh). Metabolic flux analysis showed high biomass concentration in the cultures of *A. succinogenes* induced in both xylose and nanofiltrated synthetic alcohol sulphite, while *B. succiniciproducens* cultures were more potent in terms of efficacy of SA production in any xylose or nanofiltrated dissolved sulphite alcohol.

Soul sulphate alcohol (SSL) was employed by Dimitrios et al. (2018) like a carbon resource for producing SA by means of the excluded cultures of *Actinobacillus succinogenes* and *Basfia succiniciproducens* in two different bases, delignified cellulosic materials (DCM) and alginate beads. Fed-batch cultures immobilised *A. A. succinogenes* in alginates result in a higher sugar to SA converter yield (0.81 g/g) than when DCM ineffective cultures were used (0.65 g/g). The final extraction of SA and yield obtained from fed-batch by weak cultures of *B. succiniciproducens* in alginates (45 and 0.66 g/L) were greater than *A. succinogenes* incompatible culture (35.4 g/L and 0.61 g/g) using nanofiltrated SSL as fermentation unprotected cultures of *B. succiniciproducens* alginate beads recycled in successive batch-batch sequence nanofiltration SSL leading to the production of 64.7 g of SA with a conversion range of 0.42–0.67 g/g and a production range of 0.29–0.65 g/Lh. Immovable cultures improved the efficiency of SA production compared to free cell cultures.

Spul sulphite alcohol (SSL) was used by Maria et al. (2017) and tested in the ongoing culture for SA production using *Actinobacillus succinogenes* and *Basfia succiniciproducens*. Continuous concentrations were initially performed at a constant mixing rate (0.04 h^{-1}) with various commercial xylose variants (23–55 g/L) or

continuous concentrations of xylose (40 g/L) and dilution levels (0.02–0.25 h^{-1}) indicating that the mixing levels of 0.02–0.15 h^{-1} resulted in satisfactory production of fatty acids in both species. In continuous cultures using nanofiltrated dissolved sulphite alcohol, the highest yield was obtained at a mixing rate of 0.02 h1 (0.48 g/g of A. succinogenes and 0.55 g/g of B. succiniciproducens), while higher yields were obtained. The highest concentrations were obtained in *A. succinogenes* culture values of 0.04/h (0.67 g/Lh) and 0.1/h *B. succiniciproducens* culture values of 1.6 g/Lh. Metabolic flux analysis showed high biomass concentration in the cultures of *A. succinogenes* induced in both xylose and nanofiltrated synthetic alcohol sulphite, while *B. succiniciproducens* cultures were more potent in terms of efficacy of SA production in any xylose or nanofiltrated dissolved sulphite alcohol.

Spent sulphite alcohol (SSL) is used to produce lignosulphonates (LS), antioxidant and biologically created SA. SSL-only extraction by C3H8O resulted in a partition of roughly 80% of the whole LS contents, while fermentation using isopropanol-based SSL resulted during producing approximately 19 g/L of SA by *Actinobacillus succinogenes* and *Basfia succiniciproducens*. SSL separation by nanofiltration for separating LS and extracting the solvent by the use of C4H8O2 to split phenolic compounds created a rich sugar precipitation that resulted in the manufacture of 39 g/L of SA using *B. succiniciproducens.* The above separation process has also led to the creation of 32.40 g LS and 1.15 g phenolic-rich extraction per 100 g of S.S.L. Both of these therapies have removed a lot of heavy metal and heavy metal. This novel concept of bio-refinery can be incorporated into acidic sulphite pulping mills.

Ultrafiltration and nanofiltration of the alcohol sulphite (S.S.L) were used by Chrysanthi et al. (2016a, b) to test the concurrent manufacture of lignosulphonates and bio-based SA using *Actinobacillus succinogenes* and *Basfia succiniciproducens*. Decreases made in layers of 10, 5 and 3 kDa weight loss results in significant loss of lignosulphonates (26–50%) in full dispersal, while nanofiltration uses a layer of 500 Da cut-off sound effects resulting in high fruit retention lignosulphonates (95.60%) in recurring broadcasts. Fed-batch bio-reactor culture by means of fillers from unadulterated SSL showed in similar concentrations of SA (27.50 g/L) and production (0.40 g/Lh) at all both the pressures. In the presence of non-nanofiltrated SSL, type *B. succiniciproducens* produces high levels of SA (33.80 g/L), production (0.58 g/g of sugar utilized), and production (0.50 g/Lh) when grown in permeates. From 1000 kg of sulphite-containing alcohol, more than 300 kg lignosulphonates and about 53 kg of SA was recovered by nanofiltration. But, employing a 3 kDa ultrafiltration membrane for filtration of same volume of feed produced 237 kg and 72 kg of respective products.

Sun et al. (2014) investigated an experimentation where the principle of sugar extracting crystal forming was used for SA downstream and refinement. Tert-butanol/glucose mix indicated the expected predominance in the partition behaviour of all the components. The optimum parameters impacting the extraction effectiveness, like tie line length, acidity, temperature and constituent concentrations, were assessed. Outcomes demonstrated that partition coefficient of succinic acid increased linearly as tie line length increased, and firmly rely upon the acidity. 88% of succinic

acid and 97% of t-butanol were conveyed into the top stage while 94.69% of glucose into the bottom stage at a system comprising 27% (w/w) glucose and 40% (w/w) t-butanol under pH 3.0 and temperature 25 °C. There is a strong influence of SA concentration on its partition behaviour because of its wide range of phase creation. It has been observed that the temperature influences the t-butanol/glucose system, but doesn't have any significant role for partition of succinic acid. In that extraction system, by the addition of ammonium sulphate salt (1% to 9% by weight), showed higher recovery of succinic acid (87% to 90%). Applying combination of sugar extraction with gradient crystal formation process, SA yield 73% with 98% purity were achieved which proves it to be an efficient and economical methodology for purification of biologically derived succinic acid.

The most efficient natural producer of SA is *Actinobacillus succinogenes*, a wild microbial species isolated from bovine rumen (Chrysanthi et al. 2016a, b). The factors which are influencing the bio-transformation of SA by *A. succinogenes* were studied. The type of the raw materials, culture conditions, significance of carbon dioxide availability and downstream separation and purification plays an important role in determination of SA production. According to the metabolic abilities and genome analysis that led to SA synthesis, the mutant *A. succinogenes* species may present a better potential in the not-too-distant future.

Li et al. (2010a, b) achieved high and economic yield of SA production by *Actinobacillus succinogenes*; a commercial bio-production technique using lignocellulose-based hydrolysate, waste yeast hydrolysate and mixed alkali was developed. The referred ingredients were used as raw material for carbon, nitrogen and alkaline sources, respectively. In addition, it is a novel method which was first used for pH regulation using mixed alkali ($Mg(OH)_2$ and NaOH instead of expensive MgCO3 for SA production. Through this process, about 57 g/L SA with 73% product yield was achieved. The economics of the bio-reaction was decreased by 56% as compared to the present conventional method.

An alternative approach has been developed by Foulet et al. (2019) using renewable resources to overcome non-living supply exhaustion with different ecological concerns caused by the technologies using classical fuel-dependent chemical manufacturing. The organic wastes and agro-wastes could be lucrative substitutes which will also promote environmental responsible practices.

To manufacture different types of bio-products from waste biomass, BIORARE technology stands out to an extremely cutting edge one which combines a non-aerobic digestion processing plant with biological electrosynthesis. Though the biological electrosynthesis process is still immature and yet to be implemented, it confirms high consistency as an emerging technique with high sustainability. The concept of such eco-friendly scheme is founded on the life cycle assessment (LCA) approach and further it was coupled with sensitivity analysis for LCA of bio SA manufacturing process. The main approach confirms in identification and adjustment of complex to turn the approach as highly environment-friendly one, with critically good economic and time management efficacy in commercial scale. The current density applied during the bioelectrosynthesis and the hydrolysis yield during the pretreatment of the waste stream plays an important role in the optimization between

the production efficiency and environmental footprint. The eco-efficiency ratio was applied for the detail study of environmental efficiency of the process. Despite a number of disadvantages, the overall eco-friendly efficacy of the BIORARE technology could be observed as compared to the current scenario for the production of SA, as demonstrated in this study. This technique was also employed for bioethanol manufacturing process.

Of late, SA has been widely researched due to increasing demand in speciality chemical industries. The biosynthesis of SA from biomass is advantages from the environmental point of view. The transformation of sweet potato waste (SPW) to SA may lead to high-value exploitation of biomass and cutting down the cost of fermentation process also reducing the risk of polluting the environment (Meihua et al. 2019). Engineered *Escherichia coli* (*E. coli*) strain HD134 under the control of anaerobically induced nirB promoter from *Salmonella enterica* (PSnirB) could produce about 16.30 g/L SA with a yield of 0.83 g/g after 48 h on glucose. By using SPW hydrolysate as the substrate, 18.65 g/L SA with a yield of 0.94 g/g after 48 h fermentation was achieved. Compared to SD134 under Trc control induced with Isopropyl β-D-Thiogalactoside (IPTG), this concentration and yield represented an 8.56% and 6.82% increase, respectively. The use of anaerobically induced PSnirB not only attains higher production of SA than IPTG-induced Trc promoter, but also cuts down the cost of expensive exogenous inducers. In the recent study, it was observed that by anaerobically induced PSnirBcontrol the efficient production of SA was achieved from SPW. It was relatively a low-cost method as compared to other techniques where glucose was used as substrate and IPTG as the inducer. Succinogenes is likely to be contaminated by other lactate producing bacteria. This contamination will result in decrease of stability of *A. succinogenes* and makes the fermentation process difficult. The use of xylose as the source of carbon in the present study was employed to rescue the contamination of *A. succinogenes* culture, where the contaminant was shown to be an abundant Staphylococcus epidermidis. The continuous fermentation process was carried out using packed bed reactor verifying the stability of purified *A. succinogenes* and also the performance of acid production was studied. Continuous fermentation process employed $MgCO_3$ as the CO_2 source and pH buffer, resulting in clogging of the packed bed, which was later remedied by the addition of oleyl alcohol to the mixture.This has also enhanced the operation time from 344 to 1788 h. The usage of 30 g/L glucose and 40 g/L xylose as carbon sources resulted in a main metabolite yield of 91.7 $\pm$ 3.2% lactate and 55.3 $\pm$ 3.0% succinate with a stable productivity of 16.9 $\pm$ 1.7 and 1.5 $\pm$ 0.1 g/Lh.

17.5 Conclusion

Based on the exhaustive literature review-based analysis, it could be culminated that, fermentation-based processes coupled with membrane technology would be a preferred pathway for the production of bioactive compounds like succinic acid. Fermentation of bio-feedstock helps to produce desired product in an environmentally benign green pathway. Moreover, integration of membrane technology along

with suitable tailor made membranes of working regimes in wisely developed membrane modules confirms the complete mitigation of the impurities. However, substantial tuning and intensive experimentation at the main bench-scale are required before such systems may be scaled up to the pilot-scale and industrial-scale levels of operation. Excellent scopes are still left towards mathematical modelling-simulation, and optimization of different non-conventional systems aimed towards predictive outcome generation and advanced operational control. A primary economic analysis is always preferred to establish the novelty of such processes to gain the scale-up confidence for practical implementation from green field to commissioning. Extensive review points out that still there is a huge gap in all the referred issues and are critically needed to be fulfilled. Till-date, though different approaches were taken forward towards the development of novel processes for non-conventional systems, the reports are extremely scanty in number regarding the development of a complete process containing all the aspects of process development, optimization and scale-up. Membrane-integrated-fermentation techniques accomplish all the ways of sustainable production and purification of bioactive compounds, which is required to be critically studied, optimized and scaled up for bio-processing and bio-refining such novel materials.

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Recent Advancements and Challenges in Supercritical Fluid Extraction Methods and Their Applications in Food Industries

18

Bharat Singh Irom and Nishant Rachayya Swami Hulle

Abstract

Supercritical fluid extraction (SFE) is a green extraction technology, which is looked as an alternative to chemical solvents in separation processes. SFE has been effectively used for the extraction of bioactive compounds from plant sources, and a number of commercial-scale units are now available. The extracts obtained from SFE are free from chemical residues and are utilized in food formulations. Apart from extraction, SFE also can be applied for the transformation of extracts into different forms using the concepts of particle formation and extrusion. The chapter attempts to summarize recent developments in SFE with emphasis on food industry applications in fruit and vegetables, spices and oleoresins, coffee and tea, marine, dairy microalgae and other applications of SFE with industrial potential.

Keywords

Supercritical fluid · Bioactives · Co-solvents · Carbon dioxide · Food industry · Extraction

18.1 Introduction

The demand for food with good nutritional quality made from environmentally friendly process and natural ingredients is increasing across the world. Using green technologies for extracting plant or non-plant bioactive ingredients from natural sources is one of the sustainable ways of ingredient production. Natural

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extracts obtained from these processing technologies have huge demand in food formulations and pharmaceutical and other sectors.

Supercritical fluid extraction (SFE) is one such green technology in which extraction fluids are used as solvent for extraction, and after extraction above their critical temperature and critical pressure, the solvent is converted to normal atmospheric conditions in gaseous form, leaving behind extracts with no residues. However, extracting or separating natural ingredients from biological and plant sources is a complex process and requires a lot of experimental trials and understanding of the chemistry of interaction of different components of the materials before processing. Due to its superior quality extracts, it is looked upon as an alternative to distillation, chemical solvent extraction, and microwave-assisted extraction (Molino et al. 2020). The conventional techniques used for extraction possess disadvantages like thermal degradation and solvent residues which are of concerns of food industry applications.

The industrial utilization of this technology has been reported since the 1970s for caffeine separation from tea and coffee, cooking oil refining, flavor and pungencies from spices, and other plant components. Due to its wide popularity and demand, the advanced supercritical extraction systems were developed keeping in view the commercial, environmental, and safety aspect of the extracts. The chapter reflects on recent advances and scientific results on potential applications of SFE systems in food processing domain and different areas for possible commercialization (Fig. 18.1).

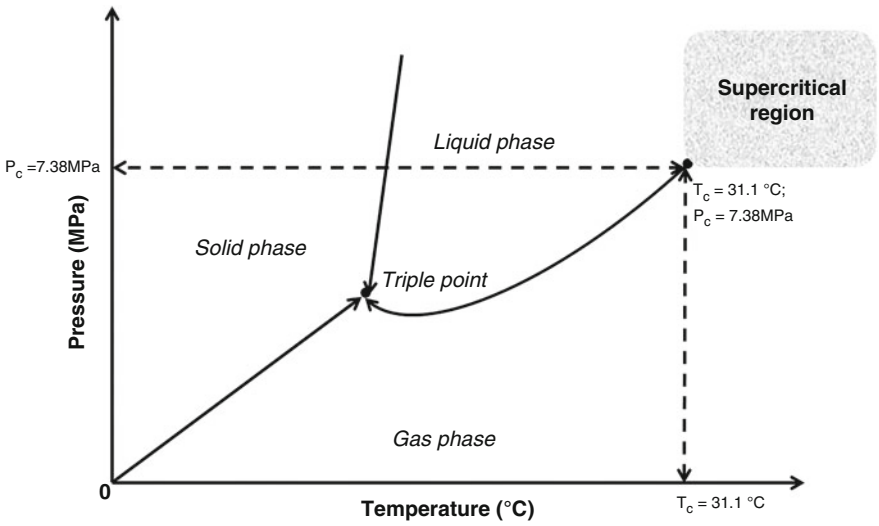

Fig. 18.1 Carbon dioxide supercritical pressure-temperature diagram

18.2 Carbon Dioxide as Supercritical Fluid Extraction

Some fluids like ethylene, methane, nitrogen, fluorocarbons, etc. can be used as supercritical fluids, however, CO_2 is more commonly used because it is low cost, innocuous or non-toxic, non-flammable, non-corrosive, and easy to control, allowing operation at low pressure and at room temperature. Carbon dioxide has been experimentally investigated and reported to have a negligible effect on the chemical properties of bioactive constituents (Rozzi and Singh 2002).

SC-CO_2 is a widely accepted option to replace organic solvents because it is capable of solubilizing lipophilic compounds and is readily removable from the finished products; it is therefore harmless, non-explosive, and economical relative to other solvents. CO_2 is, above all, "generally recognized as safe" by the US and European regulatory agencies. Carbon dioxide is abundantly available naturally and generated in many chemical industries as a by-product; hence, it has less environmental impact. In the recent decade, there have been huge developments in the extraction techniques and instrumentations, which has further eased the use of SFE as extraction. There are now systems available wherein CO_2 can be recycled, which further reduced the environmental emissions and helps in cost reductions (Da Silva et al. 2016).

18.3 Supercritical Fluid Extraction (SFE) Principle

The solubility of target compound is the main driving factor for extraction, and it further depends on the solute-solvent interactions. With the alteration of pressure and temperature, the SFE method can modify the solvent property such as CO_2 can satisfy the extraction requirement of the target compounds.

The extracting ability of the solvent in SFE is high because the solvents show both liquid- and gas-like properties. Higher diffusion rate and reduced viscosity give a desirable penetrating power in the solid matter, and its higher density gives it a higher solvation power (Reverchon et al. 2000). Rising pressure results in liquid-like CO_2 density, thereby increasing the probability of solutes and solvent interaction. However, temperature elevation contributes to the reduction in the density of SC-CO_2 and corresponding increase in the vapor pressure of the solute.

The reduction in the density of SC-CO_2 is more noticeable at lower pressures around the critical point, so that an increase in temperature results in a reduction in solubility, but the vapor pressure effect rules over at elevated pressures as the decrease in density is relatively small, leading to improved solubility (Temelli et al. 2012). The increase of the extracting temperature gives higher solubility of the target components, but it expands the concern over the stability of bioactive extracts as it may degrade the bioactivity of the natural extract when exposed to high operating temperatures (Ye et al. 2019). Further, the solute solubility in SC-CO_2 is impacted due to the interaction of solute and solvent like hydrogen bonding.

18.4 Modifier or Co-Solvent

SC-CO_2 is non-polar in nature, and non-polar compounds are solubilized in SC-CO_2 faster than the polar components of a comparable molecular weight. Even though SC-CO_2 is mainly used as an extracting solvent, its polarity can be altered by the addition of polar solvents (ethanol, ethanol-water mixtures) as modifier, which can also be termed as co-solvent. Using this method, selective extraction can be achieved, and only desired compounds are extracted. Ethanol has been the co-solvent commonly used in food applications since it is considered as a safe solvent for food processing due to its non-toxicity and CO_2 miscibility. There are several studies and reports available on utilization of ethanol while extraction of biomolecules for food processing applications (Chai et al. 2020; Wenzel et al. 2020; Caballero et al. 2020).

18.5 Applications in Food Processing

The market for food ingredients free from chemical residues is increasing rapidly, and with the availability of advanced systems, it has become feasible for the industrial production of many ingredients (tea, coffee extracts, oleoresins, essential oils, flavors). The extracts have good commercial value and applications in food and nutraceutical. Standardizing the extraction of supercritical fluid for ingredients involves testing under various process conditions. To increase the extraction, yield different pre-treatments and variables are applied to raw materials like enzyme treatment (Passos et al. 2011), ultrasound (Vaeli et al. 2020), moisture (Idris and Markom 2020).

In several reported literature, the pressures varying from 15 to 40 MPa and temperatures varying from 40 to 80 °C have been used in most SC-CO_2 extraction methods to remove natural products or bioactive compounds. For the extraction of bioactive ingredients in food and pharmaceutical grade, co-solvents are used in the range of 3% and 20% to improve the extracting efficiency in SC-CO_2. It is also reported by many studies that the optimum conditions identified using laboratory setups are scalable and shown to perform well at pilot-scale and industrial-scale setup with minor variations (Kotnik et al. 2007; Ko et al. 2016; Salea et al. 2017; Martin et al. 2015; Hassim et al. 2019; Fernandez-Ponce et al. 2016).

18.5.1 Extraction from Fruits and Vegetables

Fruits and vegetables are abundant sources of bioactives, including vitamins, phenolics, edible oils, and pigments. During processing, enormous waste is produced as by-products (peels, seeds, pomace, etc.) that can be used as a substrate to recover the bioactives in supercritical fluid extraction. The volatile compounds obtained from fruit extract can be added in different beverage formulations for enhancing flavor. They are also added to thermally processed fruit beverages or

concentrates after the thermal treatment to replenish the natural flavor compounds lost during the thermal processing at higher temperatures. Most of the compounds responsible for the flavor of the fruit juice (esters, alcohols, aldehydes, ketones, etc.) have good solubility in SC-CO_2, whereas water, salts, proteins, and sugars have less solubility which helps in the better extraction of flavor compounds. The stability of the volatile compounds depends on the process variables, form of solvent, and machine design, during the extraction process. The condition of the raw material can also affect the extraction due to biochemical reactions like fermentation, enzymatic, degradation, etc.

Caballero et al. (2020) used SC-CO_2 on three distinct olive residues with 60 percent ethanol to improve the production of polyphenolic compounds from biomass, leaves, and pomace. Ethanol (60%) as a co-solvent was used at pressures 200, 250, and 300 bars. In order to achieve a high-activity antioxidant extract, with the major polyphenolic compounds like hydroxytyrosol (the prominent compound), chlorogenic acid, caffeic acid, and ferulic acid, samples extracted at 30 MPa showed the highest values. Pavlić et al. (2020) used SFE to extract raspberry seed oil and developed kinetic models for variables including strain, flow rate of solvent, temperature, and mean particle size. They optimized processing conditions by applying artificial neural network (ANN) and reported optimum conditions as 35 MPa/40 °C and 0.4 kg SC-CO_2/h flow rate and mean particle size of 200–400 μm for maximum recovery.

Many fruit and vegetable waste include certain bioactive ingredients, such as carotenoids, which are commonly found in many fruit and vegetable waste, such as potato (sweet), apricot, tomato, pumpkin green, yellow and red pepper flesh and waste. The SFE can be used efficiently to remove such ingredients, which can be further used for different food applications (de Andrade et al. 2019). The effect of refined (oven-dried and freeze-dried) and unprocessed (fresh) apple pomace on the SC-CO2 extraction of phenolics and antioxidants was reported by Ferrentino et al. (2018). They observed that the extracts of freeze-dried apple pomace obtained from SFE had greater antioxidant activity than the traditional extracts. It was also confirmed that active polyphenols, as shown by HPLC-DAD-MS detection, were selectively extracted by SFE.

During supercritical fluid extraction, co-solvents can be used to remove polyphenolic compounds. The SC-CO_2 extraction of cacao pod husk was analyzed by Valadez-Carmona et al. (2018), and the standardized extraction conditions were 60 °C/30 MPa and 14% ethanol, resulting in 0.5% yield, 13 mg GAE/g TPC extract (total phenolic content), and 0.213 mmol TE (Trolox equivalent)/g extract of TAA (total antioxidant activity). Da Porto and Natolino (2017) also studied grape seeds for polyphenol extraction using SC-CO_2 and aqueous ethanol (57% v/v) as a modifier at 40 °C. Optimal conditions were reported as 8 MPa/CO_2 flow rate of 6 kg/h with 20% co-solvent, and 7132 mg GAE (gallic acid equivalent)/100 g DM (dried matter) were reported in confirmation experiments.

In another study by Shrigod et al. (2017), optimum processing parameters for mint processing were reported as 48 °C/15.1 MPa/0.40 mm particle size/0.625 h extraction period, resulting in a 1.4% yield and about 1000 mg/100 g carvone

content of volatile oil extract. The quality of SC-CO_2 extract was higher than the hydrodistilled essential oil with higher carvone content. Przygoda and Wejnerowska (2015) extracted the oil from quinoa seeds (*Chenopodium quinoa* Willd.) with a maximum tocopherol concentration. They reported optimum process parameters for the extraction of oil enriched with tocopherol from the selected sample, i.e., seeds of quinoa as 18.5 MPa, 130 °C, and 3 h yielding tocopherol concentration of 336.0 mg/100 g which is 25.65%. The tocopherol content in SC-CO_2 was four times higher than the conventionally extracted samples with hexane.

In 2015, Maran and Priya extracted oil using SC-CO_2 from muskmelon seed (*Cucumis melo*) and reported optimum conditions as 44 MPa/49 °C/0.6 g/min CO_2/1.35 h extraction time leading to 48.11% oil recovery (Maran and Priya 2015). They found that the SFE approach, relative to the Soxhlet process, helped to minimize the rate of solvent usage and extraction time. It was also concluded that extracted oil has the characteristics of edible oil and should be used as a supplement to food oils. Chronopoulou et al. (2013) performed a study on grape pomace for extracting oleanolic acid using the SC-CO_2. They compared SC-CO_2 with solid-liquid extraction. They reported that the ratio of extraction yields was roughly 1/5 between the supercritical and solid-liquid extraction methods. When the sample was transferred to dry grape pomace, it creased up to 1/3 with the application of ethanol as a modifier. The findings showed that SC-CO_2 extraction was efficient with similar extraction yields with solid-liquid extraction in extracting oleanolic acid from grape pomace.

Generation of sub-micrometer particles of carotenoids from emulsions was attempted by Santos et al. (2012) using SC-CO_2. They regulated emulsion flow rate, surfactant/carrier content concentration, and pressure as process variables during the processing. Standardized conditions for the development of carotenoid suspension with 344–366 nm particle size, 34–89% encapsulation efficiency, and 0.02–15 degree of isomerization were recorded. They stated that the flow rate of emulsion affected the development of carotenoid suspensions, the quality of encapsulation, and the degree of isomerization; the final size of the particles was also impacted by the surfactant/carrier content concentration. The enrichment method of tocopherols and tocotrienols from rice germ oil was standardized by Ko et al. (2012) using SC-CO_2. They observed that the most efficient tocol enrichment was accomplished with esterified RGO (rice germ oil) at a 13.8 MPa/60 °C obtaining tocotrienol level of 1270 mg/100 g.

Arnáiz et al. (2011) used broccoli leaves for lipid extraction using SC-CO_2 and fractionation techniques. The best results were obtained at 60 °C, 30 MPa pressure, 3 mL/min (SC-CO_2 flow rate), and 1.5 h process time. Two different extracts of lipids were obtained by using only CO_2 and with 15% of methanol as modifier. They found a higher polyunsaturated 18:3 n-3 concentration in extracts of SC-CO2. The major fatty acids found in the extract were 18:3 n-3 (alpha-linolenic acid), 18:3 n-6 (linoleic acid), and palmitic (16:0). Jokić et al. (2010) separated soybean oil using SC-CO_2 with optimum operating parameters as 30 MPa of pressure, 50 °C, 1.629 L min^{-1}, and 4 h of extracting time by giving maximum yield of 6.59% of soybean oil. The kinetics modeling of the extracted oil was done by one-stage

diffusion model. This extraction method had produced higher level of linolenic acids and linoleic acids with minimal quantity saturated fatty acids when compared to the extracts produced by organic solvents.

Yi et al. (2009) while extracting lycopene using SC-CO_2 from tomato skins observed that the antioxidant activity in extract remained consistent below 70 °C, but due to the higher temperature, it steadily declined above 70 °C mainly due to isomerization. Optimum conditions were 100 °C/40 MPa pressure and solvent flow rate of 1.5 mL/min with a maximum yield of 31 μg/g raw tomato. The increase in temperature from 40 to 100 °C led to the alteration of the ratio of all-trans-lycopene, and the cis-isomers ranged from 1.7 to 1.3, showing an improved bioavailability attributed to the formation of the cis-isomers. Yepez et al. (2002) extracted essential oils from coriander (*Coriandrum sativum*) at SC-CO_2 conditions of 17.7 MPa and 45 °C and Ribeiro et al. (2001) from lemon balm (*Melissa officinalis* L.) at SC-CO_2 conditions of 35 °C and 10 MPa for 4 h. The extracts from coriander and lemon balm both reported to have high antioxidant activity. Baysal et al. (2000) extracted carotenoids from tomatoes by the application of SC-CO_2 with ethanol (5%) as entrainer, and optimum conditions reported were 2 h dynamic extraction at 4 kg/h, 30 MPa pressure, and 55 °C temperature.

18.5.2 Extraction of Flavors and Fragrances

The food and beverage industry prefers to use natural flavor and fragrance rather than synthetic compound for various applications in edible products. However, availability of natural extracts is limited and has higher prices which may lead to using synthetic chemical as flavor compounds. The essential oils largely give the flavor and fragrance to the plant extracts. They are volatile in nature and usually found in very low concentration in plants. Due to their volatile nature, the process conditions during the extraction need to be precisely controlled to retain their stability. Steam distillation is conventionally used to recover essential oil; however, many volatile compounds are lost in the distillation process, and some undergo degradation reactions like oxidation, hydrolysis, etc. SC-CO_2 extraction has shown potential to minimize such degradation reactions and achieve superior quality extracts.

A study was conducted on Zhenjiang aromatic vinegar using SC-CO_2 for recovering aroma compounds by Lu et al. (2011). The optimal conditions for extracting the desired aroma were reported to be 35 MPa/25 L/h of SC-CO_2/2 h/50 °C. They identified 49 aroma compounds from the vinegar and 44 compounds from its SC-CO_2 extract. It was concluded that the aroma from the vinegar could be recovered by SFE effectively (Lu et al. 2011).

Bhattacharjee et al. (2003) worked on the recovery of aroma constituents in basmati rice by comparison between the Likens-Nickerson extraction and SC-CO_2 fluid extraction. Optimum conditions for SFE were found to be 50 °C and 12 MPa for 2 h which have provided maximum volatile constituent extraction. They also found that the SC-CO_2 extract had close to the original basmati flavor as compared to

the Likens-Nickerson extraction technique. In a study reported by Reverchon and Poletto (1996) to extract volatile oils through fractionation of flower concrete on rose and tuberose (SFE), they reported that the quality obtained using SC-CO_2 extraction was better than that of the extract obtained by the traditional techniques.

18.5.3 Extraction of Spice Oils and Oleoresins

Spices are known for having strong flavor and aroma and used as condiments due to their characteristic sensory attributes and the preservation potential in food processing. Spices are grown globally and commercially traded in different forms. The oleoresin fraction of spices is responsible for taste and spiciness. Different methods are employed for the extraction of spices including hydrodistillation, solvent extraction, and SC-CO_2 extraction. Commercially, these fractions are available as spice oil, liquid or oleoresin, encapsulated form, or emulsion form of seasonings. SC-CO_2 is now commercially accepted for spice oils and oleoresin extraction. The advantage of using SC-CO_2 is the selectivity of extracting the desired compounds, and it has superior quality for blending with other compounds. However, the extraction of desired quality oleoresins requires understanding of the different factors like particle size, moisture content, and physiological stage of the raw material to achieve maximum extraction. The extracts obtained in SC-CO_2 generally contain mixture of essential oils, waxy compounds, triglycerides, and other compounds in small quantities. These compounds can be separated by collecting different fractions at variable times so that time can be optimized.

Oleoresin extraction with higher gingerol content using SC-CO_2 followed by fractionalization of dry ginger was studied by Shukla et al. (2019). The standardized process parameters were particle size 253 μm/40 °C/27.6 MPa pressure/30 g/min flow rate and 2.55 h dynamic extraction. They further reported that the optimum condition of SC-CO_2 fractionation resulted in the recovery of 96 wt% (operating at 17.5 MPa/40 °C) pure oleoresin and 95.94 wt % (operating at 4 MPa/40 °C) pure volatile oil. Dang and Phan (2014) standardize black pepper (*Piper nigrum* L.) oleoresin extraction of using SC-CO_2 and also evaluated the antioxidant capacity of the extract. The effects of process variables on the extract yield were analyzed using response surface method using central composite orthogonal design, and the optimum conditions were 26.6 MPa, 150 minutes of duration, and extracting temperature of 47 °C giving the yield of 5.33%. The piperine content of the oleoresin extracted ranged from 26% to 48%. They reported that the extract's high antioxidant potential was comparable to that of ascorbic acid in black pepper. Li et al. (2011) reported the method for extracting oil from red pepper seeds using SC-CO_2. The maximum yielding conditions were 27.17 MPa/46.67 °C/8.11 volume % of modifier giving the oil yield of 18.4%. A linear response with $>$0.9991 correlation coefficients was reported to give the free fatty acids (FFA) from extracted oils. The research concluded that this method of extraction had a strong potential to examine short- and long-chain FFA from edible oils.

Perakis et al. (2005) carried out a study for the separation of black pepper oil using SC-CO_2. It was reported in their study that the extraction rate was enhanced with pressure and decreasing temperature. Furthermore, the same effect occurred when solvent rate was increased. Linear driving force (LDF) approximation-based model and an extended Lack's plug flow model were used to analyze the obtained data. Catchpole et al. (2003) using SC-CO_2, dimethyl ether, and propane attempted to extract the selected pungent components from ginger, chili powder, and black pepper. To assess the pungency of the extracts, the NMR method was introduced. They reported highest yield in dimethyl ether solvent extract with pungent principles, while propane was the least effective solvent. Carbon dioxide and dimethyl ether gave similar yield of capsaicin content with approximately two times higher than samples extracted by propane. Piperine content of black pepper obtained using propane was lower compared to dimethyl ether and dioxide extracts.

18.5.4 Extraction of Coffee and Tea Compounds

Caffeine is a commonly available compound in tea and coffee and added to many beverage products. Overconsumption of caffeine leads to certain health conditions like palpitations, gastrointestinal disturbance, anxiety, escalated blood pressure, and so on (Ashihara and Crozier 2001; Mejia and Ramirez-Mares 2014). There have been studies conducted on the reduction of caffeine level in tea using different methods including hot water extraction, ultrasound, and solvent and SC-CO_2 extraction. However, while reducing the caffeine, other flavor compounds which are characteristics of tea may be lost which need to be taken care of. SC-CO_2 has shown potential in the extraction of caffeine from tea and coffee.

Sokmen et al. (2018) extracted caffeine from tea waste using SC-CO_2 wherein they reported optimum conditions to be 25 MPa/60 °C/3 h as pressure, temperature, and time combinations while keeping constant flow of ethanol (0.5 mL/min) during the extraction. Caragay (1981) conducted SC-CO_2 extraction experiments with coffee beans and reported operating conditions to be 7–22 MPa/31 °C. They reported that soaking treatment before the extraction improved the decaffeination process (Peker et al. 1992). Ilgaz et al. (2018) reported the complete extraction of caffeine from black tea leaves under SC-CO_2 conditions at 37.5 MPa/62.5 °C/5 h with CO_2 flow rate of 2 L/min and 5 mol% modifier concentration.

Hurtado-Benavides et al. (2016) separated the aromatic oil from roasted Colombian coffee beans. They achieved a maximum yield of 8.9% at optimum condition of 33 MPa and 36 °C. The major fatty acids reported were palmitic, linoleic, oleic, stearic, and arachidic. They found that the volatile coffee oil compounds primarily belonged to furan and pyrazine family and showed the characteristic flavor of the drink and roasted coffee beans.

18.5.5 Extraction from Marine Sources

The SC-CO_2 is effective in reducing the fish oil oxidation and removing impurities like arsenic. Rubio-Rodriguez et al. (2012) extracted eicosapentaenoic acid and docosahexaenoic acid using SC-CO_2; they reported optimum condition of 25 MPa/40 °C to perform coupled extraction-fractionation enhancing fish oil quality. The scallop viscera lipid had higher PUFA content mainly DHA and EPA. A technique was developed to extract lipids from the scallop viscera using enzyme-assisted method and supercritical method. The lipid recovery rate was 61% for enzyme-assisted method and 78% for SC-CO_2 extraction. The GC-MS analysis of the fatty acid and sterol and their compositions were found slightly different for the lipids extracted by both the extraction methods. It was concluded that this extraction technique could be used for extracting lipids from shellfish viscera; however, the low extraction yield indicated that further optimization methods were required to enhance the yield (Zhou et al. 2010).

Amiguet et al. (2012) recommended a technique to derive PUFAs from the production of shrimp by-products. Using SC-CO_2, they extracted omega-3 PUFA-rich oil at processing condition of 35 MPa/40 °C. They reported 137 mg of oil/g from dried shrimp waste with the main components of PUFAs were 8% DHA and 7.8% EPA. In another study, eicosapentaenoic acid (50% purity) and docosahexaenoic acid (90% purity) were extracted using supercritical fluid chromatography from tuna oil with SC-CO_2 as solvent. The study suggested that to produce 1000 kg DHA and 410 kg of EPA concentrates/year would require 160 kg of stationary phase with 2.6 tons/h carbon dioxide eluent recycle (Alkio et al. 2000). Hardardottir and Kinsella (1988) found that supercritical-CO_2 fluid extraction methods with and without ethanol recovered 97% and 78% of the lipids and 99.5% and 97% of cholesterol from trout muscle, respectively.

18.5.6 SFE in Dairy Products

The dairy processing industries are now looking after different ways to reduce the cholesterol content in the dairy fat. There have been various methods attempted, and SC-CO_2 is one of the methods for reducing the cholesterol in dairy products as reported by many researchers. Sánchez-Macías et al. (2013) reported the effect of different pressures during SFE on the two varieties of goat cheese, Majorero cheese and goat Gouda-type cheese. For Majorero cheese, they achieved fat percent reduction from 57 to 50% and 55 to 48% for goat Gouda-type cheeses. They found that sphingomyelin and phosphatidylcholine contents of Majorero cheese were higher than control and goat cheeses. However, the microbial load decreased in both cheeses after supercritical fluid extraction (SFE), and as pressure is elevated in Majorero cheese, the lethality was also increased, most significantly on *Lactococcus* and *Lactobacillus* bacteria. Ghosh et al. (2018) used SC-CO_2 to minimize the cholesterol content in dairy cream powder; the processing conditions used were 40–75 °C/10–25 MPa/2.5–3 h, and the optimum conditions obtained were 75 °C/

20.4 MPa/3.5 h with up to 39% cholesterol reduction and 10.6% total fat reduction. The SC-CO_2 has also been used as an alternative to HPP technology as it can be utilized in continuous systems for microbial and enzyme inactivation reducing the processing time and operating cost (Amaral et al. 2017). Zhong and Jin (2008) studied the SC-CO_2 effect on the functional properties of whey proteins, wherein they found that SC-CO_2-treated whey protein isolates (WPI) formed stinger gels; also the powders of WPI and whey protein concentrates (WPC) were free-flowing compared to the control samples.

18.5.7 SFE of Microalgae

Microalgae are photosynthetic organisms present widely in different environments and can grow in different aquatic environments. There have been several studies reporting potential benefits of microalgae as a food ingredient source. Microalgae are good source of carotenoids, astaxanthin, lutein, and fatty acids and find applications in functional and nutraceutical food products. SFE has been reported to extract bioactives from microalgae; the complex intracellular structures may affect the mass transfer of bioactives during extraction. This may be enhanced by using appropriate pre-treatments (chemical, enzymatics, mechanical, physical, etc.) prior to extraction so that the recovery of bioactives can be enhanced (Lorente et al. 2017; Kim et al. 2016). Sanzo et al. (2018) reported increased yield and extraction of astaxanthin and lutein at 55 MPa and 50 °C from *Haematococcus pluvialis* microalgae. They also mentioned that co-solvent further improved the extraction due to swelling of biomass which increased the surface area of contact and enhanced mass transfer due to hydrogen bonding with biomass compounds and enhanced the polarity of the solvent (Nobre et al. 2006).

18.5.8 Other Applications of SC-CO_2

Apart from the extraction as the primary aim of SFE process, however, recently, other applications in the transformation of extracts into different forms using concepts of particle formation and extrusion have been reported. The extracts can be encapsulated using SFE, wherein thermal degradation occurring in other encapsulation technologies can be prevented and also the particle characteristics can be precisely controlled. The control of particle size can be achieved by altering the pressure, temperature, and nozzle diameter (Temelli et al. 2012). The rapid expansion of SC-CO_2 is also reported to form particles of varying sizes and functionalities (Jung and Perrut 2001). Supercritical antisolvent (SAS) technology is another process which is used when the required solute is not soluble in SC-CO_2 but soluble in organic solvent. SAS technology may be used for controlling the particle morphology in varying ranges of particle size (Fahim et al. 2014).

SC-CO_2 has also been beneficial in processing operations like extrusion and drying. During extrusion process, it is used to impart plasticizing effect and

expansion of the extrudate with improved mechanical and physical properties. The benefits include processing at milder temperatures, which may help in preventing loss of heat-sensitive ingredients which otherwise is more in conventional extrusion process (Chauvet et al. 2017; Rizvi et al. 1995). SC-CO_2 has also been studied and found to improve the drying of food. The advantage includes simultaneous inactivation of microflora during drying (Bourdoux et al. 2018). Further, minimal shrinkage and structural changes are reported. The sensory quality of the SC-CO_2-dried products is also reported to be comparable with the freeze-dried products (Zambon et al. 2020).

18.6 Conclusion

Supercritical fluid extraction is a green technology with potential applications in many operations in food processing. The extraction of different biomolecules from plant sources has been a well-researched area and commercially now in practice. However, the extraction needs to be standardized for each product and the target compounds, which requires prior experience and experimentation to efficiently extract the target compounds. The published literature suggests that many laboratory-scale studies are helpful in determining the processing conditions at pilot- or commercial-scale operations. The supercritical fluid extraction has also shown other applications like particle formation, drying, extrusion, and preservation that may be helpful in food processing and ingredient innovations.

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Genetically Modified Food (GMF) and its Challenges

19

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Abstract

Within the next 35 years, the earth's global population is estimated to reach 9 billion. Satisfying all 9 billion people's nutritional requirements will be challenging by using conventional farming and breeding techniques. The main focus in this article will be on the significance, hazards, and consumer perception of genetically modified food (GMFs). Since GM food offers superior quality, gives a higher yield, and can be grown in a variety of environmental conditions compared to conventionally grown foods, they are the only way to meet the ever-growing food demand. Recombinant DNA technology is combining genes from different organisms, and the subsequent product is "transgenic" and genetically modified (GM). GMFs are produced by incorporating the desired genes into the host genome using techniques of genetic engineering and, thus, inducing desired changes in the host. However, the practice of biotechnology has also raised concerns about its potential risks to the environment as well as to human beings. Controversies and public concerns surrounding GM food mainly focus on human and environmental safety, consumer perception, ethics, and food security. Therefore, the production of GMF pays particular attention to the quick and lifelong effects due to GMF such as allergies, production of lethal agents, and endurance to antibiotics. In this paper, we have summarized up-to-date knowledge about the GMF production methodology, recent genetics-related technological developments, and its impact in the field of agriculture and have also addressed major topics such as safety and environmental concerns, health hazards, and religious issues regarding GMF.

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GMF · Genetics · Crops · Health benefits · Risk factors · CRISPR-Cas9

19.1 Introduction

Genetically modified food (GMF) has been a prevalent topic ever since we started witnessing advancements in DNA modification technology. WHO has defined GMF as a category of a food, which are developed via the insertion of new gene from another organism (World Health Organization n.d.). Introducing a new gene uses genetic engineering principles and alters the genome and its working in an organism. This is called a genetic modification. Genetic engineering has been a promising field for not only the pharmaceutical sector but also the agricultural industry. GMF scientists have promised genetically modified food to have a higher nutritional value, better texture, longer lifespan, virus resistance, and better flavor (Gatew et al. 2019).

Gene can be taken from a cell or artificially synthesized and combined with genetic elements, such as promoters, selectable markers, and terminators, and then inserted into the target genome. Here, genome editing employs four different families of nucleases such as meganucleases, TALENs, zinc finger nucleases, and CAS RNA systems for this process (Zhang et al. 2013).

GMF also promised substantial cost reductions for farmers. Mainly because GMF is designed to resist herbicides, hence, they would cut costs on pesticides majorly (Hug 2008). Also, herbicide-tolerant GMFs decrease soil erosion due to "no-till" agriculture and improve water quality by reducing sedimentation, runoff nitrogen, and phosphorus. No-till farming practices improve carbon storage, cut on-farm fuel consumption, and reduce greenhouse gas footprints (Friedrich and Kassam 2012).

Even though GMF has numerous health and economic benefits, it is not widely accepted and used worldwide. Currently, there are many debates, and it is a highly controversial topic. It is mainly related to the potential health risks and their adverse effects on the environment. Another factor under consideration is consumer acceptance. Consumers mainly express disbelief in GMF's increased benefits due to the lack of awareness and knowledge regarding the topic. It is currently unclear whether the consumer skepticism is due to GM producing techniques or GM safety. It has been observed that there are not enough studies regarding the public's acceptance of GMF. Very few studies have taken place regarding this, and significant studies only took place in metropolitan cities. Rural areas have not been taken much into account (Cui and Shoemaker 2018). The production of GMF also has religious objections. People are against GMF as many consider it as "playing with God's creation." They think modifying anything natural is going against God's will and is violating in nature (Gatew et al. 2019).

Apart from the concerns mentioned earlier, there are many risk factors involved in the production of GMF. The factor that is being modified here is a living organism. The organism can grow, can reproduce, and can interact with other organisms. Due

to this, there is always room for unexpected gene interactions, and the way it expresses will differ from person to person. This unpredictable nature of genetically modified organisms makes it even more dangerous than wet lab experiments that involve the use of chemicals. Apart from health risks, other risk factors such as allergic reactions and cancer risks were also studied due to GMF.

Apart from health factors, GMF also causes environmental problems such as antibiotic resistance, and it also poses a threat to biodiversity. GMF also increases autonomy and may even cause an increase in unemployment. GMF policies are also unfavorable in developing cities; thus, it may also lead to a rise in social differences. It is also a potential threat to farmers who want to grow non-GM crops as they may spread through pollen-mediated gene transfer or horizontal gene transfer (Hug 2008). Another major ethical issue involved in the production of GMF is animal testing. It has many controversies going on because, as explained by PETA, "They languish in pain, suffer from extreme frustration, ache with loneliness, and long to be free." They have further described that animal testing is simply an inhumane behavior and does not play a vital role in the experiment's scientific results (PETA n.d.). GMFs pose a potential ecological threat by increasing the minor pest population by reducing the major pests as they are insect-resistant plants. This shift in population disrupts the entire food chain (Bawa and Anilakumar 2013). GMFs are able to express viral or bacterial antigens in the plants, such as transgenic plants, that are capable of producing antibodies and also act as oral vaccines (Zhang et al. 2013).

Some of the genetically modified crops that are majorly studied:

1. Maize (*Zea mays*)
2. Rice (*Oryza sativa*)
3. Sugarcane (*Saccharum* spp.)
4. Tomato (*Lycopersicon esculentum*)
5. Eggplant (*Solanum melongena*)
6. Potato (*Solanum tuberosum*)
7. Wheat (*Triticum* spp.)
8. Bean (*Phaseolus vulgaris*).
9. Melon (*Cucumis melo*).
10. Soybean (*Glycine max* L.)

The future of agriculture is very promising once the world is more aware of GMFs' benefits and once it comes to accept the GM techniques of breeding crops (Oliver 2014). This review aims to examine all the issues and myths around genetically modified crops, the major benefits of GMF, the adverse effects of GMF and possible resolutions, and the extent to which GMF could be used for human service in the future.

19.2 How Are Genetically Modified Crops Synthesized?

The characteristics of an organism are defined by its genetic makeup, i.e., its genome. It contains the entire genetic information of the organism. The genome for living organisms is stored in DNA molecules called chromosomes. The place where genomes are stored differs in prokaryotes and eukaryotes. In prokaryotes, the genome is stored in the cytoplasm, whereas, in eukaryotes, the genome is contained within a membrane called the nucleus.

Genetic modification is the insertion of external DNA into the genome of an organism. This is done by using external sources such as plants, animals, and viruses. This genetic modification is aimed to change a specific trait of the plant. Advancements in biotechnology have enabled us to cross all barriers, and the exchange of genetic materials between organisms has been easier than ever.

The production of transgenic crops is a two-step process:

1. Transformation: The gene of interest has to be successfully inserted into the plant cell.
2. Regeneration of transgenic plant.

Transformation can be done in two methods: by using *Agrobacterium tumefaciens* method or by using the particle gun method (Hwang et al. 2015).

19.2.1 *Agrobacterium tumefaciens* Method

Agrobacterium tumefaciens is a natural genetic engineer used in the production of transgenic crops. It is a soil bacterium that infects plants at a site where the stem and the roots first meet. That site is called the crown. This bacterium causes rapid cell proliferation and induces crown gall disease in the plants. *A. tumefaciens* is able to work due to the presence of a tumor-inducing Ti plasmid. The two most essential components present in the Ti plasmid are the T-DNA and the virulence region. The T-DNA contains 25 base pair repeats at the ends of the repeat called border sequences, known as right border (RB) sequences and left border (LB) sequences. The rest of the plasmid contains virulence genes such as virA, virB, virC, virD, virE, virF, and virG (Ghimire 2017). The process starts when bacteria's receptors aid in recognizing and attaching to the plant cell. Once recognition and attachment have taken place, the bacterium infects the plant, and then the wounded plant releases chemical compounds.

A. tumefaciens recognizes the chemicals released. virA detects the signal and gets activated. It binds to the chemical compound. This interaction, in turn, activated virG via phosphorylation. This activated virG acts as a transcriptional activator for other vir genes such as virD1 and virD2. These virD genes act as endonucleases and cleave the border sequences present in the T-DNA. This results in a single-stranded T-DNA with virD2 attached to its end. T-DNA is then exported across the cell envelope of the host plant cell. This is done using a conjugation system called the type IV

secretion system (T4SS). Once the T-DNA is inside the nucleus, it gets integrated into the plant's genome. This integration of T-DNA into the nucleus is entirely. Any kind of specification does not mediate it. Then, it expresses specific encoded genes, some of which are responsible for the synthesis of plant hormones such as cytokinin and auxin. They are the genes responsible are the genes responsible for cell proliferation, tumor production, and opine production. The T-DNA also makes the plant produce "opines," a modified amino acid that serves as a nutritional source (carbon and nitrogen source) for the bacteria (Ghimire 2017).

Once the T-DNA is successfully incorporated into the plant's genome, the DNA modification part of the GMF production is complete. Lastly, it is bred just like other typical plants, and it grows normally, but once it is fully grown, the exhibited characteristics will differ from that of typical plants. This depends on the function of the chosen gene of interest inserted into the plant's nucleus.

19.2.2 Particle Gun Method

This method aims to introduce a new gene into the nucleus of a plant cell. In this method, the gene of interest does not need to be cloned into a unique transformation vector as we do with the *A. tumefaciens* method in order to be transformed into plant cells. This method uses microscopic gold or tungsten particles to deliver the gene of interest inside the nucleus (Oliver 2014). Numerous gold particles are coated with many copies of our gene of interest. Another gene called the marker gene is also inserted along with the gene of interest. This is inserted for easy identification of whether our target gene has been successfully transformed into the cell or not. This is done on a plastic disc present at the exit area of the firing piston. The coated gold particles are then accelerated at high air pressure and are shot at the plant cells such that the gene of interest penetrates the nucleus. There is a stopping plate just before the gene penetrates the plant cell. This stopping plate blocks the disc and allows only the DNA particles coated with gold or tungsten to penetrate. When a new gene is inserted into the chromosome, the chromosomal DNA separates the new gene's insertion without replacing existing genes. This can be compared to someone cutting a queue. Once the target gene is inside, it is a part of the cell's DNA and will grow, multiply, and be passed onto the offspring (Ghimire 2017).

The *A. tumefaciens*-mediated gene transfer method is more widely used and is a comparatively more popular gene transfer method than the particle gun method. This bombardment process causes loss of integrity of the DNA because the target gene is shot into the nucleus at the high air pressure, and there are also size limitations on the target gene of interest. On the other hand, the *A. tumefaciens*-mediated gene transfer method has several advantages such as it is easy to use, it is not too expensive and results in a low copy number, and, most importantly, the integrity of the DNA is not lost when this method is used. Due to these reasons, the *A. tumefaciens* method is more popular (Ghimire 2017).

19.3 Gene Editing Using CRISPR-Cas9 Technology

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) technology is a recent advancement; it is a genome-editing tool that is faster, cheaper, and more accurate than the existing techniques for genome editing (Anders et al. 2014).

CRISPR-Cas9 technology has been successfully implemented for editing the genome in animals (Fan et al. 2018). It is now being experimented in creating transgenic plants as well. As discussed before, in Agrobacterium-mediated transformation, the gene of interest is inserted into the T-DNA, which is then inserted into the plant cell. By combining the CRISPR-Cas9 system with the Agrobacterium-mediated transformation method, we can achieve a more targeted T-DNA integration in the genome. This technique creates a double-strand break (DSB) at a specific genomic site. This double-strand break is then restored by either the non-homologous end-joining (NHEJ) method or homologous recombination (HR). CRISPR-Cas9 consists of two essential molecules that help to induce a change in the DNA (Oliver 2014).

- *S. pyogenes* Cas9 enzyme: This is a molecular scissor. It cuts the double-stranded DNA at a specific location in order for a gene to be added or removed.
- Guide RNA: The guide RNA finds and binds to the target sequence on DNA, and it acts as a guide for the Cas9 enzyme and helps it to cut at the right point of the genome.

The target sequence is generally present immediately adjacent to the protospacer adjacent motif (PAM) sequence. The PAM sequence serves as a signal for the Cas9 sequence. The end result of Cas9-mediated breakage is a double-strand break (DSB) within the target DNA (Raska and Turanek 2015).

19.3.1 Use of CRISPR-Cas9 on Disease Resistance

Nature is established in a way that plants and microbes live and evolve together. Plants are prone to many pests including bacteria, fungi, and viruses, which results in huge financial expenditure and threatens food safety. During the evolution, plants have developed several defensive strategies for attacking pathogens such as some secrete antimicrobial products that can recognize the pathogens or pathogen-derived compounds and can destroy it either by detoxification. Strategies like these are not always 100% effective because pathogens have also evolved to overcome these plant defense strategies and have made themselves capable of taking over the plant system and can spread infection; thus, farmers resorted to pesticides, but they also have their own disadvantages. Even though they prevent pests, they are harmful to both plants and humans alike for several reasons which will be discussed in the benefits section of this paper. CRISPR-Cas9 technique has addressed this issue and has been successful in creating pesticide-resistant plants.

There are many variants of CRISPR-Cas9 that scientists have discovered to create disease-resistant plants such as:

- Gene disruption via indels in coding sequences
- Gene interruption via indels in promoter regions
- Gene removal via multiplex sgRNAs
- Gene inclusion via homology-directed repair

19.3.2 Gene Disruption Via Indels in Coding Sequences

The most robust technique of editing the genome to make plants disease-resistant is using the CRISPR-Cas9 system. NHEJ machinery is not always 100% accurate and is prone to error. This method takes advantage of that. At the sgRNA-guide site, a frameshift mutation happens because of the insertion or deletion of one or additional nucleotides. This approach has been used in many crops, including corn, cereals, and wheat, to introduce various genes of interest. For using this method for disease resistance, the plant susceptibility gene, the S gene, has to be disrupted. This modifies the plant-pathogen relationship, resulting in disease-resistant plants (Hickey et al. 2020).

19.3.3 Gene Interruption Through Indels in Promoter Regions

In this approach, indels (nucleotides) are inserted or deleted in a plant's promoter region rather than the coding region. CRISPR-Cas9 can be used for this purpose of promoter editing in many ways such as it can be used to disrupt the promoter sequence by blocking gene expression or by disrupting the site where an effector binds. This disrupts the plant's susceptibility to pathogens by preventing the attachment of pathogen effector to the promoter region (Hickey et al. 2020).

19.3.4 Gene Removal Via Multiplex sgRNAs

Many sgRNAs are used in CRISPR-Cas9 method, to establish double-strand breaks at specific positions in the genome. Before the NHEJ machinery fixes the DSBs, these DSBs will delete the DNA fragment containing the gene of interest. Since sgRNAs can be designed in any area containing a suitable PAM sequence, this method can be used to remove both huge chromosomal fragments and individual genes. In S gene clusters, where several S genes are located on the same chromosome, removing the S gene chromosomal fragment results in long-term resistance to the target.

19.3.5 Gene Inclusion by Homology-Directed Repair

In all the abovementioned techniques, the S gene is altered/disrupted to generate disease resistance. However, the S gene is multifunctional, and if this gene is disrupted, other functions of the plant will also be affected, so in this method, instead of S genes, resistance genes or R genes are used. In this method, once the CAS9 protein produces the double-strand break as directed by the sgRNA, the CRISPR-mediated gene insertion works in a different way. Rather than NHEJ machinery, it employs cellular homology-directed repair machinery. The sgRNAs and Cas9 are joined by a fragment containing a R gene surrounded by a sequence homologous to the DSB ends. This directs the insertion of the R gene between the two DSB sites via HDR. It has been used to insert one or more genes at relevant positions in the genome pathogen (Hickey et al. 2020).

Plant diseases, as mentioned before, are mainly caused by infection due to pathogens such as bacteria, viruses, and fungi. CRISPR-Cas9 method has been applied widely for studies such as the inclusion of desired characteristics such as drought resistance and improved grain size and number. CRISPR technique has been used to increase resistance against plant pathogens and has also been used to introduce desirable traits to plants.

There are also other gene-editing techniques such as zinc finger nucleases and transcription activator-like effector nucleases (TALENs). A new protein is required every time followed by its validation whenever an experiment needs to be performed, thus making it difficult as well as expensive to be used. However, CRISPR-Cas9, on the other hand, is more preferred due to its specificity, multigene-editing capability, minimal off-target effects, higher efficiency, and simplicity (Jones 1999).

19.4 Benefits of GMFs

By improving the quantity and quality of agricultural production for the producers and consumers, genetic engineering is often performed to make the crops herbicide-resistant rather than to make them disease-resistant. Examples of GMFs includes, change in flower color, delayed senescence of fruits and flowers, male sterility as an aid to crossbreeding, and modification of lipid biosynthesis for specialized oil production.

Further benefits of GMSs includes, producing edible vaccines or medicines in milk, eggs, or fruit to facilitate distribution of therapeutic needs. The ability to genetically modify animals to produce pharmaceuticals in their milk has been one of the most innovative applications of genetic modification techniques and below are some of the examples with their applications:

1. *Producing edible vaccines*—Medicines or vaccines made from milk may be mass-produced, sold cheaply, and made more affordable to people all over the world. Injected vaccines are costly, necessitate qualified medical personnel for

administration, and necessitate continuous cooling during transport and storage, posing challenges in many developed countries. The use of needles also increases the chance of infection transmission. Researchers have also produced a variety of transgenic potatoes that carry a limited amount of the cholera toxin in order to immunize against the disease upon ingestion (Hug 2008).

2. *Producing functional or nutraceutical foods* with added traits for consumption is more beneficial for health or for preventing diseases, and consumers who have allergies to some foods or intolerances can safely consume this GM food. Nutraceuticals include tomatoes with improved lycopene (an antioxidant that can help prevent and cure prostate cancer and heart disease) or a soybean protein (alpha-glycinin) that has been mutated to have antihypertensive effects. The mutant protein was isolated from soybeans and shown to be effective in lowering blood pressure in hypertensive laboratory animals (Hug 2008).
3. *Genetically modified rice*—Known as "golden rice" which supplements the vitamin A pathway. The most famous of such foods is the invention of golden rice containing beta-carotene, a precursor to vitamin A.
4. *Economic benefits*—From 2006 to 2012, the global growth in farm income from GM foods grew to $116 billion (almost triple than that of the previous 10 years). According to James and Brookes, improved yield due to weed and pest resistance and advanced genetics accounted for about 42% of the economic benefit. The remainder 58% was attributed to lower manufacturing costs (Friedrich and Kassam 2012).
5. *Reforms in the chemical composition of food*—Some genetic alterations were specifically designed to increase the amount of certain nutrients or compounds of high medicinal and pro-health benefit, such as vitamins A, C, and E, unsaturated fatty acids, cellulose, and other probiotics. Transgenic plant's nutrient importance was increased by modifying their carbohydrate content (Friedrich and Kassam 2012).
6. *Food processing enhancement*—Genetic engineering innovation is nowadays exclusively used in food processing. The "Flavr Savr" tomatoes are one such prominent achievement. The genetic change in this case is the introduction of an antisense gene that inhibits the enzyme polygalacturonase. This delays the ripening process and, as a result, has a longer shelf life. Furthermore, gene editing was used to change the structure of potato bulbs. Potatoes with cyclodextrin glycosyltransferase genes from bacteria provide better durability and a more desirable shape (Wunderlich and Vecchione n.d.).
7. *Improves water quality*—The sustainability of agricultural production is regaining much importance, in view of the predicted population rise. Tillage farming practices cause much soil erosion. So, when GMFs are used, it decreases soil erosion due to "no-till" agriculture and improves water quality by reducing sedimentation. This helps to reduce greenhouse gases (Friedrich and Kassam 2012).

19.5 Risks of GMFs

The big problem associated with the consequences of GMF on the health and lives of consumers is the synthesis of toxic products, which increases the risk of activation of tumor processes in the body. These are the abnormal growth of defective cells in which specific genes give rise to tumor cells.

One such event in 1983, in Spain, was the introduction of modified rapeseed oil in the markets. Oil consumption has resulted in the fatality of a large number of consumers. The poisoning was found to cause toxic oil syndrome (TOS), which reflects contamination of the oil with aniline and its derivatives. In the United States, the poisonous effects of genetically modified crops were also identified, in which, particularly, transgenic tryptophan leads to death and pain in muscles and joints (Kramkowska et al. 2013).

The risk of augmented illness to tumor, causing from GM food's utilization, was to be equally alarming. In the year 2002, milk from genetically modified cows causes proliferation of IGF-1 in the users, hence leading to the emergence of tumors in the breast, colon, and lungs. Experiments were done on rats wherein they were served with different doses of NK603-modified maize. Their experiments' results concluded that there were disruption in the role of the liver and kidneys, manifestation of palpable tumors, and a higher mortality rate (Kramkowska et al. 2013).

Traces of lectin were found in genetically modified potatoes, which were synthesized due to the expression of a particular gene, which was lethal to the evolution of mammals. The existence of the transgene and the genetic architecture, as well as the transformation of genetic information, were thought to be associated with the emergence of abnormalities (Goodyear-Smith 2001).

Another potential risk is that they develop resistance to antibiotics. Antibiotics are commonly used in genetic engineering processes, usually as selection markers, to differentiate effectively modified bacteria from those in which the transfecting genes did not take effect. Thus, genetically modifying an organism carries the possibility of introducing antibiotic-resistant genes into benign bacteria. Antibiotics should be used as markers to certain marker genes, such as nptII, which pose no harm to people or animals, in order to avoid negative health impacts (Friedrich and Kassam 2012).

A food allergy is a severe risk factor. New protein expression and synthesis arise from the transfer of genes from one organism cells to the other cell nuclei. The amino acid sequence that forms a specific protein structure is the most significant risk factor for the development of food allergies as a result of transgenic food intake. The term allergy refers to a pathological immune reaction triggered by a reaction to the antigen of a specific dietary component. The most common allergens are assumed to be alimentary proteins, which when consumed can cause skin reaction, respiratory and circulatory system changes, and anaphylactic shock, all of which can have serious consequences on the health of consumers. Additionally, multiple customer claims of food allergy symptoms such as headaches, diarrhea, nausea, and vomiting were allegedly reported after consuming goods containing genetically modified maize (Kramkowska et al. 2013).

Allergen databases are used to predict the type of allergen present. Some of them are given below (Kadam et al. 2016):

1. Allergome (Allergome n.d.)—for proteins
2. Central Science Laboratory (Central Science Laboratory n.d.)—for proteins
3. National Center for Food Safety and Technology (NCFST) (National Center for Food Safety and Technology n.d.)—for gluten
4. Swiss model (Swiss Model n.d.)—for proteins
5. WHO/International Union of Immunological Society (WHO/International Union of Immunological Society n.d.)—for proteins

19.6 Ethical Concerns in GMFs

Genetic engineering is also thought to be problematic because of its consequences.

It is aberrant to manipulate plants, animals, and foods genetically. The so-called playing God argument is the most well-known example of this type of argument. Another such argument is that "God has placed an invisible boundary between God and man," so those who transcend these boundaries are guilty of excessive pride or self-confidence.

Any such arguments would depend more on religion about the relations of God, humans, and animals. According to Partridge's statement, environmental ethics is held responsible for our conduct toward environmental surroundings, all natural resources, and species. He also states that many of the arguments against genetic engineering revolve around whether it is right to change creatures and the natural environment.

19.6.1 Animal Benefits Via Genetic Engineering

Many have also objected to the experiments on animals in the field of genetic engineering. This indicates that humans should not treat animals as they have a will to live. Many non-animal test methods can be used in the place of animal testing. These tests are more humane; they also have the potential to be cheaper, faster, and more relevant to humans. Examples of animal tests include forcing mice and rats to inhale toxic fumes and force-feeding GM foods (Gatew et al. 2019).

19.6.2 Religious Concerns Toward Genetic Engineering

Certain religious skeptics see genetic engineering as "playing with God's creation" and oppose it because life is holy and should not be impeded by the humans. It is also wrong to alter the DNA of one organism as it lowers the dignity of human minds. Many individuals believe that genetic engineering places humans in the role of the Lord. Some argue that the universal applicability of transferring genes from one

species to another is debatable. This implies that mankind have violated and crossed divine's boundaries. According to this reasoning, any design of nature based on the insertion of additional genes is ethically reprehensible (Gatew et al. 2019).

19.6.3 Materialistic Concerns Against Genetic Engineering

Secular opponents of this GM technology must support the notion that "the dignity of an individual member of a species, or the dignity of the species itself, is linked with evolution to its current condition." They must also know that the present state of all species is due to the changes which occurred in evolution. So by modifying the environment, we cannot interrupt our self-esteem. In fact, it glorifies us to use the abilities; we have to alter our nature by the application of biological techniques to progress our lives (Gatew et al. 2019).

Some might also argue that overcoming innate limitations is a violation of our inherent dignity. Any invention that is utilized to reduce fundamental human abilities, such as cognitive functioning, is immoral. Genetic engineering needs specific consideration for some challenges, as well as limitations on its usage in areas where it may endanger certain species.

19.7 How GM Differs from Conventional Plant Breeding Techniques

The role of both GM and conventional plant breeding is to produce crops with enhanced characteristics by changing their genetic makeup.

GM achieves this by adding a new gene or genes to the genome of a crop plant. Conventional breeding achieves it by crossing together plants with relevant characteristics and selecting the offspring with the desired combination of characteristics, as a result of particular combinations of genes inherited from the two parents. Both these techniques give genetic crop improvement (Royal Society 2016).

Conventional breeding employs processes that occur in nature, such as sexual and asexual reproduction. The product of conventional breeding has certain characteristics. But it is not new for the species. These characters are present within the plant's genetic material.

But, genetic modification works through the insertion of genetic material. This "insertion" process does not occur in nature. This is done using a particle gene gun, which was discussed above, and also through chemical and electrical treatments. By doing this, we can insert the genetic material into the host plant cell.

Along with certain genetic elements, the genetic material inserts itself into the chromosome of the host plant. As a result, we can create what can be regarded as synthetic life forms, something which could not be done by conventional breeding.

As for the scope, genetic engineering allows the movement of genetic material from any organism to another organism. It also offers the ability to create genetic material and expression of products that have never existed before (Hansen n.d.).

19.8 Conclusion

With the world population growing exponentially every day, genetically modified food seems to be the only way to satisfy people's nutritional requirements. It has been discovered that malnutrition problems and environmental pollution are challenges for scientists as well as governments which can be combated by the application of genetic engineering in crops.

The recent growth of genome-editing technology such as CRISPR-Cas9 is also proving plants to have great potential. Such modern techniques are being used to identify and extract biologically active genes from food crops, such as natural toxicants and allergens. These developments are in the early stages, but in the long run, they will most certainly lead to the upgradation of food that does not have undesirable components. An example could be given with respect to the deterioration of fruits and vegetables.

Genetic modification and other biochemical techniques are being used to discover the biochemistry of food ripening and deterioration to find new methods of preserving these foods, without the use of chemical preservatives.

GMF raises many issues, such as economic, technological, environmental, political, and ethical, and many more. In this chapter, some of the benefits and risks were discussed in detail, and moreover, the main opponent of GMO is people questioning their effect on the consumer's health only to realize that those that entered the supermarket have undergone rigorous safety assessment.

It is right to take the development of GMF seriously, to question their risks and benefits, and also to debate their uses, but at the same time, it is important to avoid hysteria, and the issues/questions should be tackled rationally on an informed basis. With emerging research and innovation in gene-integration technologies, GM crops are forecasted to bring productivity and profitability in agriculture and more preservative-free food which also has a higher nutritional value for the consumers in the future.

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Genome Editing Crops in Food and Futuristic Crops

20

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Abstract

The advent of Second Green Revolution for nutritional security to feed the burgeoning world population amidst the challenges is related to climate change, resource availability, and farmers' income. In this scenario, the next-generation breeding technology or genome editing (GE) technique is promising and gaining wider acceptance and importance for improving economically important traits in crops. The meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) are important tools of GE. Among these, CRISPR is the most recent tool and revolutionizing the field of crop improvement. Advancements in the basic CRISPR/Cas system made it a more accurate, precise, and versatile tool. At present, a variety of genome-edited crops are available at

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different parts of the world, which are approved for cultivation. In this book chapter, we have mainly discussed about available genome-edited food crops and ongoing GE research work on food crops including cereals, horticultural and futuristic crops for specific traits. We have also presented an overview of the advancement in GE technique, regulation of GE food crops, and scope of GE in futuristic crops.

Keywords

New breeding technique · Genome editing · CRISPR/Cas · Base editing · Food security · Futuristic crops · Climate change

20.1 Introduction

Plant breeding has a great impact on human civilization, and both are co-evolving in nature. The Green Revolution, an important landmark in the history of agriculture, was achieved by using high-yielding varieties of staple food crops such as wheat and rice along with increased inputs, viz., fertilizers, irrigation facilities, etc., and helped many countries to achieve self-sufficiency in food production. However, over a period of time, there is stagnation in crop yield in some of the important staple food crops due to excess use of fertilizers and irrigation making hectors of the cultivable land barren. The world population is increasing at a tremendous rate with projection of 9.6 billion by 2050 (Tilman et al. 2011). In the context of the diminishing agricultural resources and mounting agricultural challenges like global warming, climate change, water scarcity, etc., there is an urgent need of a next Green Revolution for sustainable food and nutritional productivity. Such a next Green Revolution should have the potential to overcome all the limitations and barriers associated with the First Green Revolution and should help to achieve nutritional security with self-sufficiency and economic stability of farmers.

Genetic engineering involves the modification of the genetic makeup of plant by inserting foreign gene for improving particular traits. In 1982, the first genetically modified (GM) plant was developed (Fraley et al. 1983), and in the year 1994, the first GM crop that is Flavr Savr was approved for commercialization (Bruening and Lyons 2000). Since then, many GM crops are approved for commercialization and are presently available in the market. Nevertheless, out of the total area under cultivation, very less area is under GM crops, and issues associated with GM crops such as health risk, environmental and social issues, etc. have resulted in a huge rejection of GM crops by consumers and farmers as well. In this scenario, genome editing (GE) or new breeding technique (NBT) is gaining importance in the field of crop improvement (Barbadikar et al. 2019; Aglawe et al. 2018) along with clinical therapeutics and medicine because of its ability of precise site-specific changes. The product of GE is non-GM which leverages its widespread use and application in food crop improvement. The meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered

regularly interspaced short palindromic repeats (CRISPR) are some of the important tools of GE. In the process of genome editing, the cell utilizes either of the two DNA repair mechanism homologous recombination (HR) or non-homologous end joining (NHEJ) to repair the DSBs. The former mechanism is more specific as it makes use of long homologous DNA sequence for the precise insertion at the target site, while the latter NHEJ, although is more common in nature, is non-specific, and it simply utilizes the broken ends and performs the error-prone repair which results in non-specific mutations. CRISPR is the latest tool of GE which has wider utilization in genome editing in plants. The natural present CRISPR/Cas system is a three-component system which involves tracrRNA, crRNA, and Cas9 endonuclease. The CRISPR is the complex transcript processed into the simpler ones that can sight to its target. The CRISPR/Cas9 system comprises of two components, the Cas9 endonuclease and the sgRNAs which is the fusion of crRNA and tracrRNA and here the crRNA is responsible target specificity while the tracrRNA is kept same in all sgRNA. The target-specific sgRNA gets bound to Cas9 endonucleases and starts scanning for its target on the basis of the homology and cleaves the sense strand 3 bp and antisense strand 3 bp upstream of the protospacer adjacent motif (PAM) sequence and creates the DSBs. Several advancements have been done in native CRISPR system or its better deployment in GE. The CRISPR system has optimized for multiplexing which means targeting multiple genes simultaneously. These multiplexing approaches include tRNA-, Drosha ribonuclease-, and Csy4 endoribonuclease-based approaches. Of late, variants of CRISPR/Cas9 with different PAM, CRISPR/Cpf1, PAM-less editing strategy, and CRISPR for base editing are available (Liu et al. 2020).

In this book chapter, we have discussed the available GE food crops and ongoing GE research work on food crops. We have also focused on deploying GE for improving the futuristic crops. The underutilized or neglected or forgotten crops like minor millets, pseudo cereals, and minor pulses and some of the tuber crops have the potential to replace today's major food crops which will help to overcome the barriers of stagnated yield and nutritional requirements. Apart from this, the available biodiversity/gene pools can be explored for economically important traits and understanding the process of domestication. GE has a potential to improve available major food crops, but unlocking its potential for wider acceptability of neglected crops will assist in broadening the nutritional arena and the genomic resources.

20.2 Why Genome Editing (GE)?

Conventional plant breeding, mutation breeding, genomics-assisted breeding, transgenic breeding, and GE are the main methodologies in use for crop improvement currently (Fig. 20.1). Conventional plant breeding has been contributing a lot in crop improvement; nevertheless, it suffers with some of the major limitations. It is generally used to improve trait which is already present in the gene pool as it

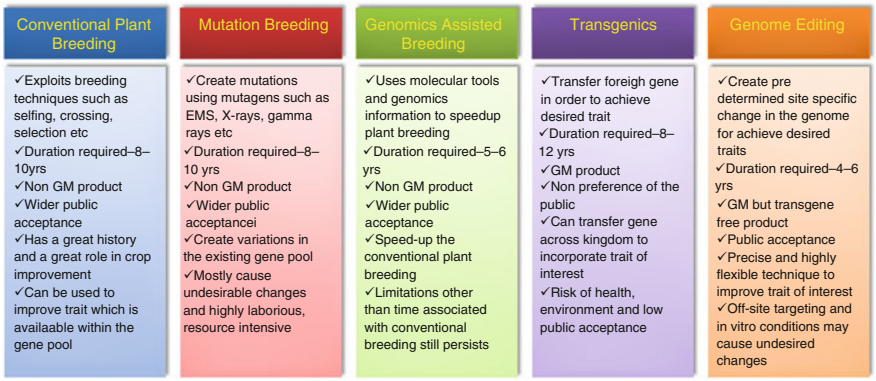

Fig. 20.1 Comparative overview of different available methods of crop improvement

needs sexually compatible species. Conventional plant breeding is laborious, time-taking, and resource-intensive and usually takes 8–10 years to develop a variety. Mutation breeding helped to overcome the biggest limitation associated with conventional breeding and helps to generate natural variation within germplasm using mutagens like EMS, MMS, X-rays, gamma rays, etc. Most of the induced mutations are deleterious, and phenotyping for the desired traits usually takes a long time which makes it laborious and time-taking. The next advancement in crop improvement is the application of molecular markers and genomics information in plant breeding. It helped to solve the problem associated with duration required to release a new variety and made it cut short for 6–8 years; nevertheless, the problem associated with the requirement of cross-compatibility still persists. During the 1980s, there was a breakthrough in crop improvement field that is transgenic technique, where a gene of interest can be transferred even across the kingdom to incorporate/improve trait of interest. In spite of its huge potential to revolutionize crop improvement field, this technique cannot be exploited much for crop improvement because of its huge rejection by farmers and consumers. Due to the health risk and environmental and social issues associated with GM crop, there is great hue and cry among consumers. GM crops need to undergo many testing and trials before releasing as a variety to secure its safety, which require a long period of 10–12 years. Recently, GE technique has been gaining immense importance in the field crop improvement as it is a very much precise, accurate, versatile, and flexible technique to improve the trait of interest. This technique allows precise editing, insertion, or deletion within the targeted gene. Even though GE resembles mutation breeding, its precise editing at targeted size eliminates the risk of deleterious effect of other unknown mutations in the crop genome, which is very common in mutation breeding product. Contrary to GM crops, GE crops are transgene-free as well as free from positional effect of transgene due to which there is less public concern for GE crops. Due to all these advantages, regulation of GE crops needs not to be as stringent as GM crops, which takes 4–6-year duration to release a variety for commercial cultivation. All these advantages associated with GE made it the technique of choice

of breeders and plant researchers for crop improvement. This technology has the potential to accelerate crop improvement process than what was expected before and has revolutionized the field of genome engineering for modifying gene/genomes for desirable trait.

20.3 Historical Perspective and Advancement in GE

The gene editing field was explored way before the arrival of CRISPR; however, over the past several years, CRISPR/Cas9 has transformed the field of genome engineering as an accelerated and efficient system for modifying gene/genomes for desirable trait. It is the greatest innovation of the last decade making the pioneers of CRISPR gene editing won the 2020 Nobel Prize in Chemistry. Over the past several years, there has been a rapid advancement in the GE technology from the meganucleases, ZFN, and TALEN to the most powerful CRISPR/Cas9.

Meganucleases known as molecular scissors were used for creating large deletion at the specific cleavage site, but the introduction of the large deletions might sometimes remove the essential genes or functional elements that are responsible for the normal plant growth and function (Khan et al. 2019). The ZFNs possess protein-binding domains which attach with target DNA fragment followed by the excision with *Fok*I endonucleases and creation of the double-stranded DNA break. Further, the targeted fragment of gene is inserted and integrated into the DNA sequence. The latter transcription activator-like effector nucleases (TALENs) possess almost similar mode of action as ZFNs; here, the DNA-binding protein domain is called TAL effector; however, the TALENs are slightly more site specific as it can target the third nt with fewer off-target effects unlike ZFNs which target the first nt (Chandrasegaran et al. 2016). The ZFN and TALEN are artificial nucleases that involve the protein engineering which is not easy as compared to engineering as nucleic acid. These transcription factors bind to DNA sequence where the fingers are designed to bind specific DNA sequence link with the unspecific *FoK*I nuclease. In comparison to ZFNs, TALENs are more powerful and effective. After this protein-based nuclease, GE system, the CRISPR/Cas9, was introduced which is RNA-DNA based.

The CRISPR/Cas9 is a bacterial adaptive immune system against foreign virus or plasmid DNA found in bacteria. The CRISPR/Cas9 is an RNA-guided endonuclease; here, the long RNA is transcribed and further processed to crRNA that binds to target. The CRISPR/Cas9 is much more powerful than the artificial nuclease as it requires the construction of target-specific sgRNA which is much easier to design than artificial nuclease which is continuously being improvised (Miao et al. 2013; Ma et al. 2015). It can target any sequence ending with PAM sequence NGG by designing specific sgRNA. In CRISPR/Cas9, there were challenges which hindered its PAM dependency for the recognition of target as it recognizes NGG PAM site for editing. To overcome this challenge, numerous Cas9 orthologs have been developed with altered PAM specificities; this includes *Staphylococcus aureus* Cas9 (SaCas9) and Cas9-VQR (D1135V/R1335Q/T1337R) which recognize and perform GE at

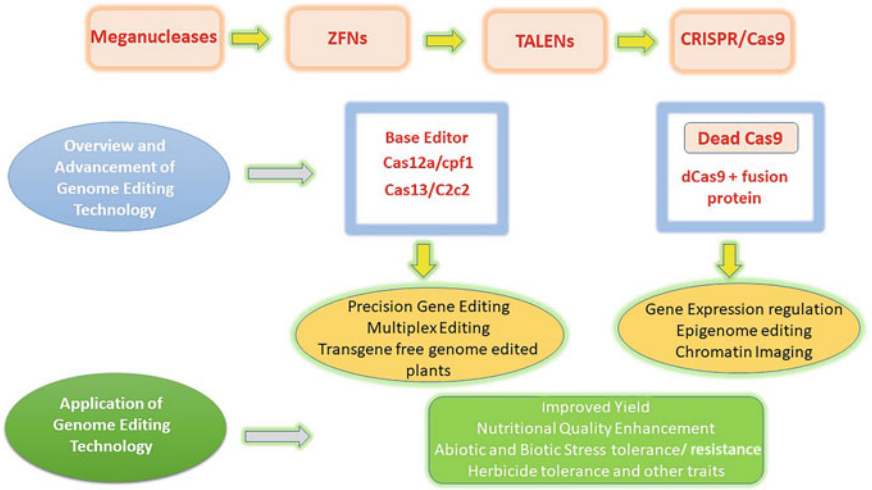

Fig. 20.2 Tools of GE and advancement done in CRISPR/Cas

NAG motif and NGA PAM site, respectively, with relatively low off-targets (Hu et al. 2014).

To increase the target specificity and reduce the off-target, many advancements have been made in this technology such as CRISPR/Cpf1 system, which recognize the target with T-rich PAM sequences and produce cohesive ends after cleavage (Yang et al. 2020) (Fig. 20.2). There have been several variants of the CRISPR/Cpf1 isolated from different species. The major difference between the CRISPR/Cas9 and CRISPR/Cpf1 lies in the absence of the HNH domain in the latter. Additionally, there are various efficient tools like Cms1 (CRISPR from Microgenomates and Smithella) which generates InDel mutations in rice and the most recent one CRISPR-Cas12b system from *Alicyclobacillus acidiphilus* (AaCas12b) (Teng et al. 2017). There is a CRISPR/Cas13 system or C2c2 which performs RNA editing. The Cys4 from the *Pseudomonas aeruginosa* is the powerful system for the multiplexed GE and has proved to possess the ability to regulate the 20 genes in a fast and effective manner in *S. cerevisiae* (Mao et al. 2018; Ferriera et al. 2018). Besides generating mutation, CRISPR GE systems, with the help of inactivated Cas9 (dCas9) nuclease, regulate the gene expression, epigenetic modification, and chromosomal imaging (Chen et al. 2012).

With the introduction of base editing for genomic modification, the precision has been enhanced, as this editing technology circumvents the creation of DSBs (double-strand breaks) for performing the targeted modifications at precise position (Hua et al. 2018b; Li et al. 2018a; Yang et al. 2020). The base editing technology mainly requires the Cas9 nickase fused with cytidine deaminase enzyme; the fusion further facilitates the alteration of bases such as cytidine (C) to uridine (U), allowing C → T or G → A substitutions which is known as cytidine base editor (CBE) and adenine base editor (ABE), respectively. This allows the creation of specific modifications at

desired locations in the genome. Using the ABE, several endogenous rice genes have been targeted for traits such as herbicide tolerance gene and genes responsible for ideal plant architecture. The modification in gene *OsSPL14* results in the enhanced grain yield in rice.

Multiplexing, i.e., targeting multiple genes simultaneously, can also be performed using these tools. The multiplexing approaches include tRNA-, Drosha ribonuclease-, and Csy4 endoribonuclease-based approaches (Woo et al. 2015). The PTG (poly-tRNA-gRNA) unlike sgRNA does not require any specific nucleotide to target specific sequence; additionally, the combination of PTG with dead Cas9 can be used to manipulate and regulate the gene expression. So, along with the various PAM-less editing strategies, multiplexing editing methods have reduced dependency on the PAM sequence. Anzalone et al. (2019) have deployed prime editing guide RNA (pegRNA) to reduce the off-target effects. Hence, this GE technology has been progressive and is being employed for improving various traits of agronomic importance like yield/yield-related traits, biotic and abiotic stress resistance/tolerance, quality traits, and nutritional and economically important traits in plants. In the section below, we have described the various traits improved using CRISPR/Cas and their possible implications in future crops/underutilized crops.

20.4 GE for Trait Improvement in Crops

GE has ever-increasing applications in trait improvements in crops; few of them are discussed here (Table 20.1 and Fig. 20.2). For in-depth reading, kindly read the review by Xu et al. (2019).

20.4.1 Quality- and Nutrition-Related Traits

20.4.1.1 GE for Improved Oil Quality

Oil is composed of saturated fatty acids, monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA), and is an essential component of food used in day-to-day life. Rancidity of oil is a major problem that occurs via oxidation of PUFA making it unsuitable for human consumption. Hence, efforts have made to reduce the amount of PUFA in oilseed crops while increasing the amount of oxidative more stable MUFA, which reduces the risk of lifestyle diseases. *Camelina sativa*, is being utilized for biofuel and animal feed, contains more PUFA, and hence is more prone to oxidation (Frolich and Rice 2005). Using the CRISPR/Cas9, the enzyme $FADH_2$ involved in PUFA production was targeted which reduced the production of PUFA and thereby increased the amount of oleic acid (MUFA) in the crop than its wild-type species; there were also subsequent reductions in the amount of linoleic acid and linolenic acid production in the seeds (Jiang et al. 2017). Similarly, the quality of rice bran oil was improved by targeting the same enzyme which doubled the amount of oleic acid as well as decreased the amount of PUFA (Abe et al. 2018). Soybean lipoxygenases are involved in the production of beany

Table 20.1 Application of GE for trait improvement in crops

Sr. no.	Crop	Method	Trait studied	Targeted genes	References
GE for improvement of quality and nutritional traits					
1	*Camelina sativa*	CRISPR/Cas9	Increased volume of oleic acid	*FAD2*	Jiang et al. (2017)
2	*Oryza sativa*	CRISPR/Cas9	Improvement of rice bran oil	*FAD (OsFAD2–1)*	Abe et al. (2018)
3	*Brassica napus*	CRISPR/Cas9	Increasing size of oil bodies	*BnLPAT2* (7 homologous genes), *BnLPAT5* (4 homologous genes)	Zang et al. (2020)
4	*Glycine max*	TALEN	Increasing oleic acid	*FAD2–1a FAD2–1b*	Huan et al. (2014)
5	*Glycine max*	TALEN	Increasing oleic acid	*FAD3-a*	Demorest et al. (2016)
6	*Glycine max*	CRISPR/Cas9	Increasing oleic acid and decreasing linoleic and linolenic acid	*Gm-FAD2–1a gm-FAD2–1b*	Do et al. (2019)
7	*Brassica napus*	CRISPR/Cas9	Increased seed oil and protein content	*BnTT8*	Zhai et al. (2020)
8	*Brassica napus*	CRISPR/Cas9	Increased seed oil content	*BnSFAR4,5*	Karunarathna et al. (2020)
9	*Glycine max*	CRISPR/Cas9	Reducing beany flavor to improve oil and protein quality	*LOXs* (*Gmlox1, Gmlox2,* and *Gmlox3*)	Wang et al. (2020)
10	*Solanum lycopersicum*	CRISPR/Cas9	Fruit ripening	*lncRNA1459*	Li et al. (2018d)
11	*Oryza sativa*	CRISPR/Cas9	Beta-carotene improvement	*OSOR*	Endo et al. (2019)
12	*Oryza sativa*	CRISPR/Cas9	Beta-carotene improvement	*CAROTENOID CASSETTE*	Dong et al. (2020)
13	*Musa acuminata Cavendish*	CRISPR/Cas9	Beta-carotene improvement	*PDS*	Kaur et al. (2018)
14	*Musa acuminata Cavendish*	CRISPR/Cas9	Beta-carotene improvement	*LCY*	Kaur et al. (2020)

15	*Actinidia chinensis*	CRISPR/Cas9	Carotenoid biosynthesis	*ACPDS*	Wang et al. (2018a)
16	*Citrus sinensis*	CRISPR/Cas9	Carotenoid biosynthesis	*CSPDS*	Jia and Wang (2014)
17	*Apple*	CRISPR/Cas9	Carotenoid biosynthesis	*PDS*	Nishitani et al. (2016)
18	*Glycine max*	CRISPR/Cas9	Carotenoid biosynthesis	*GmPDS11* and *GmPDS18*	Du et al. (2016)
19	*Solanum lycopersicum*	CRISPR/Cas9	Lycopene biosynthesis	*SGR-1 LCY-E, LCY-B1, LCY-B2*	Li et al. (2018e)
20	*Oryza sativa*	CRISPR/Cas9	Red rice	*Rc*	Zhu et al. (2019)
21	*Oryza sativa*	CRISPR/Cas9	Starch improvement	*SBE I, SBE II*	Sun et al. (2017)
22	*Oryza sativa*	CRISPR/Cas9	Starch improvement	*OsGBSSI*	Xu et al. (2020)
23	*Oryza sativa*	CRISPR/Cas9	Starch improvement	Promoter of *OsGBSSI*	Huang et al. (2020)
24	*Oryza sativa*	CRISPR/Cas9	Starch improvement	Promoter and 5'UTR of *OsGBSSI*	Zheng et al. (2020)
25	*Hordeum vulgare*	CRISPR/Cas9	Starch improvement	*HvGBSSIa*	Zhong et al. (2019)
26	*Ipomoea potato*	CRISPR/Cas9	Starch improvement	*IbGBSS, IbSBEII*	Wang et al. (2019)
27	*Solanum tuberosum*	CRISPR/Cas9	Starch improvement	*GBSS*	Andersson et al. (2017)
28	*Solanum tuberosum*	CRISPR/Cas9	Starch improvement	*StSBE1, StSBE2*	Tuncel et al. (2019)
29	*Zea mays*	CRISPR/Cas9	Starch improvement	*GBSS*	Gao et al. (2020)
30	*Manihot esculenta*	CRISPR/Cas9	Starch improvement	*MePTST1, MeGBSS*	Se et al. (2018)
31	*Solanum lycopersicum*	CRISPR/Cas9	Shelf life	*ALS*	Yu et al. (2017)
32	*Solanum lycopersicum*	CRISPR/Cas9	Fruit ripening	*lncRNA1459*	Li et al. (2018e)

(continued)

Table 20.1 (continued)

Sr. no.	Crop	Method	Trait studied	Targeted genes	References
33	*Oryza sativa*	CRISPR/Cas9	Storage life	*Lox*	Ma et al. (2015)
34	*Solanum tuberosum*	CRISPR/Cas9	Storage life	*Vlnv*	Clasen et al. (2015)
35	*Zea mays*	CRISPR/Cas9	Phytate reduction	*ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4*	Liang et al. (2014)
36	*Oryza sativa*	CRISPR/Cas9	Phytate reduction	*OsPLDα1*	Khan et al. (2019)
37	*Brassica napus*	CRISPR/Cas9	Phytate reduction	*BnITPK*	Sashidhar et al. (2020)
38	*Thlaspi arvense*	CRISPR/Cas9	Erucic acid reduction	*TaFAE1*	McGinn et al. (2019)
39	*Triticum aestivum*	CRISPR/Cas9	Allergen reduction	*WTAI-CM3 WTAI-CM16*	Camerlango et al. (2020)
40	*Triticum aestivum*	CRISPR/Cas9	Allergen reduction	*a-gliadin*	Sanchez-Leon et al. (2018)
41	*Oryza sativa*	CRISPR/Cas9	Cd reduction	*OsNramp5*	Tang et al. (2017)
42	*Solanum lycopersicum*	TALEN; CRISPR/Cas9	Antioxidant production	*ANT-1*	Cermak et al. (2015)
43	*Solanum lycopersicum*	CRISPR/Cas9	Parthenocarpy (seedless)	*SlIAA9*	Ueta et al. (2017)
44	*Solanum lycopersicum*	CRISPR/Cas9	Parthenocarpy (seedless)	*SlIAA9*	Hara et al. (2020)
45	*Lactuca sativa*	CRISPR/Cas9	Ascorbate content	*LsGGP2*	Zhang et al. (2018)
46	*Solanum lycopersicum*	CRISPR/Cas9	Increased γ-aminobutyric acid content	*SlGABA-TP1, SlGABA-TP3, SlCAT9, SlSSADH*	Li et al. (2018c)
47	*Oryza sativa*	TALEN	Fragrance improvement	*BADH2*	Shan et al. (2015)
48	*Solanum tuberosum*	CRISPR/Cas9	Antibrowning	*PPO*	Gonalez et al. (2020)
49		CRISPR/Cas9	Antibrowning	*PPO*	Waltz (2016a, b)

	Agaricus bisporus				
50	*Solanum lycopersicum*	CRISPR/Cas9	Domestication	*SP, SP5, SICLV SIWUS, SIGGP1*	Li et al. (2018a)
51	*Solanum lycopersicum*	CRISPR/Cas9	Fruit ripening	*RIN*	Ito et al. (2015)
52	*Glycine max*	CRISPR/Cas9	Carotenoid biosynthesis	*GmPDS11* and *GmPDS18*	Du et al. (2016)
53	*Triticum aestivum*	CRISPR/Cas9	Fe content	*TaVIT2*	Connorton et al. (2017)
54	Barley	CRISPR/Cas9	Increased glutenin content	*D-hordein*	Yang et al. (2020)
GE for biotic stress tolerance					
55	*Oryza sativa*	CRISPR/Cas9	Bacterial blight resistance	*OsSWEET11* and *OsSWEET14*	Jiang et al. (2013)
56	*Oryza sativa*	CRISPR/Cas9	Bacterial blight resistance	*OsSWEET13*	Zhou et al. (2015)
57	*Oryza sativa*	CRISPR/Cas9	Bacterial blight resistance	*OsSWEET14*	Li et al. (2012)
58	*Citrus sinensis (L.) Osbeck*	CRISPR/Cas9	Bacterial resistance	*CsLOB1*	Peng et al. (2017)
59	*Citrus paradisi*	CRISPR/Cas9	Bacterial resistance	*CsLOB1*	Jia et al. (2016)
60	*Malus prunifolia*	CRISPR/Cas9	Bacterial resistance	*DIPM-1, DIPM-2, DIPM-4*	Osakabe et al. (2018)
61	*Solanum lycopersicum*	CRISPR/Cas9	Bacterial resistance	*SlJAZ2*	Ortigosa et al. (2019)
62	*Malus domestica*	CRISPR/Cas9	Bacterial resistance	*MdDIPM4*	Pompili et al. (2020)
63	*Solanum lycopersicum*			*SIDMR6–1*	Paula de Toledo Thomazella et al. (2016)
64	*Oryza sativa*	CRISPR/Cas9	Fungal resistance	*OsSEC3A*	Wang et al. (2016)
65	*Triticum aestivum*	TALEN&CRISPR/Cas9	Fungal resistance	*TaMLO-A1, TaMLO-B1,* and *TaMLOD1*	Wang et al. (2014)

(continued)

Table 20.1 (continued)

Sr. no.	Crop	Method	Trait studied	Targeted genes	References
66	*Triticum aestivum*	CRISPR/Cas9	Fungal resistance	*TaEDR1*	Zhang et al. (2017)
67	*Solanum lycopersicum*	CRISPR/Cas9	Fungal resistance	*SlMlo1*	Nekrasov et al. (2017)
68	*Solanum lycopersicum*	CRISPR/Cas9	Fungal resistance	*SlDMR6–1*	Paula de Toledo Thomazella et al. (2016)
69	*Hordeum vulgare*	CRISPR/Cas9	Fungal resistance	*HvMORC1*	Kumar et al. (2018)
70	*Triticum aestivum*	CRISPR/Cas9	Fungal resistance	*TaNFXL1*	Brauer et al. (2020)
71	*Brassica napus*	CRISPR/Cas9	Fungal resistance	*BnCRT1a*	Pröbsting et al. (2020)
72	*Grape*	CRISPR/Cas9	Fungal resistance	*VvWRKY52*	Wang et al. (2018b)
73	*Brassica napus*	CRISPR/Cas9	Fungal resistance	*BnWRKY70*	Sun et al. (2018)
74	*Citrullus lanatus*	CRISPR/Cas9	Fungal resistance	*Clpsk1*	Zhang et al. (2020b)
75	*Cucumis sativus*	CRISPR/Cas9	Viral resistance	*eIF4E*	Chandrasekaran et al. (2016)
76	*Hordeum vulgare*	CRISPR/Cas9	Viral resistance	*MP, CP, rep/RepA LIR*	Kis et al. (2019)
77	*Oryza sativa*	CRISPR/Cas9	Viral resistance	*OseIF4G*	Macovei et al. (2018)
78	*Oryza sativa*	CRISPR/Cas9	Insect resistance	*CYP71A1*	Lu et al. (2018)
79	*Solanum lycopersicum*	CRISPR/Cas9	Resistance to broomrapes	*SlCCD8*	Bari et al. (2019)

80	*Hordeum vulgare*	CRISPR/Cas9	Resistance to WDV	*WDV genome*	Kis et al. (2019)
81	*Solanum tuberosum*	CRISPR/Cas13a	Resistance to PCY	*PVY genome*	Zhan et al. (2019)
GE for abiotic stress tolerance					
82	*Oryza sativa*	CRISPR/Cas9	Drought tolerance	*SRL1* and *SRL2*	Liao et al. (2019)
83	*Oryza sativa*	CRISPR/Cas9	Drought and salt tolerance	*OsDST*	Santosh Kumar et al. (2020)
84	*Zea mays*	CRISPR/Cas9	Drought tolerance	*ARGOS8*	Shi et al. (2017)
85	*Oryza sativa*	CRISPR/Cas9	Salt tolerance	*OsRR22*	Zhang et al. (2019b)
86	*Solanum lycopersicum*	CRISPR/Cas9	Salt tolerance	*SlARF4*	Bouzroud et al. (2020
87	*Oryza sativa*	CRISPR/Cas9	Cold tolerance	*OsSRFP1*	Fang et al. (2016)
88	*Oryza sativa*	CRISPR/Cas9	Cold tolerance and yield	*OsSRFP1, GS3, OsMYB30*	Lv et al. (2017)
89	*Lactuca sativa*	CRISPR/Cas9	Heat stress	*NCED4*	Bertier et al. (2018)
90	*Solanum lycopersicum*	CRISPR/Cas9	Heat stress	*SlMAPK3*	Yu et al. (2019)
91	*Brassica napus* L	CRISPR/Cas9	Yield	*BnaMAX1*	Zheng et al. (2020)
92	*Oryza sativa*	CRISPR/Cas9	Yield and improved aroma	*Os03g0603100, Os03g0568400,* and *GL3.2* and *OsBADH2*	Usman et al. (2020)
93	*Oryza sativa*	CRISPR/Cas9	Yield	*Gn1a, DEP1, IPA1*	Li et al. (2016b)
94	*Oryza sativa*	CRISPR/Cas9	Yield	*LAZY1*	Miao et al. (2013)
95	*Solanum lycopersicum*	CRISPR/Cas9	Yield	*SELFPRUNING, OVATE, FASCIATED* and *FRUIT WEIGHT 2.2, LYCOPENE BETA CYCLASE,* and *MULTIFLORA*	Zsögön et al. (2018)
GE for herbicide tolerance					
96	*Zea mays*	ZFN	Herbicide tolerance	*IPK1*	Shukla et al. (2009)

(continued)

Table 20.1 (continued)

Sr. no.	Crop	Method	Trait studied	Targeted genes	References
97	*Glycine max* (soybean)	CRISPR/Cas9	Chlorsulfuron	*ALS1*	(Li et al. 2015)
98	*Zea mays*	CRISPR/Cas9	Chlorsulfuron	*ALS2*	Svitashev et al. (2015)
99	*Oryza sativa*	CRISPR/Cas9	Chlorsulfuron and bispyribac-sodium tolerance	*ALS*	Sun et al. (2016)
100	*Oryza sativa*	CRISPR/Cas9	Imazamox	*ALS*	Shimatani et al. (2017)
101	*Oryza sativa*	CRISPR/Cas9	Bispyribac-sodium	*ALS*	Butt et al. (2017) Kuang et al. (2020) Ali et al. (2020)
102	*Solanum lycopersicum*	CRISPR/Cas9	Chlorsulfuron	*ALS1*	Veillet et al. (2019)
103	*Solanum tuberosum*	CRISPR/Cas9	Chlorsulfuron and bispyribac-sodium tolerance	*ALS1*	Butler et al. (2015) Veillet et al. (2019)
104	*Triticum aestivum*	CRISPR/Cas9	Nicosulfuron, mesosulfuron, imazapic	*ALS*	Zhang et al. (2019a, b)
105	*Citrullus lanatus*	CRISPR/Cas9	Tribenuron	*ALS*	Tian et al. (2018)
106	*Capsicum annuum*	CRISPR/Cas9	Glyphosate	*EPSPS*	Ortega et al. (2018)
107	*Manihot esculenta*	CRISPR/Cas9	Glyphosate	*EPSPS*	Hummel et al. (2018)
108	*Oryza sativa*	CRISPR/Cas9	Glyphosate tolerance	*EPSPS*	Li et al. (2016)
109	*Linum usitatissimum*	CRISPR/Cas9	Glyphosate tolerance	*EPSPS*	Sauer et al. (2016)
110	*Oryza sativa*	CRISPR/Cas9	Herboxidiene (GEX1A)	*SF3B1*	(Butt et al. 2019)

111	Dicotyledonous crops	CRISPR/Cas9	C17	*CESA3*	Hu et al. (2019)
112	*Oryza sativa*	CRISPR/Cas9	Haloxyfop-R-methyl	*ACCase*	Li et al. (2018a) Li et al. (2020)
113	*Oryza sativa*	CRISPR/Cas9	Haloxyfop	*ACCase*	Li et al. (2020)

flavor that hampers the eating quality of the oil. On a commercial level, it is eliminated by heat, microwave processing, and organic solvent extraction which adds on in the cost of post-harvest processing and production (Nishiba et al. 1995). Using CRISPR/Cas9 mutants was generated for lipoxygenase gene, and transgene-free mutants were created in soybean which showed reduction in beany flavor (Wang et al. 2019).

20.4.1.2 GE for Improvement of Pigments

Over the years, scientists have been focusing on the bio-fortification of crops, which includes increasing the amount of β-carotene, a precursor of vitamin A. These efforts will also help to overcome the vitamin A deficiency and its associated diseases. In the process of bio-fortification for vitamin A, the genetic engineering of the β-carotene pathway served as a milestone. However, these genetically engineered crops faced regulatory issues, which hinder its commercialization. Rice has always been an important cereal as a prime target for bio-fortification. Another such genetically modified crop is the cauliflower (*Brassica oleracea* var. botrytis) mutant Orange (Or), which produces high β-carotene due to mutation in transcript splicing (Lu et al. 2006). Based on this idea, the CRISPR/Cas9 technique was used to knock down the orthologous gene in rice associated with *Osor* (*Oryza sativa*); this mutation resulted in the high accumulation of β-carotene as compared to the wild type (Endo et al. 2019). To increase the percent of β-carotene, carotenoid biosynthesis cassette (5.2 kb) was introduced at a defined target location by CRISPR/Cas9 which increased the accumulation of β-carotene with no off-target effects and marker (Dong et al. 2020). Similarly, Kaur et al. (2020) enhanced the accumulation of beta-carotene in banana by mutating the *lycopene epsilon cyclase* (*LCYE*) leading to a sixfold more accumulation of beta-carotene by inhibiting the production of lutein from trans-lycopene.

Lycopene, a bioactive compound found in tomato, is associated with many health benefits including lowering risk of chronic diseases, cancer, and cardiovascular diseases (Tang et al. 2014; Poucheiu et al. 2014). The amount of lycopene was successfully increased in tomato using CRISPR/Cas9 approach, where the accumulation of lycopene was increased along with inhibition in the conversion of lycopene to α- and β-carotene. Homozygous mutants obtained were carried forward, and the expression of lycopene was observed to increase 5.1-fold (Li et al. 2018b). Moreover, consumption of several other pigments such as anthocyanin and proanthocyanidins is reported to have a beneficial effect on human health. High levels of such pigments are present in red rice. Production of red pericarp color in wild rice *Oryza rufipogon* is the result of the expression of two complementary genes *Rc* and *Rd*. However, most of the cultivated rice varieties have white pericarp which is a result of 14 bp deletion at the seventh exon of *Rc* gene. The CRISPR/Cas9 was used to restore the functional recessive allele of *Rc* gene that resulted in the production of red pericarp in three elite varieties and ultimately led to the accumulation of proanthocyanidins and anthocyanidins without affecting any agronomic trait (Zhu et al. 2019).

20.4.1.3 GE for Good Starch Quality

Since the nineteenth century, efforts have been made to improve the amylose content in cereal crops, as it has been found that the high amylose content is associated with an increase in resistant starch and has a beneficial health effect (Regina et al. 2006; Jiang et al. 2010). Earlier, this improvement in the amylose content was made by cloning as well as by introgression of mutant allele of starch debranching enzymes (Stenard et al. 1993). But later on, in the crops such as wheat and barley, higher amylose content was produced by the downregulation of *SBEII* (Satoh et al. 2003; Regina et al. 2006, 2010). In another important cereal that is rice, higher amylose content was obtained by introducing RNA interference-mediated gene silencing of *SBEII* via the formation of hairpin RNA (Wei et al. 2010; Butardo et al. 2011). In spite of several efforts, there was no satisfactory result; rather, incomplete and variable expression of transgene was observed in different lines. Also, the regulatory process for GM crops is time-consuming and costly, making this approach less significant (Shan et al. 2015). Targeted mutation in *SBEI* and *SBEII* resulted in the significant increase in amylose as well as resistant starch content (Sun et al. 2017). Similarly, in another experiment, modification of amylose and amylopectin was studied in *Ipomoea potato*. By mutating two starch biosynthetic genes (*GBSS* and *SBEII*) which are involved in amylose and amylopectin synthesis pathway, it was observed the increased level of amylose and reduced level of amylopectin as in these lines, the starch debranching enzyme was knocked out. Though the level of starch content in *GBSS*- and *SBEII*-mutated lines was not significantly differed than the wild type, the percent of amylose was found increased in *SBEII* mutant lines and found decreased in *GBSS* mutant lines (Wang and Chen 2019).

The branched part of starch, amylopectin, breaks down easily during digestion as well as holds an industrial importance. So, for increasing the content of amylopectin by reducing the amylose content, a single gene coding for the formation of amylose has been mutated. In plants, amylose is produced with the activity of enzyme, i.e., granule-bound starch synthase (GBSS). Hence, by mutating three different regions of *GBSS1* gene in potato using CRISPR/Cas9, the amount of amylopectin was successfully increased (Andersson et al. 2017). Efforts have been made to increase the amylopectin due to its great industrial demand in maize. This has been achieved by mutating waxy gene locus which codes for *GBSS1* (Pioneer 2015; Gao et al. 2020), and this genetically modified maize with high amylopectin is named as the waxy corn, by DuPont (Xiantao et al. 2020). The waxy corn was among the first CRISPR-modified products that were cultivated and sold without any restrictions in the USA (Waltz 2016a, b).

20.4.1.4 GE for Increased Shelf Life

Improving shelf life has been an important factor in reducing post-harvest losses and ultimately to reducing food wastage. Perishable crops, such as tomatoes, are more susceptible to post-harvest deterioration. Though naturally available mutant lines of tomato (never ripe, alcobaca, ripening inhibitor, and non-ripening) have been used to improve tomato, these mutations affect the organoleptic quality of tomato (Kopeliovitch et al. 1982; McGlasson et al. 1987). Among all, *acetaldehyde Alc*

mutant line was significant than other mutated lines. Based on this idea, the CRISPR/Cas9 system mutation was created in *Alc* gene, which results in the desired homozygous mutation without T-DNA insertion, and it was further demonstrated to decipher good shelf life on phenotypic characterization (Yu et al. 2017). In another approach, the shelf life of rice was enhanced by knocking down genes involved in the deoxygenation of fatty acids by TALEN system (Ma et al. 2015). The resultant mutants were progressed for the next generation, and agronomic characteristics of the same were evaluated which revealed the impact of lipoxygenase on the storage capability of rice. In tuber crops such as potatoes, the sprouting during storage is one of the major constraints for its long-time storage. This problem can be addressed by subjecting the tuber crops to cold storage. However, the cold storage results in the enhancement in the amount of reduced sugars which reacts with free amino acids in potato and produces bis-acrylamide which is a potential carcinogen, when these potatoes are subjected to high-temperature processing. Hence, efforts have been made to reduce the bis-acrylamide concentration in potato; for this, the candidate gene *Vlnv* (*Vacuolar sugar invertase*) was knocked out using TALEN. The gene is reported to code for vacuolar sucrose invertase enzyme which produces glucose and fructose from sucrose, and mutant plants were generated with the reduced amount of bis-acrylamide, and ultimately, the long shelf life potatoes devoid of carcinogen were obtained (Clasen et al. 2015).

20.4.1.5 GE for Inhibiting Anti-Nutritional Factors

Anti-nutritional factors reduce the absorption of available nutrients and have an adverse effect on human health (Gemede 2014). An anti-nutritional compound, phytic acid (PA), represents almost 75% of the total phosphorus content in seed and is indigestible for monogastric animals. The TALEN system was utilized to target four different genes in the PA synthesis pathway, and one gene was targeted using CRISPR/Cas9 which resulted in the reduced amount of phytate (Zhan et al. 2019). The amylase trypsin inhibitor (ATI) is a potential allergen found in wheat, now recognized as a cause of baker's asthma and allergies related to wheat (Zevallos et al. 2017). Wheat mutants were generated at two subunits of ATI using CRISPR/Cas9. Marker-free gene approach was used, and homozygous transgenic plants were obtained in T_0 generation, and successful knockdown of immunogenic protein was observed (Camerlengo et al. 2020).

Heavy metals in crops have been reported to pose health hazards like cadmium (Cd) found in rice (Clemans et al. 2013). Elimination of heavy metals in the soil results in lowering the accumulation of heavy metals in plants. Targeting genes for heavy metal transport is one of the important solutions to address the problem of heavy metal accumulation. Using CRISPR/Cas9, the cadmium metal transporter, *OsNramp5* gene (for the natural resistance-associated macrophage protein), was targeted for low cadmium content in grains. The mutated plants for *OsNramp5* gene showed drastic reduction in the accumulation of Cd in grains, roots, and shoots in rice (Tang et al. 2017).

20.4.1.6 GE for Increased Fruit Quality

Fruits are an important part of diet and a rich source of vitamins and dietary fibers. Improving the quality of fruits is considered as an important aspect in the development of agriculture. Seedless fruits have made an impact on the acceptability of fruits at global level. Parthenocarpy is the process of the development of fruit without fertilization, and it is considered as the most important process involved in the development of seedless fruit. By utilizing the above idea, the auxin/indole-3-acetic acid *SlIAA9*, an important gene in the parthenocarpy process, was mutated and showed a similar phenotype as that of parthenocarpy plant with no off-target effects (Ueta et al. 2017). Similarly, null-segregant parthenocarpic tomatoes were generated using CRISPR/Cas9 by targeting *SlIAA9* gene, and transgene-free plants were obtained (Hara et al. 2020).

A leafy vegetable *Lactuca sativa* (lettuce) has been edited using a single-guide RNA targeting to endogenous ORFs which modulated the expression of different mRNAs. Edition of upstream open reading frames (uORFs) of *LsGGP2*, a gene encoding key enzyme in vitamin biosynthesis, lead to increased oxidative stress tolerance, and content of ascorbate by ~150% (Zhan et al. 2019). Ascorbic acid (vit. C) is one of the important vitamins and possesses many beneficial health effects by mitigating oxidative stress. The antioxidants in plants have beneficial health effects as it reduces the chance of lifestyle diseases. Anthocyanin pigments are known as effective antioxidants. Production of anthocyanin in plants is mainly regulated by anthocyanin action Myb transcription factor. To increase the anthocyanin production, tomato plants were edited using TALEN and CRISPR/Cas9 for Myb transcription factor gene *ANT-1*. The strong promoter CaMV35s was placed upstream of *ANT-1*. The resultant plants showed increased anthocyanin production as a distinct purple color was observed in fruits, stems, and leaves (Cermak et al. 2015).

20.4.1.7 GE for Enhancing Fragrance

Aroma in plants is the result of many volatile and semi-volatile components. The fragrant rice has great global importance, and hence, its associated studies attract many breeders. Fragrance in rice is mainly contributed by a compound 2-acetyl-1-pyrroline (2-AP), and the genetic analysis of 2-AP has proved that its expression is controlled by single recessive gene *fgr* which codes for betaine aldehyde dehydrogenase 2 ($BADH_2$) (Bradbury et al. 2005). Novel alleles of *OsBADH2* were created in non-aromatic elite rice variety ASD16 leading to aroma using CRISPR/Cas9 (Ashok Kumar et al. 2020). Further, the molecular analysis of BADH2 revealed its role in inhibiting synthesis of 2-AP by diverting upstream promoter GABald. Hence, the *BADH2* mutants are found to have the accumulation of 2-AP (Bradbury et al. 2008; Chen et al. 2008).

Incorporating natural *BADH2* requires more time, labor, and cost in terms of developing desirable mapping population. The RNAi-mediated approach was utilized to downregulate *BADH2* expression, but variable expressions were observed in the transgenic lines, and moreover, the plants developed through RNAi are considered as transgenic (Niu et al. 2008; Chen et al. 2012). All these constraints significantly reduce the successful commercialization of the generated transgenic

plant varieties. TALEN was successfully utilized to create *BADH2* mutants more efficiently in a short time. Six heterozygous mutants were obtained from 20 transgenic lines (T_1), and 4 lines which were forwarded to T_2 generation also showed efficient mutation. The two AP contents varied from 0 to 0.35–0.75 mg/kg equivalent to positive control (Shan et al. 2015).

20.4.1.8 GE for Controlling Browning in Fruits and Vegetables

Browning of fruits and vegetables is a major problem in the agriculture sector as it damages organoleptic value as well as nutritional losses. This ultimately leads to the lowering down of market price. Browning occurs due to the formation of quinones from phenolic as a result of the action of enzyme polyphenol oxidase. The potato mutants were successfully generated for the *polyphenol oxidase* (*PPO*) by CRISPR/Cas9. It was observed that among all, 68% of plants showed mutation in at least one allele of gene, while 24% showed mutation at four alleles. Moreover, there were no off-target effects found, and about 69% reduction in the activity of *PPO* was observed along with the reduction in enzymatic browning up to 75% (Gonalez et al. 2020). Similarly, browning has been prevented in mushrooms using the above approach and approved by USDA for consumption (Waltz 2016a, b).

20.4.2 GE for Herbicide Resistance in Crops

Weeds are the major competitor for crops with the resources such as nutrients, water, space, and light which results in decrement in crop yield. So, effective techniques for weed management are used to achieve maximum yield and crop productivity. Generally, there are two methods: First is the spraying of herbicide which usually targets proteins involved in plants' metabolic pathways. These pathways are essential for plant's cellular functions; non-functioning of the pathway ultimately kills the weed plant. But, it may cause damage to crops and shows the negative impacts on human health and environment too. Second is the use of biotechnology to transfer herbicide tolerance genes into the crop plants to make them herbicide tolerant/resistant and more environmentally friendly (Lombardo et al. 2016).

The *ALS* (*Acetolactate synthase*) is a key enzyme for the biosynthesis of branched-chain amino acids such as valine, leucine, and isoleucine (Lee et al. 1988; Chipman et al. 1998). The herbicides, viz., sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthio (or oxy)-benzoates, and sulfonylamino-carbonyl-triazolinones, act by inhibiting the enzymatic activity and blocking the synthesis of amino acids (Mazur et al. 1987; Zhou et al. 2007).

The GE platform has been successfully deployed for the development of herbicide-tolerant crops. In *Linum usitatissimum*, mutations were created by CRISPR/Cas9 using single-stranded oligo repair template at phosphoenol pyruvate site, which introduces two SNPs in *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene and substitutes T178I and P182A (TIPA) that ultimately results in inhibiting the binding of herbicide glyphosate (Sauer et al. 2016). Similarly, herbicide resistance rice is developed by replacing the second rice exon encoding the

endogenous gene *EPSPS*, with the new exon which consists of several nucleotide substitutions followed by the amino acid substitution (T102I and P106S), and this finally results in herbicide resistance rice cultivar (Li et al. 2016a). Additionally, by making use of GE technology, the gene replacement has been performed in tobacco for alternating the conserved region of *ALS* gene which results in the ineffective inhibition by these herbicides. In tobacco, the *ALS* gene mutation is found to confer the resistance for sulfonylurea herbicides using ZFN-mediated gene targeting method (Cai et al. 2009; Shukla et al. 2009; Townsend et al. 2009), while the TALENs and CRISPR/Cas9 have been deployed in crops like potato (Butler et al. 2015), maize (Svitashev et al. 2015), and rice (Li et al. 2015; Sun et al. 2016) for generating the herbicide-resistant crops targeting the same gene.

Chlorsulfuron and bispyribac-sodium (BS) herbicides are used to control a wide variety of grasses and dicot weeds, which mainly target the *OsALS* and block the synthesis of branched-chain amino acids. TALEN-based GE was exploited to create point mutation in the *OsALS* with an efficiency of 6.3% (Li et al. 2016a). Similarly, CRISPR/Cas9 was used to introduce point mutations into targeted regions of *OsALS* (Sun et al. 2016). In a study by Zhang et al. (2019a), base editing was done for the development herbicide tolerance in wheat with new selectable marker gene (Zhang et al. 2019a).

20.4.3 GE for Biotic Stress Tolerance

Significant productivity losses in crops have been reported due to biotic and abiotic stress (Vats et al. 2019). The GE promotes efficient, target-specific gene modifications for developing resistance against both biotic and abiotic stresses (Chen and Gao 2014; Zhang et al. 2019b). The CRISPR/Cas is being used for creating alleles and knocking out the genes for bacterial and fungal diseases. In this section, we have brought up few of the GE applications and the way by which these biotic and abiotic stresses can be tackled and crop improvement can be achieved. Over the years, the plant diseases are gradually being recognized as a global threat, as there is reduced yield of food crops due to biotic stress, in spite of executing advanced agricultural practices (Haque et al. 2018). Bacterial infection, fungal pathogens, viruses, nematodes, and pests are the biotic stresses encountered by the crops that hinder their productivity. Therefore, the development of resistant crops toward these stresses by gene modification would ensure the improvement in crop yield and viability (Vats et al. 2019).

20.4.3.1 GE for Bacterial Resistance

One of the biotic stresses includes bacterial infection caused in plants. Unfortunately, bacterial phytopathogens go undetected many times and are difficult to control; thus, prevention and exclusion of these pathogens via inducing genetic resistance is one of the sustainable ways to control these diseases (Zafar et al. 2020; Borrelli et al. 2018). The gene editing techniques are being used to eradicate this susceptibility to diseases. The CRISPR/Cas9-mediated silencing of rice bacterial

blight susceptible genes *OsSWEET11* and *OsSWEET14* and *OsSWEET13* have been reported to impart resistance (Jiang et al. 2013; Zhou et al. 2015). In another report, resistance against rice bacterial blight disease was reported using TALEN-based mutagenesis of *OsSWEET14* promoter leading to the disruption of the gene in rice (Li et al. 2012).

One of the most damaging bacterial diseases that negatively affects fruit productivity is citrus canker (Peng et al. 2017). Peng et al. (2017) have reported the improvement of citrus canker resistance by targeting EBE of CsLOB1 promoter using CRISPR/Cas9. The five pCas9/CsLOB1sgRNA constructs modified the EBE PthA4 of the CsLOB1 promoter in Wanjincheng orange (*Citrus sinensis* (L.) Osbeck) giving resistance. Recently, there has been another report wherein CRISPR/Cas9-mediated modification in CsWRKY22 gene has shown a reduction in susceptibility to the citrus canker disease caused by *Xanthomonas citri* subsp. citri in Wanjincheng oranges (Wang et al. 2018b). A mutant tomato plant was generated by employing CRISPR/Cas9-mediated deletion in *SIDMR6–1* gene, resulting in frameshift mutation and premature truncation of the protein. This tomato *SIDMR6–1* gene is usually observed to be upregulated when infected with *Pseudomonas syringae* pv. tomato and *Phytophthora capsici*. The mutated transgenic tomato plants showed broad-spectrum disease resistance against pathogens, such as *P. capsici*, *P. syringae*, and *Xanthomona* species (Paula de Toledo Thomazella et al. 2016).

20.4.3.2 GE for Fungal Resistance

Fungal infections have been impacting on the crop production, as they cause massive destruction. Thus, many genotypes have been developed with the aid of gene editing techniques in order to combat these infections. Likewise, worldwide rice blast (CO: *Magnaporthe oryzae*) has been known to extensively destruct fungal infection. A plant ethylene responsive factor (ERF) is a subfamily of the APETALA2/ethylene response factor (AP2/ERF) which is involved in multiple stress tolerance. The mutant rice lines developed by targeting OsERF922 imparted the enhanced blast resistance (Wang et al. 2016). Similarly, rice mutant lines developed by *OsSEC3A* gene knockout using CRISPR/Cas9 technology were reported which showed an increased resistance against rice blast disease. Further destruction of *OsSEC3A* has demonstrated resistance with increased levels of salicylic acid imparting pathogen resistance (Ma et al. 2018).

Bread wheat is one of the staple crops; thus, it is economically feasible to have improved biotic-resistant wheat varieties. In wheat, powdery mildew is caused by *Blumeria graminis* f. sp. tritici (Bgt). Wang et al. (2014) introduced improved hexaploid bread wheat variety with heritable resistance against powdery mildew disease by targeting all three (TaMLO-A1, TaMLO-B1, and TaMLOD1) homo alleles in wheat that encodes mildew resistance locus O (MLO) proteins. They have achieved this by using TALEN as well as CRISPR/Cas9 technique, respectively. In another example of wheat, three homologs of wheat *TaEDR1* gene, a negative regulator for the defense response, were targeted to achieve TaEDR1 mutant lines with increased tolerance against powdery mildew (Zhang et al. 2017).

There are 16 *MILDEW RESISTANT LOCUS O* (*Mlo*) genes, i.e., *SlMlo1* to *SlMlo16*, in tomatoes. Nekrasov et al. (2017) targeted the *SlMlo1* locus using double sgRNA strategy which resulted in the mutant line with increased resistance against powdery mildew (CO: *Oidium neolycopersici*). In another report, CRISPR/Cas9 technology was used to incorporate small deletions in the *SlDMR6–1* gene. This gene was reported to upregulate when the plant is infected by *Pseudomonas syringae* pv. tomato and *Phytophthora capsici*. Thus, the small deletions in the gene resulted in frameshift mutation leading to premature truncation in the protein and made the tomato mutant lines resistant toward *Phytophthora* spp. and *Pseudomonas syringae* (Paula de Toledo Thomazella et al. 2016).

20.4.3.3 GE for Viral Resistance

Viral infections have been a topic of concern from the past few years, because they have quite a serious impact on plants. Therefore, having significant loss in terms of food and economy, GE could aid to gain resistance and overcome these stresses. GE techniques were used in the case of cassava brown streak disease caused by Ugandan cassava brown streak virus (UCBSV) and cassava brown streak virus (CBSV), which is the main concern in the case of cassava yield. These viruses mainly require the host eukaryotic translation initiation factor 4E (eIF4E) and interaction of the viral genome-linked protein (VPg) to cause disease. CBSV and UCBSV encode five eIF4E and novel cap-binding protein-1 (nCBP-1) and nCBP-2 (Kropiwnicka et al. 2015). Gomez et al. (2019) demonstrated that the CRISPR/Cas9-based multiple gene modification of cassava elF4E isoforms nCBP-1 and nCBP-2 simultaneously has developed tolerance against the virus in cassava. Ali et al. (2015) have shown significant attenuation of viral infection by delivering sgRNAs for sequences of tomato yellow leaf curl virus (TYLCV). This caused overexpression of Cas9 endonuclease in *Nicotiana benthamiana* and conferred resistance toward TYLCV. Chandrasekaran et al. (2016) develop cucumber-resistant line against cucumber vein yellowing virus, potyviruses, zucchini yellow mosaic virus, and papaya ringspot mosaic virus-W. They have achieved this by disrupting eIF4E and generating transgene-free heterozygous eIF4E mutant plants. They design gRNA to target the N- and C-terminals of eIF4E. Wheat dwarf virus (WDV) belonging to the *Geminiviridae* family infects both wheat and barley resulting in severe yield loss. By employing CRISPR/Cas9 system, Kis et al. (2019a, b) designed four gRNAs targeting MP and CP, Rep/RepA coding sequence, C-terminus of Rep, and LIR region. These gRNAs were further cloned and transferred into the barley crop using *Agrobacterium* sp.-mediated gene transfer technique. Their experiment confirmed the barley crop resistant to WDH in T_0 and T_1, respectively.

20.4.3.4 GE for Pest and Insect Resistance

Introducing insect-resistant plants via genetic modification is crucial in agriculture, because insect damage is a serious issue in agriculture. It reduces the crop quality, yield, and ultimately productivity (Parmar et al. 2017). The use of pesticides to solve this issue has proven to be unsafe for humans and even for the environment. Development of insect-pest-resistant/insect-pest-tolerant varieties is one of the best

possible ways to achieve good yield by maintaining environment safety too (Aswar Rao et al. 2017). In rice, tryptamine 5-hydroxylase cytochrome encoded by the gene *CYP71A1* catalyzes the conversion of tryptamine to serotonin. Lu et al. (2018) reported the knockdown of CYP71A1 gene that reduced serotonin production, increased the salicylic acid levels, resulting in the reduced growth in plant hoppers. The GE-mediated improvement to obtain insect or pest resistance in plants needs still to be explored, whereas most of the work should be focused on editing and modifying genes of insects and pests.

20.4.4 GE for Abiotic Stress Tolerance

The major environmental constraints or abiotic stresses which adversely hamper the crop growth, development, and productivity include drought, salinity, high temperature, cold, etc. Conventional plant breeding is a highly laborious, time-taking, and resource-intensive process. In this regard, GE has paved the way for developing stress-tolerant genotypes because of their simplicity, specificity, accuracy, and precise target modification. GE has wider application for improving abiotic stress tolerance, which ultimately helps for increased crop yield (Surabhi et al. 2019; Parmar et al. 2017).

20.4.4.1 GE for Drought Tolerance

Crop production and yield are drastically impacted by drought conditions (Martignago et al. 2020). However, several plants show tolerances toward drought. There are some examples where drought tolerance is achieved using GE (Mahmood et al. 2019). Leaf morphology play an important role in the drought tolerance of plant. In rice plant, *semi-rolled leaf* (*SRL*) gene encodes glycosyl phosphatidyl inositol-anchored protein that regulates leaf rolling on the adaxial side. Liao et al. (2019) create mutation in *SRL1* and *SRL2* genes by using CRISPR/Cas9 technology. The mutant *SRL1* line showed reduction in lignin contents and cellulose in the epidermis, and the mutant *SRL2* showed curved and narrow leaves which are important morphological characteristics for drought tolerance. Recently, in rice cultivar MTU1010, CRISPR/Cas9-based GE has been used to target *OsDST* genes in order to achieve drought and salt tolerance. This DST mutant line showed broader leaf width and reduced stomatal density, which resulted in drought tolerance (Santosh Kumar et al. 2020). *1-Aminocyclopro-pane-1-carboxylic acid synthase6* (*ARGOS*) is a negative regulator of the ethylene response in maize plants. Plants with overexpression of *ARGOS8* gene in drought stress have shown reduced ethylene sensitivity and improved grain yield. Shi et al. (2017) replaced the native promoter of *ARGOS8* with GOS2 promoter with the help of CRISPR, resulting in increased yield from five bushels per acre in mutant type as compared to the wild type under drought stress at flowering stage.

20.4.4.2 GE for Salinity Tolerance

Salt stress (salinity) is an important abiotic stress which affects the crop growth and yield worldwide mainly at the coastal area. To develop the salinity-tolerant cultivars through GE is the most cost-effective and environmentally friendly approach. Rice *OsRR22* gene is a transcription factor that is involved in cytokinin signal transduction and metabolism. Takagi et al. (2015) reported increased salt tolerance by loss of function of *OsRR22* gene. In another study, Zhang et al. (2019b) developed a mutant rice line with improved tolerance for salinity by Cas9-OsRR22-gRNA expression vector which was designed to knock out the *OsRR22* gene. They observed good salt tolerance at seedling stage. Under stress conditions, root development is promoted by the downregulation of *SlARF4* gene and increases soluble sugars' content and also maintains the content of chlorophyll at high level. Under normal and stressful conditions, *ARF4* shows higher salt tolerance and osmotic stress by reducing stomatal conductance along with increased leaf water content and abscisic acid (ABA). Bouzroud et al. (2020) knocked out the tomato's SlARF4 gene with the help of CRISPR/Cas9 which resulted in the increased tolerance level of tomato plants against salt and osmotic stresses.

20.4.4.3 GE for Cold Tolerance

Abiotic stresses have become a major concern in agriculture; one of the major environmental stresses is cold stress. Many tropical crops show reduced growth and yield. Therefore, this is one of the major concerns upon inducing tolerance toward this stress. Achieving cold tolerance using GE is one of the approachable solutions. In plant development and stress responses, the plant-specific RING (really interesting new gene) domain finger proteins are confined to important roles. Fang et al. (2015) showed that the suppression of *OsSRFP1* (*Oryza sativa stress-related RING finger protein 1*) gene expression conferred to cold tolerance. Fang et al. (2016) reported the knockdown *OsSRFP1*, that encodes for E3 ubiquitin ligase, using CRISPR/Cas9 which leads to cold tolerance through enhancing antioxidant protection in rice. Likewise, rice growth and yield can be affected drastically by cold stress which eventually leads to less grain production. Reducing the expression levels of *OsPIN5b* lead to the formation of longer panicles, higher tiller number, and higher rice yield (Lu et al. 2015). Under cold stress, OsMYB30 and OsJAZ9 complex suppresses the BMY gene expression and affects the degradation of starch and maltose accumulation, which resulted in increased cold sensitivity (Lv et al. 2017). To overcome this, Zeng et al. (2020) targeted *OsPIN5b* (a panicle length gene), *GS3* (a grain size gene), and *OsMYB30* (a cold tolerance gene).

20.4.4.4 GE for Heat Tolerance

Heat stress is becoming a serious problem for agriculture and becoming severe day by day in the face of climate change. Generally, at high temperature, there is high reactive oxygen species (ROS) production, metabolic instability, and membrane damage in plant cells, thus reducing the crop productivity (Iba 2002; Kumar et al. 2015, 2016). In order to combat this issue, GE is proving its potential. In the tomato plant, *SLAGAMOUS-LIKE 6* (*SlAGL6*) gene controls the transition from the state of

"ovary arrest" to fertilization-triggered fruit set. Klap et al. (2017) performed knockout of the *SlAGL6* gene using CRISPR/Cas9 with synthetic gRNA which was designed to target the second exon. Under natural heat stress conditions, fruit yield of *SlAGL6* mutant line was significantly higher. The 9-cis-EPOXYCAROTENOID DIOXYGENASE4 (NCED4) is a gene coding regulatory enzyme involved in the biosynthesis of ABA. Bertier et al. (2018) knock out the NCED4 gene by the CRISPR/Cas9 system and achieved seed germination even at higher temperatures (germinating >70% at 37 ° C). In tomato, a total of 16 putative SlMAPK (mitogen-activated protein kinases) genes were identified for development, regulation, etc. Yu et al. (2019) demonstrated knockout of SlMAPK3 that enhanced heat tolerance.

20.4.5 GE for Improved Crop Yield

Crop yield and productivity are a matter of concern with respect to population growth and climate change. Sustainable food production is challenging with competition in resources like water and land and factors like climatic changes, rise in cost, etc. (Usman et al. 2020). Efficient and innovative technique such as GE is a good aid to enhance the productivity of crops. The axillary meristem plays an important role in plant architecture which is regulated by hormonal and environmental factors. The *MAX1*, *MAX3*, and *MAX4* genes encode cytochrome P450 (carotenoid cleavage dioxygenase 7) CCD7 and CCD8, respectively, which are involved in strigolactone (SL) biosynthesis. Zheng et al. (2020) used the CRISPR/Cas9 system to knock out two rapeseed (*Brassica napus* L.) homologs (*BnaMAX1*). This experiment resulted in semi-dwarf, increased branching phenotypes and directly increased yield per plant. *Cytochrome P450s* (*Cyt P450s*) play a big role in biochemical pathways, plant metabolism, cell proliferation, and expansion. In rice, *big grain2* (*BG2*) gene encodes for Cyt P450, OsCYP78A13. The grain size and exon region variations in these genes determined the difference in grain yield (Xu et al. 2015). Usman et al. (2020) targeted three *cytochrome P450* homoeologs (*Os03g0603100*, *Os03g0568400*, and *GL3.2*) and *OsBADH2* and developed rice mutants with high yield and improved aroma. Mutation in each gene resulted in increased grain weight, and the triple mutant displayed higher yield.

In another case, Li et al. (2016b) targeted *Gn1a* (*GRAIN NUMBER 1a*), *DEP1* (*DENSE AND ERECT PANICLE*), *GS3* (*Grain Size*), and *IPA1* (*IDEAL PLANT ARCHITECTURE1*) genes that are regulators of grain number, panicle architecture, grain size, and plant architecture, respectively, in rice. The editing of the above genes via CRISPR/Cas9 resulted in *gn1a*, *dep1*, and *gs3* mutants with enhanced grain number, dense erect panicles, and larger grain size, respectively. In rice, *LAZY1* gene plays an important role in tiller-spreading phenotype, and the tiller number is directly proportional to yield. Miao et al. (2013) developed a mutant line by targeting the *LAZY1* gene using the CRISPR/Cas9 system, and 11 lines carrying mutations in the specific region of *LAZY1* gene were found. Zsögön et al. (2018) used CRISPR/Cas9 to target six genes, viz., SELFPRUNING, OVATE, FASCIATED, FRUIT

WEIGHT 2.2, LYCOPENE BETA CYCLASE, and MULTIFLORA, of tomato plant simultaneously that are involved in growth habit, fruit shape, size, nutritional quality, number, etc. The developed line with all the six mutant genes displayed threefold increase in fruit size and tenfold increase in fruit number and by 500% lycopene accumulation.

20.4.6 GE for Crop Domestication

Plant domestication has been in practice from the ancient era. For most of the crops, the domestication of wild species was carried out on the basis of very few limited traits such as ease of cultivation, harvesting, processing, etc. which resulted in the loss of genetic variability due to the lack of broad basis selection (Zhang et al. 2020a). Earlier, the traditional breeding methods were used to domesticate the wild species, but these are time-consuming processes and have many constraints. The emergence of the genome editing technologies like ZFNs, TALENs, and CRISPR/Cas9 has been a new revolution that has speed up the breeding process. Particularly, CRISPR/Cas9 creates mutation that resembles the natural mutation, and further with the advent of new technologies, marker-free plants are created. The technique was successfully used for the domestication of tomato *Solanum pimpinellifolium* which is a high stress-tolerant species but lacks in fruit production (Li et al. 2018c). Similarly, six different loci associated with yield have been edited in the same species by CRISPR/Cas9 (Zsogon et al. 2018). This technology is beneficial to domesticate some perennial crops as well as crops like banana, cassava, gooseberry, etc. In one approach, orphan crop ground cherry was domesticated by editing orthologous genes (*Solanum pimpinellifolium*) responsible for plant architecture, flower production, and fruit size (Lemmon et al. 2018).

20.4.7 GE for Hybrid Production

Heterosis breeding is a widely adapted strategy by plant breeders to improve the agronomic characters (Schnable et al. 2013). This method is challenging, and some important phenotypes are lost after subsequent generation. The clonal propagation of hybrid seed is one approach to tackle this problem. Wang et al. (2019) reported a solution to the problem by editing three genes *REC8* (*Recombination Protein A*), *PAIR1* (*protein HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS 1*), and *OSD* (*OMISSION OF SECOND DIVISION*) meiotic genes in the meiotic pathway along with one gene associated with fertilization *MATRILINEAL* (*MTL*) gene in rice. This could induce the formation of haploid seeds in hybrid rice, and heterozygous haploid plants were produced that were further clonally propagated.

20.4.8 GE for photoperiod adaptation

Day length influences the flowering in plants, but day length sensitivity of crop limits its cultivation at different places. Photoperiod sensitivity becomes one of the major constraints in the domestication of wild species (Xu et al. 2013). This problem can be tackled by editing genes related to flowering induction and repression. The florigen paralog and flowering repressor, *SELF-PRUNING 5G* (*SP5G*) in tomato was mutated using CRISPR/Cas9 that resulted in the rapid flowering and quick burst of flower production giving early yield (Soyk et al. 2017).

20.4.9 GE for the Development of Male Sterile and Transgene-Free Crops

Production of transgene-free crops is considered as an important aspect of gene editing studies. Removal of transgene ensures no unnecessary changes in genome as well as prevents off-target cuts by cas9 enzyme. There are few approaches of producing transgene-free plants. One such approach for the removal of transgene can be achieved by the use of some suicide genes like *Barnase* and *CMS*, which results in the development of male sterile plants. These genes were successfully used to produce transgene-free and male sterile *Oryza sativa* (He et al. 2016). The barnase gene was placed under the REG2 promoter (early embryo specific), and *CMS* gene was kept under the control of Camv35s promoter. Due to the toxic action of barnase and lethal action of *CMS*, transgene-free plants were obtained in single generation. This strategy is limited to plants regenerated using tissue culture.

20.4.10 The Policies, Governance, and Regulatory Landscape of Genome-Edited Food Crops

The deployment of genome editing in agriculture is largely based on how they are regulated. The governance and policies of regulating the gene-edited (GE) crops greatly vary among countries (El-Mounadi et al. 2020). These dissimilarities are due to varied definitions, legislation, and regulatory frameworks for GE crops (Zhang et al. 2020a; Lusser et al. 2013). The existing regulatory frameworks were either process based (which focus on the technique of origin in developing the new crop plant variety) or product based (focus mainly on the risk caused by the ultimate food products derived from GE crops). In countries where the GE crops are regulated as product based, they are not treated equivalent to GMO; however, they were treated similar to spontaneous or physical or chemical mutagenesis-derived products since there is no foreign gene or DNA introduction from other species (Zhang et al. 2020a). However, a process-based regulatory framework existing in the European Union treats the GE crops as GMOs (Callaway et al. 2018). In addition to the above discussed criteria, there exists regulatory classification for GE crops into three categories viz Site Directed Nuclease-1 (SDN-1) where sequence changes are

allowed with very minimum of nucleotide base changes with no foreign DNA introduced to improve the plant variety; SDN-2, where up to 20 nucleotide changes are allowed to introduce to improve the existing crop variety; SDN-3, where a large DNA template or sometimes a gene is introduced into the plant variety under improvement (Podevin et al. 2013; Wolt 2017). Based on the basis of above discussed classifications, different countries have diverse regulatory policies.

Here, in the current section, holistic views of opinion in addressing the various regulatory policies existing among different nations are discussed. Eckerstorfer et al. (2019) reviewed and compared the regulatory frameworks among European Union (EU) and non-EU countries. The study is based on an up-to-date literature analysis and interviews with the regulatory experts of different nations. The authors proposed five strategies for the nations to regulate non-GM crops. According to the author's point of view, synchronized regulatory framework of GE crops is essential for transparent international trade. Hence, the authors proposed to introduce an international public repository of biotechnology-derived products. Schiemann et al. (2020) had recently reviewed the policies and governance of GE in plants (which also includes regulatory framework, socioeconomic aspects, risk assessment, ethics, etc.). The basis of this review is based on a wide range of research articles collected and summarized till date. The authors illustrated the broad and complex landscape of ideas which need to be addressed for the success of GE in plants. They stated that it is essential to attain multiple sustainable development goals of food and nutritional security provided its scientific progress is coupled with policy and governance issues. Such issues need to be sorted out to attain synchrony at both national and international levels. Rim Lassoued et al. (2019) have conducted risk and safety considerations of GE crops through a survey of a consortium of international experts in plant biotechnology. They found that GE crops pose marginal risks for human and environment. However, the uses of technology-driven crops in agriculture were highly discouraged. Since the policymakers were driven mainly by sociopolitical factors rather than scientific principles, Steffi Friedrichs et al. (2019) had organized the conference session on regulatory aspects of genome editing, where government representatives from six nations discussed the regulatory frameworks pertaining to genome editing (The Organisation for Economic Co-operation and Development (OECD) Conference on Genome Editing: Applications in Agriculture—Implications for Health, Environment and Regulation). A detailed description of regulatory framework existing in different countries has been well reviewed and summarized by the authors (Eckerstorfer et al. 2019).

The regulatory frameworks of some important nations are discussed. In the USA, the US Department of Agriculture (USDA) exempted the GE waxy corn (endosperm starch with exclusive amylopectin) and non-browning mushrooms from GMO regulation (Waltz 2016a). In addition, USDA in 2017 proposed the regulatory framework for the exemption of SDN-1- and SDN-2-based GE crops (https://go.nature.com/2VBr64V). Argentina in 2015 made a regulatory framework for GE crops (Whelan and Lema 2015), and in 2018, it excluded the SDN-1-derived GE products from GMO regulations. However, SDN-3-derived GE crops were considered as GMO, and no regulatory criteria were framed for SDN-2-derived GE crops

(Lema 2019). The same is the case of countries like Brazil and Chile (Duensing et al. 2018). Canada's regulatory policy states that GE-edited novel products must undergo scrutiny before their commercial release for their toxicity, allergenicity, and impact on non-target organism. Canada has approved GE-edited non-browning apples and non-dark spot potatoes for commercial release, and they are available in the market after strict evaluation (Walt 2016a, b). The European Union remains politically opposed for GE crops (Walt 2016b). China is very actively involved in GE of various food crops. Though it has strict regulations for the use of GMO (except papaya; GM papaya is grown in China for domestic needs), soon it may follow the USA and exclude GE crops from GMOs. In India, the GE crops were considered as process based and have strict regulation. In January 2020, India published draft guidelines for the regulation and risk assessment of GE organisms and is still in discussion mode and yet to take decision in this regard (Table 20.2) (http://dbtindia.gov.in/latest-announcement/dbt-invites-comments-%E2%80%9Cdraft-document-genome-edited-organisms-regulatory). Based on the tiered approach, the GE plants derived from SDN-1 technique are exempted from GMO regulations; however, there is still lacuna in discriminating SDN-1- and SDN-2-derived GE crops. Further, the SDN-3 technique-derived GE plant varieties were kept under the umbrella of GMO regulations. There is still no specific regulatory framework for the regulation of GE crops in India, and all technology-driven crop improvements are still under existing regulatory framework (El-Mounadi et al. 2020) (Fig. 20.3). For further information about the extended GE crop updates, global gene editing tracker can be visited, an online platform facilitating the countrywise GE crops developed and the regulatory framework executed (https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/india-crops-food/#jet-tabs-control-9203). In order to overcome the vagueness in policy making, the global scientists had proposed reevaluation and science-based regulatory framework for GE crops (Huang et al. 2016; El-Mounadi et al. 2020).

There exist a high level of asynchrony, regulatory uncertainty, and non-harmonized policy making among nations representing a bottleneck in harnessing the technology-driven crop improvement effectively and efficiently. GE in futuristic crops has a wide scope to increase the yield potential, to obtain climate resilience varieties which can overcome biotic and abiotic stresses accompanied by high nutritive value. Further, it is the responsibility of the researchers to address the risk, safety, and environment concerns. In addition, the scientific community and the policy makers must work hand in hand to create general awareness by presenting the scientific progress and the facts of technology-driven products for consumer acceptance and purchase.

20.4.11 Futuristic Crops

According to the UN Food and Agriculture Organization, we should be able to achieve double food production by 2050 (Ray et al. 2013). The three possible solutions are available for increasing crop resources to tackle the danger of food

Table 20.2 Regulatory bodies for GMO/genome editing in India

Statutory body constituted in India	Responsibilities	Governing body	Reference
rDNA advisory committee (RDAC)	Advisory committee for policy making on emerging technologies	Department of Biotechnology, ministry of science and technology	Ahuja et al. (2018)
Institutional biosafety committee (IBSC)	Research and development; contained experiments	Registered institutions; private companies; universities	Ahuja et al. (2018)
Review committee on genetic manipulation (RCGM)	Frames guidelines and conducts scientific risk assessment of plants, animals, microbes, and pharma products	Department of Biotechnology, ministry of science and technology	Ahuja et al. (2018)
Genetic engineering appraisal committee (GEAC)	Apex committee for environmental risk assessment of GM organisms Final approval for the environmental and commercial release of GM foods or its derived products	Ministry of Environment, and Forest and climate change	Ahuja et al. (2018) Ahuja et al. (2018)
GEAC and RCGM	Event selection trials/BRL 1 trials	Department of Biotechnology, ministry of science and technology	Ahuja et al. (2018)
State biotechnology coordination committee (SBCC)	State-level monitoring and supervision	Governed by state government	Ahuja et al. (2018)
District-level committee (DLC)	District-level monitoring and supervision	Governed by state government	Ahuja et al. (2018)
Food safety and standards Authority of India (FSSAI)	Food safety assessment of GM foods (viable and processed) Approval for the commercial release of GM foods (processed)	Ministry of Health and Family Welfare	Ahuja et al. (2018)
Legislation/act	Responsibility	Governing body	Reference
Rules 1989 (Under the Environment (Protection) Act)	Regulate and monitor the entire spectrum of activities of GMOs and their derived products, which includes production, manufacturing, packing, sale, export, import, etc.	Implemented jointly by the Ministry of Environment, Forest and Climate Change and Dept. of Biotechnology and State Governments	Ahuja et al. (2018)

(continued)

Table 20.2 (continued)

Legislation/act	Responsibility	Governing body	Reference
Plant Quarantine (Regulation For Import Into India) Order 2003	Regulates the import of germplasm/GMOs/ transgenic plants for R&D purpose	Ministry of Agriculture and Farmers' Welfare	Ahuja et al. (2018)
Biological Diversity Act, 2002	Regulates the genes used in R&D for crop and livestock improvement through genetic manipulation	National Biodiversity Authority	Ahuja et al. (2018)
The Food Safety and Standards Act, 2006	Regulates the manufacture, storage, distribution, sale, and import of food which includes GM food	Food Safety and Standards Authority of India	Ahuja et al. (2018)

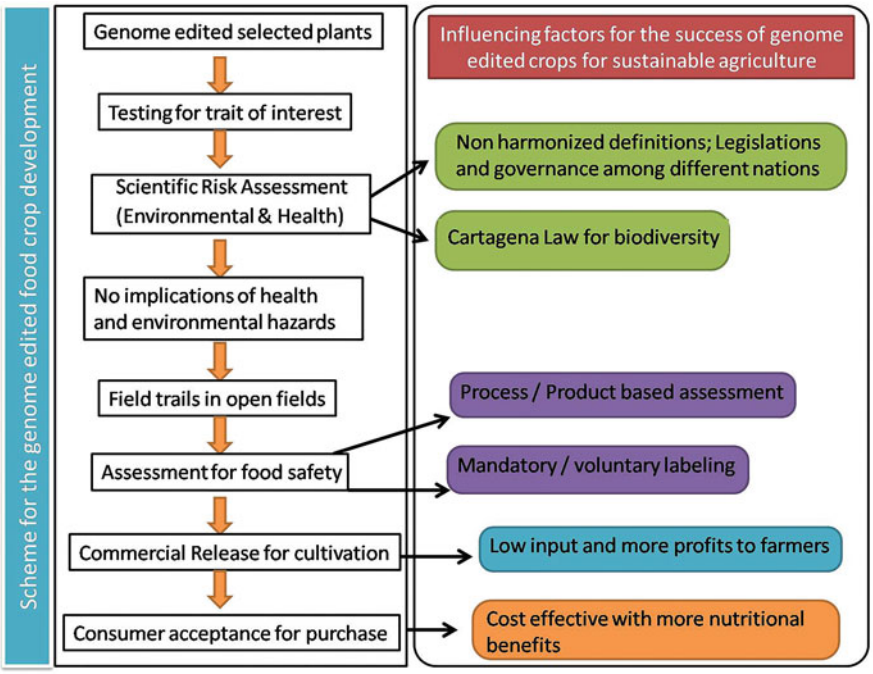

Fig. 20.3 Proposed scheme for the development of genome-edited food crops for sustainable agriculture in the near future

security: (1) increment in the production of the major food crops; (2) exploring the underutilized crops; and (3) exploring the available plant biodiversity to find completely new food crops. The first option is widely exploited to fulfill the food requirement of population. Presently, wheat, rice, maize, and soybean are the major food crops of the global population and contributing two-thirds of the calories we harvest from fields. For the future of global food security, we should be able to increase the production of these four food crops. But actual increments in these four major food crops, namely, maize, rice, wheat, and soybean, have been estimated to be 1.6%, 1.0%, 0.9%, and 1.3% per year (Ray et al. 2013). There is stagnation in the production and yield of major food crops of the world. There are many underutilized crops which are also called minor crops or orphan crops or neglected crops. Underutilized crops are locally important in many places but are not widely adopted for commercial cultivation at their place of origin and other places as well. Underutilized crops like African yam bean, lima bean, winged bean, moringa, jack bean, proso millet, foxtail millets, amaranth, quinoa, bambara groundnuts, kedondong berry, sweet potato, buck wheat, emmer wheat, sesame, safflower, etc. have a great potential as food crops, as they are highly nutritious and rich in calories. Moreover, their sturdy nature and tolerance toward many biotic and abiotic stresses make them suitable candidates for increasing crop production even in unfavorable conditions. Searching alternative food crops is also one of the best possible solutions for combating food security challenges. We have around 4,00,000 plant species on earth, but among these, only 200 species have been domesticated and used as a food (Moshelion and Altman 2015). Among the 200 domesticated crops, very few are mainly used as food crops in regular diet. *Limnanthes alba* (meadowfoam), used for a source of unique seed oils, *Taxus brevifolia,* source of Taxol (anticarcinogen), Kiwifruit (*Actinidia deliciosa*) are some of the examples of new food crops (Janick 1999). There is a great scope to extend our choice for food crops in the platter. Plant breeders and researchers need to focus on the genetic gain of underutilized crops, wild species, and unadapted germplasm.

New genomics-based techniques or new plant breeding technologies especially GE can be exploited to speed up the plant domestication process (Li et al. 2018c). Around 60 genes are listed by Meyer and Purugganan (2013) whose variants were reported to be involved in crop domestication process. Further, he reported that mutations for loss of function in the proposed genes were associated with domestication processes. Such GE mutation in the proposed genes and silencing genes for undesirable traits will help to enhance and speed up the domestication process of many potential new food crops. Traits like higher yield, insect-pest-disease resistance, abiotic stress resistance, nutritional value, anti-nutritional factors, food quality, ease of harvesting, storability, ease of propagation, reduced juvenility, maturation period, photo-insensitivity, reduced cooking time, reduced shattering, etc. can be targeted for improvement (Table 20.3). Recently, Li et al. (2018c) and Zsögön et al. (2018) have successfully used GE to target domestication-related genes

Table 20.3 List of undesirable traits associated with underutilized crops

Neglected crops	Botanical name	Traits to be improved	References
African yam bean	*Sphenostylis stenocarpa*	• Cooking time—requires long cooking time • Growth habit—requires mandatory staking • Poor seed quality • Post-harvest diseases • Pod shattering • Anti-nutritional factors	Oagile et al. (2007), Popoola et al. (2019)
Winged bean	*Psophocarpus tetragonolobus*	• Indeterminate growth habit • Pod shattering • Late maturing • Low yield • Scandent habit or climbing habit • Anti-nutritional factors	Wong et al. (2014), Popoola et al. (2019)
Dolichos bean (hyacinth bean)	*Lablab purpureus*	• Susceptibility to diseases • Susceptibility to insect and pest	Rai et al. (2018), Oboh et al. (2000), Popoola et al. (2019)
Lima bean	*Phaseolus lunatus*	• Susceptibility to diseases • Susceptibility to insect and pest • Growth habit—requires mandatory staking	Baudoin (1993), Popoola et al. (2019)
Jack bean	*Canavalia ensiformis*	• Cooking time—requires long cooking time • Anti-nutritional factors	Viera (1996), Popoola et al. (2019)
Common bean	*Phaseolus vulgaris*	• Susceptibility to insect and pest • Low yield • Susceptibility to various biotic stresses	Baldermann et al. (2016), Popoola et al. (2019)
Sword bean	*Canavalia gladiata*	• Hard seed coat • Susceptibility to insect and pest • Anti-nutritional factors	Viera (1996), Popoola et al. (2019)
Pigeon pea	*Cajanus cajan*	• Growth habit—tallness • Susceptibility to insect and pest • Cooking time—requires long cooking time	Pontes Junior et al. (2016), Popoola et al. (2019)
Bambara groundnut	*Vigna subterranea*	• Susceptibility to insect and pest • Low yield • Cooking time—requires long cooking time	Mubaiwa et al. (2018), Bamba et al. (2017), Popoola et al. (2019)

(continued)

Table 20.3 (continued)

Neglected crops	Botanical name	Traits to be improved	References
		• Difficulty in dehulling • Anti-nutritional factors	
Quinoa	*Chenopodium quinoa*	• High yield • Development of better environmentally adapted varieties • Anti-nutritional factors	Gargiulo et al. (2019), Bazile et al. (2016), Ferranti et al. (2018)
Kersting's groundnut	*Kerstingiella geocarpa*	• Post-harvest pest attack	Ayenan and Ezin (2016), Popoola et al. (2019)

in the wild progenitor of tomato, which proves the potential of GE in speeding up the domestication process of plants. Recently, the Less Shattering1 (*SvLes1*) gene was identified in green millet (*Setaria viridis*), the close relative of foxtail millets, using CRISPR/Cas.

This gene controls the shattering of plant and can be deployed to improve mechanical harvesting trait. Further, several non-functional alleles of the same gene were created using CRISPR/Cas9 (Mamidi et al. 2020). For further information on futuristic crops, readers are directed to refer to a recent good review written by Dawson et al. (2019). Experts from the international institute Crops for the Future (CFF), Malaysia, say that "There is no food insecurity in the world, there is food ignorance." They also insist the use of underutilized crops that are already more nutritious (https://www.bbc.com/future/article/20180821-are-forgotten-crops-the-future-of-food).

20.5 Conclusion

The GE has a great potential to achieve food security for growing world population. As a second alternative to achieve food security, we can expand our food crop resources by promoting the use of underutilized crops and by searching totally new food crop from the available plant biodiversity. GE can play a significant role in this aspect by involving in trait-specific improvement of underutilized crops and speeding up the domestication process of totally new food crop. Though in many countries GE crops are regulated under different rules than GM crops, still policies of many countries are not clear in this regard, which is the biggest barrier in the widespread application of GE in food crop improvement. GE in plants play a key role in addressing the global food security provided policies, governance, and regulatory-related issues are sorted out to attain synchrony at both national and international levels.

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Part V

Food Quality and Management

Bacteriocins as Biological Components for Managing Food Quality

21

Vandana Bharti, Neha Jain, and Archana Mehta

Abstract

In today's time, the only demand for the consumer is disease-free, health-promoting foods without any chemical preservatives or biological contamination. To fulfill these demands, many researchers are trying to identify novel approaches to preserve the food by using bacteriocin as a biological preservative. Bacteriocins are heat-stable peptide, are synthesized ribosomally, have antimicrobial properties, are produced by one bacterium, and prevent the growth of other bacteria. Bacteriocins produced by lactic acid bacteria (LAB) have antibacterial activity against gram-positive bacteria and food poisoning bacteria such as *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., and *Clostridium* spp. Spoilage of food includes some alteration which reduces food quality, and these foods are unacceptable for consumption. The disease that occurs in human after consumption of spoiled food is called foodborne disease. Bacteriocins are best-known examples used for biopreservation, because of their safety parameter in foods as well as in low amounts, they inhibit microbial growth. This book chapter aims to discuss the application of bacteriocins in food preservation as a biological agent to maintain the quality of food.

Keywords

Food quality · Preservation · Bacteriocins · Functional foods

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21.1 Introduction

In the present time, people are conscious of their health and the health benefit of food; they are more aware of chemical food additive or natural preservative and more prefer food without any chemical additive. Therefore, the food industry is focused on producing uncontaminated good-quality food due to consumer demand. However, these industries are using a large number of chemical preservatives to maintain food quality. The government also made some rules and regulations for the food manufacturing industries to decrease the ratio of chemical additives in processed foods. From years ago in fermented foods, lactic acid bacteria (LAB) are used to enhance the flavor, texture, and nutritional value of the food products because of their metabolic properties. LAB are mainly used for the protection of food by producing hydrogen peroxide, carbon dioxide, diacetyl and ethanol, phenyllactic acid, and bacteriocin as metabolite products. At present, bacteriocin metabolites of LAB are considered as natural food ingredients; it is a ribosomally synthesized, extracellular low molecular mass protein that has bactericidal or bacteriostatic activity (Vieco-saiz et al. 2019). Food industries commonly used bacteriocins in foods as an additive in a semi- or purified form or as a starter culture and as an ingredient of fermentation of a bacteriocinogenic strain. This chapter highlights the factor involved in the spoiling of food, the disease caused by spoiled food, properties of bacteriocin, as well as its classification and mode of action. Furthermore, bacteriocin applications in the control of food quality by inhibiting microbes are reviewed, and regulation issues and safety regarding bacteriocin use in the food industry are also discussed (Settanni and Corsetti 2008).

21.2 Factors Affecting Food Quality

In today's time, the quality of food has been well defined by scientists in two ways: scientific status and consumer preferences. Food quality depends on its spoilage, nutrients, additives, composition, functional ingredients, flavorants, colorants, and general safety and on contamination by scientific status. However, the smell, color change, slime or rough surface, change in test, or any type of change in the original appearance of food is directly linked to consumer preferences. The quality of food is spoiled by many factors, and these are the naturally occurring factors causing deterioration (Hui 2007). There are so many factors that include enzymes, parasites, insects, air, rodents, light, temperature, physical damage, microorganisms, time, and other creatures (Fig. 21.1). To preserve food quality and inhibit spoilage in food, we must identify the factors.

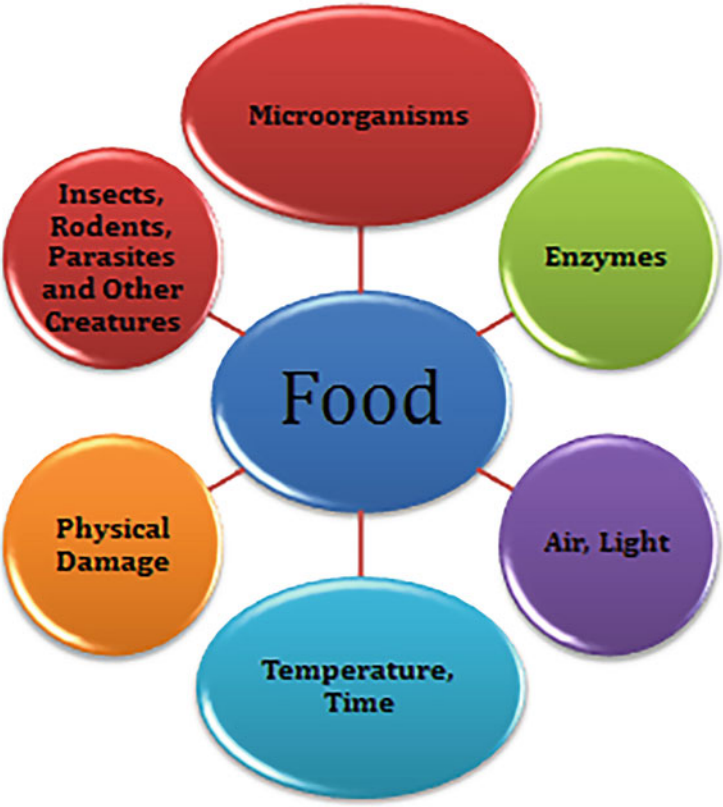

Fig. 21.1 The main factors causing food spoilage

21.3 Disease Caused by Spoiled Food

Food poisoning occurs due to eating contaminated food, and it causes nausea, fever, and stomach pain, headache, feeling weak, vomiting, or sweating in healthy persons. When microorganisms (bacteria, viruses, fungi) or any poisonous metals such as cadmium or lead come in contact with food, it results in contaminated foods. Foods that are not properly cooked are more prominent to cause food poisoning (Hayes 1995; Dudley 1988). These include seafood, eggs, cooked rice, meat, ham, chicken, milk, and all dairy products. Some common foodborne disease and their causal organisms were mentioned in Table 21.1.

Table 21.1 Diseases caused by spoiled foods

Organisms	Foodborne diseases	Causal organisms
Bacteria	Salmonellosis	*Salmonella sp.*
	Enteric fever	*Salmonella enterica*
	Diarrhea	*E. coli, Shigella dysenteriae*
	Cholera	*Vibrio cholera*
	Typhoid	*Salmonella typhi*
	Food poisoning	*Clostridium perfringens*
	Botulism	*Clostridium botulism*
	Listeriosis	*Listeria monocytogenes*
	Yersiniosis	*Yersinia enterocolitica*
	Campylobacteriosis	*Campylobacter jejuni* and *Campylobacter coli*
Fungi	Candida spp.	*Candida albicans*
	Fusarium sp.	*Fusarium verticillioides*
	Alternaria sp.	*Alternaria alternata*
Viruses	Norovirus	Norwalk virus
	Hepatitis A	Hepatovirus A
	Polio	Poliomyelitis virus

21.4 Food Preservation Methods

Food preservation involves a number of food processing stages to keep food quality at a preferred level so that maximum nutrition values can be benefit and achieved. It is defined as the techniques in which we trying to minimize the spoiling factor those can be external or internal responsible for spoiling of food. The main objective of this method is to maintain the nutritional value of food without changing its flavor, color, and texture as well as enhance its lifetime. Food preservation techniques include physical methods such as drying, pasteurization, refrigeration, freezing, smoking, canning, irradiation, Pascalization, pulse electric field electroporation, oscillating magnetic field, hurdle technology, nonthermal plasma (NTP) technology, modified atmosphere packing (MAP) have been used and adopted from old techniques besides this some are very costly; whereas in chemical methods include salting/sugaring, antimicrobial compounds, antioxidants, natural/artificial additives are used as well as in biological methods. Fermentation and biopreservation are used to preserve food quality. Biological preservation is the most used technique from the abovementioned because it is safe and reported no side effect on human (Jeevaratnam et al. 2005). Bacteriocins are best alternate of chemical preservatives or antibiotics newly discovered biological techniques for preservation and to maintain the food quality by inhibiting the growth of food spoilage bacteria. Its use in food as preservatives is safe (Gálvez et al. 2007). Bacteriocins' use as natural preservatives in food satisfies the demand of safe and high-quality foods because they are easily digested by the gastrointestinal tract (Ananou et al. 2007; Kalam and Ahmad 2016; Mills et al. 2011). In Table 21.2, we summarized the different

Table 21.2 Food preservation methods

Methods	Description	Advantages	Disadvantages
Physical methods			
Drying	Reduce water content to prevent bacterial growth	Produces concentrate type of food Microbial growth and autolytic enzymes are inhibited	Loss of some nutrients, thiamin, and vitamin C Individuals sensitive to SO_2
Pasteurization	High heat processing	Inactivates autolytic enzymes Destroys microorganisms	Loss of heat-sensitive nutrients
Refrigeration	Low temperature	Decreases microbial growth Slows enzymes' autolysis	Loss of nutrients
Freezing	Low temperature causes dehydration	Prevents microbial growth at low temperature and unavailability of water	Reduces product quality and decreases the synthesis of vitamin B
Smoking	Incorporation of substances from smoke	Preserves meat, fish, and some foods	Health hazards; sometimes causes cancer
Canning	Heating and sealing of the food in airtight container	Destroys microorganisms and autolysis of enzymes	Loss of water-soluble nutrients
Irradiation	Exposure of food to short-wave radiation energy causes DNA damage and destruction of microbes	Inhibits sprouting potatoes Increases strawberries and mushrooms shelf life	Loss of nutrients
Pascalization	High hydrostatic pressure	High retention of nutrients and vitamins Inactivates microbes and minimizes the chemical reaction in food	Inactivates spores
Pulse electric field electroporation	Short high-voltage pulses	High retention of nutrients and vitamins Disrupts microbial cell wall	Toxicity risk Limited to liquid food only Spores are not sensitive
Oscillating magnetic field	High-intensity magnetic field applied at moderate frequency in non-electric conductive environment	Stabilizes and preserves either solid or liquid foods Inactivation of vegetative cells of microbes	Not much effects on spores or enzymes

(continued)

Table 21.2 (continued)

Methods	Description	Advantages	Disadvantages
Hurdle technology	All the parameters of preservation are used	Minimally processed, convenient foods; low amount of salt or fat in packaged food	Costs higher
Nonthermal plasma (NTP) technology	Extremely energized matter composed of highly reactive species including gas molecules	Inactivates foodborne pathogens Used for surface decontamination Acts rapidly; no toxic residuals on processed parts	Sensitive to vitamin C and vitamin E Not suitable for high-fat−/lipid-containing food
Modified atmosphere packing (MAP)	Gaseous atmosphere maintains the changed atmosphere	Extends the shelf life of food Convenient and cheap Preserves the natural quality of food	Temperature control necessary Different gas formulation required for each product
Chemical methods			
Salting/ sugaring	Adding salt or sugar in food	Water unavailable for microbial growth Does not destroy nutrients	Increase salt or sugar content of food
Antimicrobial compounds	Benzoic acid, nitrate, citric acid, and SO_2	Prevent food quality	Toxic to health Allergic reactions may occur
Antioxidants	Prevent oxidative deterioration of food	Prevent spoiling of food	Toxic to health
Natural/ artificial additives	Sodium benzoate, nitrates	Prevent microbial growth No loss of nutrients	May cause sensitivity
Biological methods			
Fermentation	Reduces pH and produces antimicrobial	Preserves food quality Reduces microbial growth	Produces alcohol as a waste product Produces lactic acid and ammonia
Biopreservation	Lactic acid bacteria (LAB); bacteriocins	Ensures the safety and quality of food Inhibits microbial growth Increases digestibility Increases the shelf life of food Increases nutritional values	Narrow inhibitory activity spectrum Not much active against gram-negative bacteria

preservation techniques to maintain the quality and safety of food and their advantages and disadvantages.

21.5 Bacteriocins

Bacteriocins are heat-stable, antimicrobial peptides synthesized ribosomally and produced by gram-positive and gram-negative bacteria and also in some members of Archaea. They have narrow antimicrobial spectrum against closely or in other genera species. The genes that code for bacteriocins can be either chromosomally or plasmid coded. Usually, they range from 30 to 60 amino acids in length and have been associated with numerous species of bacteria (Bharti et al. 2015). However, the bacteriocins of lactic acid bacteria (LAB) have received much attention in terms of food safety due to their generally recognized as safe (GRAS) status (Benmechernene et al. 2013). They can be readily introduced into fermented foods without prior purification or concentration, and they exhibit activity against gram-positive pathogens such as Listeria monocytogenes and Staphylococcus aureus. Although they generally don't target gram-negative bacteria, bacteriocins may be effective at killing gram-negatives if the outer membrane is destabilized. However, despite a wealth of bacteriocins that have been investigated as potential preservatives for the food industry, till date, only two LAB bacteriocins, namely, nisin and pediocin PA-1, are commercially available. Lactic acid bacteria (LAB) bacteriocins are classified into four groups on the basis of structure and mode of action (De Vuyst and Leroy 2007; Benmechernene et al. 2013; Kumariya et al. 2018; Yang et al. 2014; Table 21.3).

21.6 Mode of Action

Generally due to the cationic character of bacteriocins, it effortlessly interacts with other bacteria (gram-positive or gram-negative bacteria), and it can be bactericidal or bacteriostatic. A large number of anionic lipids in the cytoplasmic membrane affect the energetic status of the cell by formation of pores. The bactericidal phase involves the binding of the bacteriocin to specific receptors on the cell membrane of the target bacterium and the formation of a complex with membrane components, leading to pore formation, or destabilization of cytoplasmic membrane integrity with a detergent-like effect. However, no receptor or region in the bacteriocin molecule that would act as a receptor-binding site has been identified thus far. In vitro studies using lipid membranes have provided evidence that the mechanism of action most probably involves pore formation. A class I bacteriocin nisin has shown dual mode of action. The cationic nature of nisin can bind to anionic lipid (lipid II), the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and as a result prevent correct cell wall synthesis, causing cell death. Moreover, nisin can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. Among several class II bacteriocins, lacticin

Table 21.3 Classification of bacteriocins

Bacteria	Class of bacteriocin	Sub-class	Bacteriocin	Producing strain	Inhibitory spectrum
Gram-positive bacteria	Lantibiotics M.wt: <5 kDa Heat stable Contain unusual amino acids	Ia	Nisin	*Lactococcus lactis subsp. lactis*	*Staphylococcus* sp., *Enterococcus* sp., *Lactobacillus* sp., *Listeria* sp., *Mycobacterium* sp., *Micrococcus* sp., *Pediococcus* sp., *Clostridium* sp., *Bacillus* sp. *Lactococcus* sp., *Leuconostoc* sp.
		Ib	Mersacidin	*Bacillus subtilis*	*Staphylococcus aureus Streptomyces scabiei*
	Non-lantibiotics M.wt: <10 kDa Moderate heat stable	IIa	Plantaricin	*Lactobacillus plantarum*	*Lactobacillus* sp., *Pediococcus* sp.
		IIb	Lactococcin Q	*Lactococcus lactis*	*Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Listeria, Bacillus, Enterobacter, Escherichia, Salmonella, Yersinia*, and *Citrobacter*
		IIc	Lactocyclicin Q	*Lactococcus* sp.	*Bacillus* sp., *Enterococcus* sp., and *Lactococcus* sp.
	Bacteriolytic M.wt: >30 kDa Heat labile Complex bacteriocins with glycol and/or lipid moieties	III	Helveticin J	*Lactobacillus helveticus*	*Lactobacillus bulgaricus*, *Lactococcus lactis*
	Non-bacteriolytic Contain lipid and carbohydrate moieties	IV	Enterocin	*Enterococcus faecalis*	*Listeria* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., *Bacillus* sp.
	Reutericyclin			*Lactobacillus reuteri*	*Lactobacillus* sp., *Staphylococcus aureus*, and *B. subtilis*
	Pediocin			*Pediococcus acidilactici*	*Pseudomonas aeruginosa*

(continued)

Table 21.3 (continued)

Bacteria	Class of bacteriocin	Sub-class	Bacteriocin	Producing strain	Inhibitory spectrum
Gram-negative bacteria	Colicins		Colicin E2	*Escherichia coli*	Escherichia coli and Shigella sp.
	Microcins		Microcin B17	*Escherichia coli*	*Escherichia coli* and *Salmonella* sp.
	Tailocins			*Pseudomonas fluorescens*	*Xanthomonas vesicatoria*

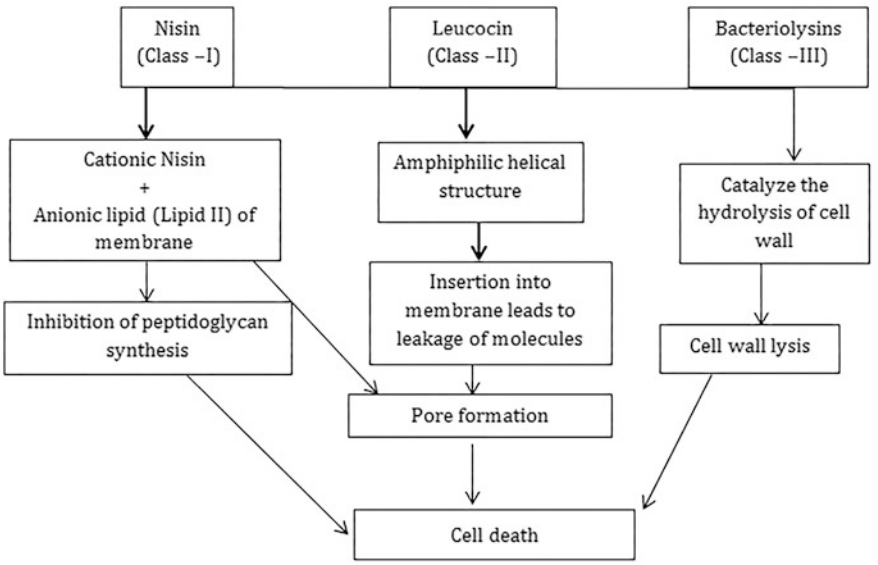

Fig. 21.2 Schematic diagram representing the mode of action of class I, class II, and class III bacteriocins

3147, a two-peptide lantibiotic, has also shown dual activities, and their mode of action is the same as nisin, whereas leucocin, a class II bacteriocin, has an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to the depolarization and death of cell. Some bacteriocin of class II like mersacidin does not form pores in the membrane, but it can bind with lipid II. However, in class III bacteriocins, such as bacteriolysins (lysostaphin), a group of large bacteriolytic proteins can catalyze the hydrolysis of cell wall of gram-positive targets leading to the death and lysis of the cell (Fig. 21.2; Cotter et al. 2005).

21.7 Application of Bacteriocin in the Control of Food Quality

Role of Nisin It is a class I A lantibiotic, pentacyclic, heat-stable peptide produced by *Lactococcus lactis*. Six different forms of nisin have been discovered and characterized, designated as A–E and Z. Moreover, nisin A is the most active type of nisin. It is industrially produced; internationally accepted food preservatives and effective bactericidal agent against gram-positive and gram-negative bacteria also inhibit the spore germination and growth of pathogenic bacteria contaminating the surface of food products. The used of Nisin in food storage decrease the spore count of pathogens in dairy products increase the self-life of food without destructive appearance, flavor and texture (Müller-Auffermann et al. 2015). Nisin can be considerably increasing the limited shelf life of such pasteurized products. Production of nisin with antioxidative agent is more effective. It is also used in pasteurized, processed cheese products to prevent the outgrowth of spores such as those of *Clostridium tyrobutyricum* that may survive heat treatments as high as 85–105 °C. Use of nisin allows these products to be formulated with high moisture levels and low NaCl and phosphate contents and also allows them to be stored outside chill cabinets without risk of spoilage. The potential application of the freeze-dried fermentation product as a preservative to improve the storage performance of meat and fruit, canned evaporated milk. Nisin is added to milk in the Middle East where shelf life problems occur owing to the warm climate, the necessity to transport milk over long distances, and poor refrigeration facilities. It can double the shelf life at chilled, ambient, and elevated temperatures and prevent the outgrowth of thermophilic heat-resistant spores that can survive pasteurization. It is commonly used in the beer fermentation and remains active during the fermentation process with Saccharomyces yeasts. The addition of nisin has no negative effects on the aroma, flavor, appearance, and physical stability of beers, and it does not have any negative impacts on the final product (Gharsallaoui et al. 2016). Metabolically engineered producing strain of nisin is a cost-effective, easily prepared, and high-performance food preservative (Liu et al. 2020).

Role of Pediocin Pediocin PA is a small, non-toxic heat-resistant bacteriocin produced by *P. acidilactici* spp. and used as natural preservatives in large scale to inhibit the growth of food-spoiling bacteria such as Listeria monocytogenes, *S. aureus*, *Pseudomonas* sp., and *Escherichia coli* (Jeevaratnam et al. 2005; Espitia et al. 2016). It is mostly used in cream, cheese sauce, and cottage cheese because of its anti-listerial effect on high temperature and pH. The semi-purified form of food-grade pediocin reduced the growth of *S. aureus* in raw milk of buffalo. Direct addition of pediocin in vacuum-packed food or in different dairy products such as yoghurt, ice cream, and cottage cheese and in milk powder has been observed to inhibit *Listeria* spp. However, its application on the surface of slices of cooked sausage decreased the *L. monocytogenes* counts from the initial (Knoll et al. 2008).

Role of Lacticin Lacticin 3147 and lacticin 481 are a two-component lantibiotic produced by *Lactococcus lactis*. Lacticin 3147 is used for making buttermilk and in

the control of Listeria and Bacillus microflora in cottage cheese, natural yogurt, and infant milk formulation (Article, 2012). Lacticin 481 has a moderate range of spectrum against *Clostridium tyrobutyricum* and *L. monocytogenes* and also on some LAB species. In non-purified form, it is used to control the microbial growth in milk during storage in refrigeration. However, the semi-purified form of lacticin 481 reduced the *L. monocytogenes* growth in stored fresh cheese. Complete removal of *L. monocytogenes* is done, but their growth can be controlled by lacticins. It is more efficient in cheddar cheese manufacture as starter (Settanni et al. 2005; Silva et al. 2018).

Role of Reutericyclin Reutericyclin is a hydrophobic inhibitory, low molecular weight bacteriocin which inhibits the food spoilage organisms by attacking on the cytoplasmic membrane.

In purified form, it prevents the growth of several groups of fungi, yeast, and gram-positive and gram-negative bacteria during the fermentation of food. Producing organism of reutericyclin is *Lactobacillus reuteri*. The use of purified form of reutericyclin, metabolically inactive cells of producing strain and direct use of *Lactobacillus reuteri* in food or pharmaceutical products is possible and more effective. The addition of *L. reuteri* bacteria during seed germination works as a protective culture and inhibits the growth of *Staphylococcus aureus*, *Enterobacteriaceae*, *Listeria* sp., and *Pseudomonas* sp. In the baking industry, the growth of *Bacillus* species is the main spoilage problem. The addition of reutericyclin-producing strain during heat sourdough fermentation is active after baking. Hence, reutericyclin-producing strains prevent the growth of rope-forming bacilli and prolong the shelf life of baked goods (Article 2012).

Role of Mesentericin Y105 Mesentericin Y105 bacteriocin produced by *Leuconostoc mesenteroides* Y105 has inhibitory spectrum against gram-positive bacteria such as *Enterococcus faecalis* and *Listeria monocytogenes* (Bharti et al. 2015).

Role of Lactococcin B Lactococcin B bacteriocin is present in three different forms, i.e., lactococcins A, B, and M. It is a low molecular weight, hydrophobic peptide, produced by *Lactococcus lactis*. These bacteria are used as starter culture for the rapid acidification of food because they produced lactic acid by utilizing exiting carbon source during the fermentation of food. The acid environment is maintaining the food quality by the inhibition of food spoilage bacteria (Singh 2018).

Role of Sakacin P Sakacin P, a bacteriocin produced by *Lactobacillus sake* Lb706, is present in two different forms: sakacin P and sakacin A; both forms have an inhibition activity as well as a bacteriostatic effect against *Listeria monocytogenes*. This is mainly used in the vacuum-packed cold-smoked salmon where *Listeria monocytogenes* grew rapidly. Sakacin will keep preserved food fresh for 4 weeks. Besides this, some other studies suggested the use of *Lactobacillus sake*, a

sakacin-producing strain, in the preservation of meat products showing the drop in viable cell counts of Listeria (Holck et al. 1992; Axelsson and Holck 1995).

Role of Acidocin Lactobacillus acidophilus produced acidocin, a plasmid-encoded (2.4 kDa) bacteriocin, which has an inhibitory spectrum against pathogenic bacteria *Listeria monocytogenes*, *Clostridium sporogenes*, *Brochothrix thermosphacta*, *Lactobacillus fermentum*, and *Lactobacillus delbrueckii* (Ahmed et al. 2010).

Role of Enterocins Enterocin, a cyclic bacteriocin, is produced by *Enterococcus* species which has an inhibitory spectrum against *Bacillus* and *Clostridium* sp. and prevents the growth of these pathogens during the storage of food and is considered safe for food consumption because of its stability on high temperature and pH (Perez et al. 2012). It prevents the growth of *Staphylococcus* and *L. monocytogenes* in half- and full-fat skimmed milk but also decreased the fat amount in milk. The purified enterocin CCM 4231 is used in skimmed milk and yogurt to prevent the growth of *L. monocytogenes* and *S. aureus* (Silva et al. 2018). The high dose of enterocin is used in the storage of fresh cheese in the reduction of *L. monocytogenes* pathogen. The combined effect of reuterin and enterocin reduced the bacterial count of *L. monocytogenes* in milk (Alvarez-Cisneros and Espuñes 2011).

In Table 21.4, we listed all the commercially available functional foods in which different bacteriocin-producing strains are used.

21.8 Combined Application of Bacteriocin with Other Technology to Maintain the Food Quality

Bioactive Packaging In bioactive packaging, bacteriocin or bacteriocin-producing strain is incorporated in food during food packaging to keep the food safe from outer contamination. In this method, immobilized bacteriocin is directly incorporated in packed food during their packaging or can be added in the food packet before food packing, which will be released during the storage of food product (Parada et al. 2007).

Bacteriocin with Heat Treatments Some food poisoning bacteria such as *Leuconostoc monocytogenes* can grow at high temperature. The heat resistance of these bacteria in milk can be reduced by heat treatment with nisin; it is a cost-saving method to provide food storage safety and decrease the endospores surviving during heat treatments. Bacteriocins nisin, pediocin ACH, and enterocin AS-48 reduced the action of some gram-negative bacteria with low heat treatments (Gálvez et al. 2007).

Bacteriocins with Modified Atmosphere Packaging (MAP) Bacteriocins and modified atmospheric packaging (MAP) are commonly used in the food manufacturing industry to extend the shelf life of fresh food products which ultimately make changes in the gaseous environment by creating barriers. Shelf life

Table 21.4 Commercially available functional foods

Brand name	Product type	Bacterial strain	Manufacturer
Actimel	Probiotic yoghurt drink	*L. casei* Immunitas	Danone, France
Activia	Creamy yogurt	Bifidus Actiregularis	Danone, France
Aciforce	Freeze-dried	*Lactobacillus lactis, L. acidophilus, Enterococcus faecium, L. acidophilus*	Biohorma, the Netherlands
Bacilac	Freeze-dried	*L. acidophilus, Lactobacillus rhamnosus* GG	THT, Belgium
Bactisubtil	Freeze-dried	Bacillus sp. strain IP5832	Synthelabo, Belgium
Bififlor	Freeze-dried	*L. acidophilus, L. rhamnosus, Bifidobacterium bifidum*	Eko-bio, the Netherlands
Gefilus	Freeze-dried powder	*Lactobacillus rhamnosus* GG	Valio, Finland
Hellus	Dairy products	*Lactobacillus fermentum* ME-3	Tallinn Piimatoostuse AS, Estonia
Proviva	Fruit drink and yogurt	*Lactobacillus plantarum*	Skanemejerier, Sweden
Rela	Yogurt, cultured milk, and juices	*Lactobacillus reuteri*	Ingman foods, Finland
Revital active	Yogurt and drink	Probiotics	Olma, Czech Republic
Soytreat	Kefir	Probiotics six strains	Lifeway, USA
Vitamel	Dairy product	*Lactobacillus casei* GG *Bifidobacterium bifidum Lactobacillus acidophilus*	Campina, the Netherlands
Yakult	Milk drink	*Lactobacillus casei* Shirota	Yakult, Japan

prolongation of food by MAP is based on the retardation of intrinsic food changes and inhibition of spoilage microbiota. These systems have greater effects when applied in combination with bacteriocins, thus ensuring dual safety against food spoilage (Quinto et al. 2019; Gálvez et al. 2007).

Bacteriocins and Pulsed Electric Fields The bacterial inactivation is succeeding using nonthermal pulsed electric field (PEF) technology in low-viscous food products. The long-term intensity of this can cause damage to bacterial cell membranes and increased bactericidal effects. A short dose of gamma radiation is mainly used to minimize undesirable changes in food for consumer acceptance. Pediocin (ALTA™ 2341) and low dose of gamma irradiation improved the bactericidal effect on *L. monocytogenes* in cooked sausages (Gálvez et al. 2007).

21.9 Rules and Regulation Status of Bacteriocins

Bacteriocin has shown some same properties with antibiotics; due to these similarities, use of bacteriocin in food is banned. The use of bacteriocin-producing strain as a starter culture requires its GRAS status (Soltani et al. 2021). Producing strain must be well characterized and identified till its species level ('Food Safety and Quality in Developing Countries: the Role of Lactic Acid', 2015). The use of bacteriocin or bacteriocin-producing strain in food application should have the following features.

- The bacteriocin-producing bacteria must have generally recognized as safe (GRAS) status.
- Bacteriocin must have a broad range of inhibitory spectrum.
- Can be stable at high temperature.
- Health beneficial effects also enhanced safety.
- No effect on food flavor and its quality.

21.10 Conclusion

It is very necessary to monitor the health of people for any side effect of consuming long-term product containing bacteriocin; for that, medical experts, food officers, and bacteriocin producers are recommended. In today's scenario, the only consumer's nutritional demand is healthy fresh food with therapeutic properties without any risk of sickness. In India, microbes have been used since ancient times to preserve the food for quality and safety such as idli, dosa, and dahi. The use of microbes in fermentation is unknown. So the scientific data authentications need to be done to popularize the biological preservation evidence with traditional knowledge. To enhance these properties, scientists have to improve or explore advanced technology for food manufacturing industries to satisfy quality, safety, and nutritional demands. Addition of bacteriocin in food has the potential to protect the consumer from many health complications. But in food industries, the availability of bacteriocin in large amounts is a major problem. This bacteriocin availability issue might be solved in the near time with the help of recombinant DNA methods. This technology will produce designer food having a high level of bacteriocin. The only drawback of this method is the food having the bacteriocin may be cleaved by gastrointestinal enzymes before showing any activity. Apart from DNA technology, bacteriocin can be added to food products at the time of fermentation of food with bacteriocin-producing strain.

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Control of Foodborne Pathogens Using Nanotechnology

22

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Abstract

Nanotechnology applications are evident in all the major scientific fields including food processing and antimicrobial potential. Nanoscale compounds are being researched and used in the control of foodborne pathogens and increase the shelf life of food products. At the same time, the nanotechnology has showed the path toward the development of sensors for the monitoring of foodborne pathogens, biodegradable nanofilms and covering protect food products from pathogens. Further, the advancement in the field of food nanotechnology led to the development of novel food packaging applications. Contributions of nanotechnologist in controlling foodborne pathogens and for both types of nanocomposites in the packaging, viz., biodegradable (polylactides, starch, protein, etc.) and non-biodegradable polymers, are addressed in this chapter.

Keywords

Foodborne pathogens · Toxin · Nanotechnology · Carbon nanotube-based sensor · Antimicrobial food packaging · Synergetic antimicrobial nano-formulations

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22.1 Introduction

Multiple disease and infection caused by the foodborne pathogens are the most common threats to humans (Chlebicz and Śliżewska 2018; Letchumanan et al. 2019). Up to 250 such disease−/infection-causing agents have been recognized by researchers across the globe (Le Loir et al. 2003). Foodborne pathogens include bacteria, viruses, fungi, and parasites (Appleton 2000; Lee et al. 2014a; Kumar et al. 2019). Moreover, in many cases, harmful toxic compounds were released by these food pathogen microbes that not only contaminate the food products but also act as a source for the foodborne illness (Martinović et al. 2016; Devleesschauwer et al. 2019). With the progression of science and technology, foodborne infections and diseases are no more unpreventable. Billions of humans around the world fall sick due to the diseases or infections caused by foodborne pathogens (Tyagi et al. 2013; Prado et al. 2016). However, numerous advancements have been made for the diseases and infections caused by foodborne pathogens, but still foodborne diseases and infections are responsible for mortality in humans. In addition to this, seeing the global situations, such foodborne pathogenic microbes are also posing a greater risk to be used by the terrorists in the situation of wars (Taylor 2008). Hence, considering the above fact, there is a need for developing new methods or techniques for the quick and cheap identification along with the removal of these disease-causing microorganisms in food items.

There are many advanced methods or techniques such as fluorescent antibody (FA), enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR) that are in use for the detection of these food pathogens. However, all these methods are time-consuming and cumbersome and have limited sensitivity for the particular foodborne pathogen (Prado et al. 2016). Nanotechnology has emerged as a boon for many sectors such as health care, wastewater treatment, drug development, biosensor, etc. (Tyagi et al. 2019, 2020; Jain et al. 2020; Garg et al. 2020). Moreover, its application in the development of nano-based sensor has emerged as a boon for the detection of food pathogen microbes or their toxin present in the food stuffs (Stephen Inbaraj and Chen 2016; Bhardwaj et al. 2018). There are multiple ways in which nanotechnology can aid in making a detection technique or kits more efficient or in the development of altogether a new approach for the detection and analysis of foodborne pathogens or their part or the toxin released by them. Nanotechnology-dependent techniques offer several advantages such as quick detection, high sensitivity, greater reliability, and simple isolation and detection of foodborne pathogens and viruses (Tyagi et al. 2021). Biosensing, one of the major techniques in nanobiotechnology, got its approval in the detection of a multiple disease-causing pathogens or their toxins (Burris and Stewart 2012; Orlov et al. 2013; Lee et al. 2014a, b). Figure 22.1 illustrates the various applications of nanotechnology in the food sector.

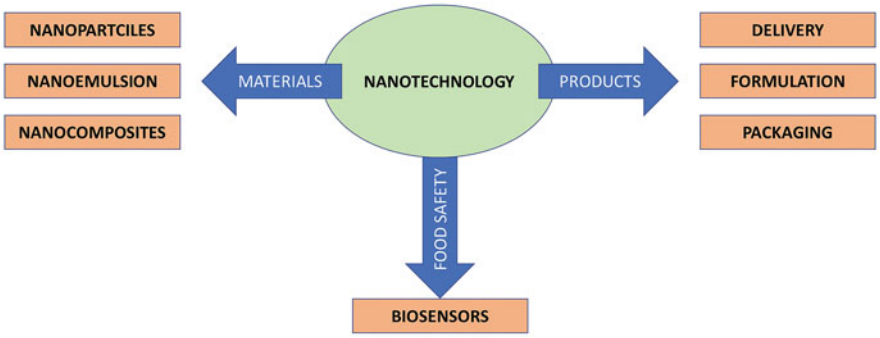

Fig. 22.1 Application of nanotechnology in the food sector

22.2 Contribution of Nanotechnology in Foodborne Pathogen Detection

Nanotechnology-based techniques include the characterization, development, and manipulation of structures at the nano-level and improved development of nano-devices/kits or materials that come in the range of 1–100 nm (Tyagi et al. 2016). Some common food pathogens and their detection or identification by nanoparticles are illustrated in Table 22.1. When the size of the particle is reduced, that is, lower than its threshold values, its properties such as optical, magnetic, and electronic get changed (Duncan 2011; Ladj et al. 2013). These new and changed properties allow a whole new variety of developments along with multiple applications in various fields. The European Union defines nanotechnology as a key enabling technology (Prado et al. 2016). Key enabling technology is accompanied with high research and development intensity, rapid innovation cycles, and high capital expenses along with highly skilled employment. Key enabling technology is applied to accomplish various small, quick, and improved tasks. It is estimated that the value of nanotechnology will rise to $3 trillion across the global economy by the end of 2020. With the more development in nanotechnology industries, the working force in this field may rise to at least six million workers by the end of this decade (Rochester 2010). Research and development in the nanotechnology area has.

showed rapid increment over the last few years, with a number of companies and research institutes specialized in the fabrication and development of new forms of nanosized material (Duncan 2011). Some of the basic use of nanotechnology comprises consumer products such as cosmetic items (face wash, skin creams, etc.) and anti-odor or antimicrobial textile products (Garg et al. 2020). Medical therapeutics and the diagnostics kits for multiple diseases are important fields in which nanotechnology is bringing new and exciting developments every day. Most recent progresses in nanotechnology are significantly impacting the development of some analytical methods, due to the improvement of new nano-structures, nano-devices, nanomaterials, and nanoparticles. Further, all these comprise nano-shells,

Table 22.1 Some common food pathogens and their identification using different types of nanoparticles

Microbial contamination	Nano-based material used	References
Escherichia coli	Gold nanoparticles	Miranda et al. (2011)
Escherichia coli-0157:H7	Gold nanoparticles	Jung et al. (2005)
Salmonella sp.	Metal and TiO_2 nanocrystal	Joo et al. (2012)
Escherichia coli *Sarcina lutea*	Amine-functionalized magnetic nanoparticles	Huang et al. (2010)
Listeria monocytogenes	Fluorescent gold nanocluster-labelled peptide	Hossein-Nejad-Ariani et al. (2018)
Listeria monocytogenes	Iron and iron oxide nanoparticles	Zhao et al. (2017)
Cronobacter sakazakii	Silica-based iron oxide nanoparticles	Zhao et al. (2013)
Salmonella enterica	Iron and iron oxide nanoparticles	Wang et al. (2015)
Staphylococcus aureus	Silica nanoparticles	Kim et al. (2011)
Listeria monocytogenes	TiO_2 nanowires	Wang et al. (2008)
Bacillus cereus	Polyaniline nanowire	Pal et al. (2007)
Escherichia coli	Glyco-nanoparticles	El-Boubbou et al. (2007)
Pseudomonas aeruginosa	Core/shell magnetic iron oxide nanoparticles	Liu et al. (2009)
Bacillus anthracis	Polyamine-functionalized iron oxide nanoparticles	Zhang et al. (2010)
Staphylococcus aureus	Streptavidin-coated magnetic nanoparticles	Abbaspour et al. (2015)
Listeria monocytogenes	Amino-modified silica magnetic nanoparticles	Bai et al. (2013)
Vibrio parahaemolyticus	Magnetic beads	Seo et al. (2010)
Salmonella typhimurium	L-aspartic acid-modified iron oxide nanoparticles	Ravindranath et al. (2009)
Escherichia coli	Iron-platinum	Gao et al. (2006)
Salmonella sp.	Cadmium selenide/zinc sulfide quantum dots	Lee et al. (2014b)
Shigella flexneri	Gold nanoparticle-based colorimetric aptasensor	Feng et al. (2019)
Salmonella spp.	Gold nanoparticles	Garrido-Maestu et al. (2017)
Staphylococcus aureus *Pseudomonas aeruginosa*	Gold nanoparticles	Pissuwan et al. (2020)
Escherichia coli, *Staphylococcus aureus*, and *salmonella typhimurium*	Magnetic nanoparticles	Wilson et al. (2019)
Salmonella enteritidis	Nitrogen-rich carbon nanoparticles	Wang et al. (2019)
Listeria monocytogenes	Gold nanoparticles	Wachiralurpan et al. (2018)

nano-wires, nanotubes, and nano-barcodes with a variety of shapes, sizes, and compositions (Marrazza 2012; Maksimović et al. 2019; Xu et al. 2019). In addition to this, recent development in nanotechnology contributed vastly in the miniaturization of nano-devices. As stated above, nanomaterials and nanoparticles present in nano-shell, nano-wires, nanotubes, etc. acquired new structural, physical, and chemical properties that differ from its bulk matter. As a result, these new technologies and developments are an interesting alternative for many conventional or classical methods (Gómez-Hens et al. 2008). Nanoparticles can be used either in solution form or in immobilized form according to the requirement in the biosensing devices (Valdés et al. 2009). Nanoparticles present many interesting advantages for multiple sensing applications as compared to classical or conventional methods using chemical reagents. Their ease of synthesis and other properties such as high stability, high surface area, and high ability to be functionalized with a variety of biological molecules such as peptides, DNA, antibodies, and aptamers make them a suitable candidate for the detection or identification of a range of biologically relevant analytes. Similarly, inorganic nanomaterials (iron oxide, semiconductor quantum dots, and gold nanoparticles) showed a variety of unique, intrinsic, physical, and chemical properties such as new and unique magnetic, optical, and electronic properties that have been exploited to generate physical recognition signals (Prado et al. 2016). Moreover, nanoparticles or nano-devices are used for food and environmental monitoring, not exclusively as labels, but also with other objectives, such as contributing to the selection and/or separation of certain target molecules (Valdés et al. 2009).

22.3 Sensor-Based Monitoring and Separation of Foodborne Pathogens

Food pathogens are the main cause of food spoilage and food poisoning and pose a serious human health risk. The early detection of such pathogens can lead to the prevention of foodborne illness and contamination. Now, the world is also facing a new threat of bioterrorism where water and food are the major security issues. As it is evident that even a small number of pathogens in food can be detrimental for humans, continuous monitoring of food is essential to ensure safe and nutritious food to every human being.

22.3.1 Conventional Methods of Detecting and Analyzing Pathogens

Conventional methods of detecting and analyzing pathogens in the food stuff include the cell culture-based assays (Gracias and McKillip 2004). There are several other methods of detection like electrochemical biosensors, PCR, and enzyme-linked immunosorbent assay (Blackburn and Mcclure 2002). The technological advancement in the field of nanotechnology has opened up a new paradigm of monitoring of

pathogens; these advanced methods of detection have several advantages over conventional methods such as less time-consuming, accuracy, reliability, reproducibility, cost-effectiveness, etc.

22.3.2 Biosensor-Based Detection of Pathogens

Now, nanotechnology is being used in the development of biosensors that have enormous applications in the field of science (Poole and Owens 2003). Biosensor is a device with a biological element (mostly antibody, nucleic acid receptor, or other ligand molecule) which interacts with an analyte and generates a response. This generated response is then transformed into an electronic signal (Miao et al. 2004). Nanosensors are generally used for the conversion of biological signals into an electronic signal. The material used in making these sensors is also of nanoscale and offers several advantages over the traditional material-based sensors (Poole and Owens 2003). The nanomaterials used in making the nanosensors are generally gold, carbon nano-structure, magnetic nanoparticles, silicon nanomaterials, graphene oxide, dendrimers, and conducting polymers.

22.3.3 Nanomaterial-Based Detection of Pathogens

One such method is based on magnetic nanomaterials. The unique property of nanoparticles exploited further as magnetic nanoparticles makes the detection method much more rapid, simple, selective, and sensitive. In one research, the biofunctional magnetic nanoparticles were used to confine vancomycin-resistant enterococci (VRE) and other gram-negative bacteria. In the same study, it is also reported that the magnetic nanoparticles can also assist in the fast separation of pathogens (Gu et al. 2003). Modified magnetic nanoparticles have the potential to detect the pathogens even from the crude samples. Such magnetic nanoparticles were also used to separate *E. coli* from beef, groundwater, and milk sample (Augustine et al. 2016). The use of magnetic nanoparticles in polymerase chain reaction, biosensors, immunoassays, and spectrometry is reported to give very quick results. These nanoparticles were also found to increase the efficiency of these techniques by 10–100 times.

22.3.4 Fluorescent Nanoparticles and Quantum Dot-Based Detection of Pathogens

Fluorescent nanoparticles, quantum dots (QD), and giant magnetoresistance (GMR) sensors are some of the recent modifications used for the detection of pathogen and toxins. Quantum dots have been used in the detection of live bacterial contamination in the food crops (Burris and Stewart 2012; Krishna et al. 2018). Every year, infected food is responsible for more than 0.4 million deaths and around 600 million infection

cases around the globe (Salvetat et al. 1999). According to a report, foodborne disease is responsible for 30% of death among children (Finger et al. 2019; Grace et al. 2020). Conventional culture-based methods are being used for the detection of the pathogen, but the process is very time-consuming, and the result takes a few days. With the use of nanotechnology, the problems related to the conventional approach can be overcome. Nanotechnology is the science where the matter is understanding at a scale of 1–100 nm offers a more sensitive, reliable, and quick method of detection and analysis.

22.3.5 Carbon Nanotube-Based Sensor for Detecting Pathogenic Bacteria

Carbon nanotubes have their applications in drug delivery and biomolecular applications (Dresselhaus et al. 2000). These nanotubes are the hollow tubes made up of one or more concentric layers of graphite (Salvetat et al. 1999). These tubes have a unique structure and physical properties (Salvetat et al. 1999). The biosensor ought to have properties, viz., specificity, sensitivity, and less detection time in accessing the presence of pathogens in a food material. Several advancements were made in the development of carbon nanotube-based biosensors (Salvetat et al. 1999). One such example is single-walled carbon nanotubes (SWCNTs) (Jain 2012). SWCNTs were reported to be used for the detection of *Salmonella* spp. and other pathogens (Zelada-Guillén et al. 2010; Yamada et al. 2014).

22.4 Biodegradable Nanomaterial for Food Protection and Packaging

Packaging of food materials is always demanded not only to be free from pathogens but also to be able to prevent them to and should not add environmental waste. Biodegradable packaging material helps in maintaining the human health and helps the ecosystem. However, such packaging materials are having problems, viz., short shelf life, poor barrier, mechanical properties, etc. With the advancement of nanotechnology, nano-polymer composites such as potato starch, corn starch, Arabic gum, carboxymethyl cellulose, chitosan, etc. are being incorporated to overcome the problems related to biodegradable packaging material (Table 22.2). The problems related to the packaging of food material (packaging and environmental waste) can be overcome with the help of nanotechnology-based biodegradable packaging materials. A good packaging also ensures the marketability and acceptability of the product and also provides the safety assurance by decreasing the contamination (Youssef et al. 2019). Each year, a number of cases of deaths and illnesses due to foodborne pathogens are being reported. It is also adding an extra burden on the economy as a substantial percentage of health-care and medical care budget is being invested in the treatment of the patients of foodborne illness (Scharff 2010). Packaging plays an important role in ensuring the safety of the food material as it protects

Table 22.2 Nanomaterials combined with polymers for the improvement of their working capability for the detection of pathogens

Nanomaterial	Polymer and type	Nanomaterial effect	Reference
TiO_2	Starch and starch	• Improved UV protection, thermal properties, and hydrophobicity • Reduction of water vapor permeability	Goudarzi et al. (2017)
Nano-graphene	Starch and chitosan-tapioca starch	• Improved thermal and mechanical properties • Reduction of water vapor permeability	Ashori and Bahrami (2014)
TiO_2	Starch and amylose starch/ polyvinyl alcohol	• Improved mechanical and water resistance properties • Showed antibacterial activity	Liu et al. (2015)
ZnO nanoparticles	Chitosan and chitosan	• Improved mechanical properties (tensile strength, thickness, and barrier properties) and improved antimicrobial activity	Sanuja et al. (2015)
Nano-ZnO	Chitosan coated on polyethylene film	• Improved antimicrobial activity with enhanced solubility	Al-Naamani et al. (2016)
MgO	Chitosan and chitosan	• Improved mechanical properties (tensile strength, thickness, and barrier properties) and improved antimicrobial activity	Sanuja et al. (2014)
MgO	Chitosan and chitosan	• Improved mechanical, thermal properties, and UV protection • Flame-retardant properties	De Silva et al. (2017)
TiO_2	Chitosan and chitosan/ polyvinyl alcohol	• Improved mechanical properties (tensile strength, thickness and transparency, barrier properties) and antimicrobial activity	Lian et al. (2016)
Ag nanoparticles and cellulose nanocrystals	Cellulose and cellulose	• Improved mechanical properties and enhanced antibacterial and antioxidant activity	Wu et al. (2019)
Nanoclay	Synthetic polymer and polylactide	• Significant improvement of oxygen barrier	Svagan et al. (2012)
ZnO nanoparticles	Protein and soybean protein	• Improved mechanical properties and enhanced antibacterial and activity	Tang et al. (2019)
Zein nanoparticles	Protein and whey protein isolate	• Improved of mechanical properties and water vapor barrier	Oymaci and Altinkaya (2016)

it from the external environment by providing isolation. It also helps in minimizing the damage due to mechanical forces and other related problems like dust, gases, moisture, microbial contamination, radiation, light, and insects. Food may get

contaminated by many reasons such as during storage, shipping, distribution, or display. Henceforth, it is the packaging which ensures that the food material does not get contaminated by any of these reasons. At the same time, it is also expected that the packaging should be impermeable, non-toxic, and inert to the microorganisms (Sung et al. 2013). Packaging also describes the quantity of food material to be consumed by the consumers, and at the same time, it also prevents the food from pollution and damage (Moustafa et al. 2019).

22.4.1 Starch-Based Films Protect Food Products from Pathogens and Increase Their Shelf Life

Starch is one of the natural bio-based polymers which offers several advantages such as huge accessibility, low cost, non-toxicity, and its biodegradable feature. The films of starch can be prepared by using extrusion or solvent casting method. The mechanical, thermal, and barrier properties of starch-based films can be altered using the content and type of plasticizer and starch, the content of amylose, and the morphology of polymer. Starch-based films show good oxygen barrier property; however, they are not equally good as the moisture barrier. Due to their barrier property, the starch films are widely used in the coating of fruits and vegetables and on some other food materials to increase their shelf life as well (Escamilla-García et al. 2018; Lermen et al. 2021).

22.4.2 Chitosan-Based Films Protect Food Products from Pathogens

Chitosan is obtained from different biological materials such as the cell wall of fungi and shells of crustaceans. However, chitosan was initially introduced by Rouget (Crini 2019), a widely found hey polysaccharide planet other than cellulose. Chitosan is made up of N-acetylglucosamine and glucosamine units joined by 1–4 glycosidic bonds. Due to its properties such as microbial resistance, biodegradability, biocompatibility, and non-toxicity, chitosan is being used by industries and has numerous applications (Youssef et al. 2019). Chitosan shows solubility in different organic acids, and it is immiscible in alkali, water, and mineral acids. However, it can be dissolved in alcohol acetone and water by adding the little acidic amount. Chitosan is also a good material for nanofiber production. Chitosan is known to avoid the various contaminating agents such as bacteria and fungi. Polycationic property of this polymer helps it in getting absorbed onto the surface of bacteria. Further, it is known to make compound with metal ions and changes the permeability of cell membrane. Chitosan has an ability to bind with the DNA which makes it a prospective material for gene delivery. It has been elucidated that it has a role also in mRNA inhibition. Research has revealed that the use of additional metal ions enhances the antimicrobial activity of chitosan. In one of the researches, Zn^{+} and Ag^{+} complex with chitosan was found to increase the antimicrobial activity (Wang et al. 2004). The tensile strength and elongation are some of the mechanical

properties of chitosan that makes it a perfect material for packaging. According to a report, the tensile strength of chitosan films was found increased at room temperature when created with acetic acid (Youssef et al. 2014). Other factors such as drying temperature and relative humidity also play an important role in the mechanical features of chitosan-based films (Fernández-Pan et al. 2010). The composite chitosan polyvinyl alcohol films were developed using the electrospraying method. In this film, the various contents of chitosan were also found to impact its morphological characteristics. The use of chitosan was found to increase the mechanical characteristic of the polyvinyl alcohol (PVA)-chitosan (CS) films as compared to the original PVA films. Chitosan/ZnO bio-nanocomposites were also used to make antimicrobial starch-based composite films.

22.4.3 Biodegradable Films of Cellulose and Synthetic Polymers Protect Food Products from Pathogens

Cellulose and its derivative such as carboxymethyl cellulose (CMC) are perfect materials to produce biodegradable films. The properties of CMC, viz., transparency, non-allergenicity, non-toxicity, low cost, etc., make it a good material for industrial use. The films made up of CMC are also having good mechanical properties and impermeable to gases. CMC is also being utilized in the paper, food, detergent, and textile printing industries. Synthetic plastics mainly used in food packaging are posing a serious environmental challenge. Henceforth, the demand of biodegradable or renewable and low-cost material is increasing (Shahbazi et al. 2016; Azzaoui et al. 2017).

22.5 Antimicrobial Packaging for Ensuring Quality and Safety of Food Products Against Foodborne Pathogens

To increase the shelf life of food products and their safety, it is obligatory to have a packaging with antimicrobial property. Antimicrobial packaging is one of the most innovative and advanced packaging concepts. Antimicrobial packaging offers several other advantages such as oxygen scavenging and moisture control. The construction of such packaging is also multifaceted (Zhong et al. 2020). Most of the packaging system consists of the food product, the headspace, and the packaging material. Any of these three may have antimicrobial agents to ensure and increase antimicrobial efficiency. The use of antimicrobial packaging material and the incorporation of antimicrobial inserts in the headspace, the innovative gaseous agents are also being used in the headspace to prevent microbial spoilage (Han 2005). To ensure the quality and safety of food and allied products, the control of both aerobic and anaerobic bacteria is very challenging and necessitates the development of new packaging technologies (Barros-Velázquez 2016).

It is researched on the shelf life of meat and meat products and found to have significant impact. The use of antimicrobial compound provides resistance against a

range of microbial population which is equally good against specific microorganisms. Several compounds such as enzymes, salts, bacteriocins, and some miscellaneous compounds, viz., triclosan, silver zeolites, and fungicides, were tested for their effectiveness an antimicrobial compound in film making (Quintavalla and Vicini 2002). In one of the researches, the effectiveness of biodegradable polylactic acid (PLA) was evaluated for its application as antimicrobial food packaging. Its antimicrobial property was tested against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enteritidis* (Jin and Zhang 2008). It was deduced that the biopolymer and the bacteriocin have a great potential as antimicrobial food packaging.

22.6 Synergetic Antibacterial Nano-Formulation Approach for Controlling the Foodborne Pathogens

Nanoparticles and oxides of metals or metals are some of the types of inorganic antimicrobial compound. Compared to the organic antimicrobial compound which includes various enzymes, organic acids, and polymers, the inorganic compounds differ in their thermostable property. This thermostable property makes them widely applicable as they can sustain the use of such materials in adverse processing situations. The second difference is observed in the antimicrobial property of the inorganic material compared to antibiotics. The biocidal spectrum of nanomaterials is broader than antibiotics along with their impact on a variety of cell types. Nanotechnology research has led to the development of many unique nanomaterials with improved antimicrobial characteristics. Further, various metal oxides (ZnO, MgO, and CaO) are also known for their antimicrobial activity due to the production of reactive oxygen species (ROS). A new dimension in the field of nanotechnology has emerged in the manufacturing of various nanosized substances for their application in molecular computing, energy production, medical diagnostic, therapeutic, etc.

Nanocomposite based on the natural bio-based polymers with either inorganic or organic nanofiller is being developed as a novel packaging material. Such a material has better biodegradability and a broader antimicrobial property. Compared to conventional bio-based polymers, bio-nanocomposites are having superior characteristics. Bio-nanocomposites can be classified on the bases of structure and type of matrix and its size, shape, and origin of reinforcement.

22.7 Conclusion

Nanotechnology applications have played an important role in food science, food processing, and microbiology with the increasing needs of nanomaterial in food processing, food packaging, food development, and food safety and the detection of foodborne pathogens and extension of food shelf life. In the past one decade, the popularity of nanomaterials' uses in the food sector is increasing; therefore, interest

and activities in this research area have greatly focused. The applicability of nanomaterials in the areas of food packaging and food safety is well known. Additionally, promising results have been achieved in foodborne pathogen detections, and food preservation using nanomaterial was discussed. This review also summarizes the potential of nanoparticles for their uses in the foodborne pathogen detections and contribution of nanotechnologist for monitoring pathogens through biosensors, nanosensors, metal nanomaterials, and carbon nanotube-based sensing and increasing the shelf life of food products by applying nanomaterial coatings and films of polymers. All in all, the food nanotechnology-based industry in order to provide consumers a safe and contamination-free food and to ensure the consumer acceptability of the food with enhanced functional properties.

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Regulation of Genetically Modified and Gene-Edited Foods: An Overview

23

Asha Martin

Abstract

This chapter provides an overview of the regulatory aspects of genetically modified organisms (GMOs). It also updates on the recent initiatives taken by leading statutory agencies on new developments in biotechnology. It is complemented with updated information. As the cultivation of GMOs enters into its landmark 25th year, the readers of this book will get a peek into the regulations governing GMOs from an Indian perspective. An overview of the approaches for GMO legislations applicable in the USA, EU, and India is presented to give the readers an idea of the similarities and differences between the GMO legislations of the three different jurisdictions.

Keywords

Genetically modified organisms · Genetic modification · Gene editing · GMO legislations

23.1 Introduction

One of the most important landmarks in the history of science is the discovery of the molecular structure of deoxyribonucleic acid (DNA) by James Watson and Francis Crick in 1953. Watson and Crick's path-breaking paper on the structure of DNA published in Nature (April 25, 1953) not only described the double helical structure of DNA but also postulated how organisms store biological information and pass it

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from one generation to the next. The unraveling of the helical structure of DNA paved the way for applications of modern biotechnology that employs gene manipulation to enhance the capability of specific organisms to produce substances for the benefit of mankind. James Watson, Francis Crick, and Maurice Wilkins were collectively conferred the Nobel Prize in Physiology or Medicine in 1962 for their elucidation of the structure of DNA and its implication for information transfer in living organisms. Scientists have been able to build on the basic knowledge of DNA, and today, there is a plethora of applications in health, medicine, forensic science, and agriculture. Almost six decades after the discovery of the DNA structure, Jennifer Doudna and Emmanuelle Charpentier in the year 2012 reported the development of the genome editing tool CRISPR/Cas which is the abbreviated form for clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (Cas) (Jinek et al. 2012). Their CRISPR/Cas9 "genetic scissors" cuts DNA at a specific location and allows researchers to modify genes with precision. To say that CRISPR-Cas9 is one of the major findings of the twenty-first century is no exaggeration. This gene editing tool has transformed biology research, enabling faster discovery of drugs, and has made the study of diseases easier. In addition to the health sector, this technology of manipulating genes has significantly impacted the agriculture sector especially the development of crops, foods, and industrial fermentation processes. The announcement of the Nobel Prize winners for the year 2020 came as this chapter was being written. The 2020 Nobel Prize in Chemistry was awarded to Jennifer Doudna and Emmanuelle Charpentier in recognition of their contribution to the development of the CRISPR technique. The emergence of recombinant DNA technology and gene editing technology are classic examples of how advances in science can dramatically transform and touch all spheres of our lives. Agricultural biotechnology is one such area wherein the application of gene editing technology for modifying crop genomes has resulted in a new generation of plant varieties in the global market. These exciting advancements in science have also resulted in concerns being voiced about their safety and the need for regulating crops and products developed using the recombinant DNA technology and gene editing technology.

The global regulatory landscape of genome-edited crops is presently not well established, and countries that have established laws or regulations are in a minority. This chapter has been written with the focus of providing an overview of the regulatory aspects of foods or products obtained from genetically modified organisms (GMOs) primarily from the Indian perspective. For this, it is important for us to know relevant GMO regulations elsewhere. As it would be impossible to cover comprehensively GMO food legislations from around the world, an overview of the approaches for legislations applicable in the USA, EU, and India is presented. This will give the readers an idea of the similarities and differences between the GMO legislations of the three different jurisdictions. US and EU regulatory systems are chosen because the USA cultivates more GM crops on the market than any other nation and is one of the first nations to come out with guidelines for research involving recombinant DNA and EU has one of the most stringent polices in place to regulate GMOs.

23.2 Genetically Modified Organisms and Gene-Edited Organisms

The term GMOs refers to microbes, plants, and animals whose genetic material is altered in such a manner that does not occur naturally either due to mating or during the process of natural recombination. The technology used for developing GMOs is often referred to as "recombinant DNA technology" or "genetic engineering." It allows the transfer of genes from one organism to another and also among incompatible species. One striking feature of this technology is that it overcomes species barrier. The techniques of molecular biology have enabled manipulation of the genetic constitution of organisms. Simple genetic traits can be transferred from virtually any other organism (bacteria, plant, animal) to crop plants using genetic engineering techniques. Foods that are produced from GMOs or which contain or consist of such GMOs are called genetically modified (GM) foods. The traditional method of breeding involves the selection of desired traits by crossing crops and their wild relatives and is often considered to be a time-consuming and relatively imprecise method. Recombinant DNA technology on the other hand has brought a new precision in crop improvement process. The genetic modification typically entails the introduction of a fragment of DNA (the insert), an assembled combination of genetic elements (promoter, enhancer, terminator) usually taken from other naturally occurring organisms into the genome of the organism to be modified. Genetic modification can also be achieved by modifying existing codes without inserting foreign DNA. Gene editing refers to techniques that utilize specialized enzymes that can add, interchange, or delete DNA from a genome with a high degree of precision and specificity. Point-specific mutations in the genome are carried out by site-directed nucleases (SDN) and oligo-directed mutagenesis (ODM). The SDN comprises of zinc-finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN), meganucleases, and CRISPR (Lassoued et al. 2019). An advantage of gene editing technology includes faster development of traits in crops at low costs. These include crops that are more nutritious and traits that enable them to withstand the vagaries of nature brought about by climate change.

23.2.1 The Need for Genetically Modified Crops and Gene-Edited Crops

The challenges to agriculture in the twenty-first century are profound (Table 23.1). Global population is projected to reach nine billion in the year 2050. With diminishing water resources and shrinking cultivable land and deteriorating soil quality, addressing food security would be a challenge (Federoff 2010). The moot question is how one can increase agricultural productivity on arable land at higher temperatures using lesser water. Prominent reasons for reduced crop productivity are losses arising from pest attack and stress resulting from drought, salinity, and frost. Increasing crop yields by cultivating plants that can withstand biotic and abiotic stresses can help achieve food security and global sustainability. The increase in the

Table 23.1 Key challenges to agriculture and the potential of GM and gene-edited crops to achieve food security

Challenges	Opportunities	Examples of GM crops	Examples of gene-edited crops
Deterioration in soil quality; diminishing water resources; shrinking area of cultivated land; climate change (global warming)	Increase the tolerance of crops to adverse growing conditions	Drought tolerance II corn (DuPont Pioneer)	Climate-resilient crops that can grow at higher temperatures and saline soils Drought-tolerant soybean
Food security Sustainable foods	Imparts resistance to crop pests and reduce pesticide usage	Insect-resistant MON 810 maize Herbicide-tolerant roundup-ready soybean	Pest-resistant crops Low input of pesticides requiring crops
Wastage	Enhance processing characteristics for reducing wastage and costs	Delayed ripening of vegetables and fruits, reduced browning and bruising of fruits and vegetables	Non-browning apples, white mushrooms, and potatoes
Malnutrition and undernourishment	Improve the nutrient composition of crops by modifying the amino acid, vitamin, and fatty acid profile	Increased beta-carotene content (golden rice)	Camelina sativa with enhanced omega-3 oil Soybeans with improved oil profiles Crops with improved nutrition and antinutrients

yield of a particular crop can be achieved by introducing tolerance to herbicides, by imparting resistance to insect pests and certain diseases, and by enhancing the processing characteristics. Flavr Savr™ tomato modified for delayed ripening so that they didn't spoil during transport was among the first GM crops to be granted approval by the US Food and Drug Administration (FDA) way back in 1994. Herbicide tolerance and pest resistance were the prominent traits in the first generation of GM plants. For example, the 5-enolpyruvylshikimate-3-phosphate synthase gene, sourced from a bacterium *Agrobacterium tumefaciens*, has been inserted in soya bean resulting in tolerance to glyphosate, a herbicide. MON 810 maize is an authorized transgenic maize event in which insect resistance has been conferred by the introduction of cry1Ab gene. A larger variation of genes and novel traits was noticeable in the second generation of GM plants that included increased stress tolerance to drought, frost, and salt, altered nutrient composition, and production of materials of industrial importance like enzymes. Food quality can be enhanced by increasing the synthesis of desired compound and/or decreasing the synthesis of undesired compounds, such as antinutrients. In spite of their promise, regulatory

hurdles and consumer concerns have resulted in very few GM crops and products being available in the global market and supermarket shelves. It is anticipated that the advent of gene-edited crops owing to its precision and accuracy will overcome concerns posed by GM technology and finds consumer acceptance (Kumar et al. 2020). Advancements in crop genetic engineering can hasten up the development of GM varieties with higher yield, superior nutrition, and tolerance to biotic and abiotic stresses (Kamthan et al. 2016). Few examples of prominent GM crops and gene-edited crops are as follows.

23.2.1.1 Golden Rice

Golden rice is rice that has been genetically modified by inserting two genes that code for phytoene synthase and carotene desaturase so as to improve the β-carotene content for addressing vitamin A deficiency which purportedly affects 250 million people globally (Potrykus 2010). In 2018, golden rice cleared successfully the safety evaluations from USFDA, Food Standards Australia New Zealand, and Health Canada, and in the year 2019, the Philippines approved GR2E golden rice to be used in food, feed, or processing.

23.2.1.2 GM Apples

Apples that were genetically modified to resist browning were granted approval in the USA in 2015. Polyphenol oxidases (PPOs) are enzymes that are naturally present in fruit and vegetables and catalyze the oxidation of polyphenols to quinones, when fruit is cut or bruised resulting in browning. The GM apples were developed by genetically engineering apples with a transgene that produced specific RNAs that silenced the expression of PPO genes that are responsible for browning (Waltz 2015a).

23.2.1.3 GM Potato

Acrylamide is a metabolite formed as a result of the reaction between asparagine and sugars when potatoes and other cereal grains are subjected to high temperatures during cooking. Acrylamide has the potential to affect human health, and efforts are underway to reduce the formation of acrylamide in the food supply chain. GM potato genetically engineered to have diminished bruising and browning has been approved for commercial planting by USDA. RNA interference (RNAi) technology has been employed to reduce the level of acrylamide in potato by suppressing the expression of asparagine synthetase-1 (Asn1) gene. It is reported that suppression of the asparagine enzyme lowers acrylamide production by about 50–70% when the potatoes are cooked (Waltz 2015b).

23.2.1.4 Transgenic Aflatoxin-Free Maize

Every year, millions of tons of crop are lost due to aflatoxin contamination. Several strategies have been adopted to reduce the levels of aflatoxins that are potentially carcinogenic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Transgenic aflatoxin-free maize was developed by employing small

interfering RNA molecules to silence the biosynthesis of aflatoxins in maize (Thakare et al. 2017).

23.2.1.5 Low-Gluten Wheat Edited with CRISPR/Cas9

Celiac disease (CD) is a common autoimmune disorder where the ingestion of gluten proteins leads to characteristic inflammation in the upper small intestine of CD patients. Strict adherence to diet which is gluten-free is the only way to overcome the disorder. The amount of α-gliadins in wheat has been reduced using the CRISPR/Cas9 technology which is beneficial for gluten-intolerant consumers (Sanchez-Leon et al. 2018).

23.2.2 Global Status of GMOs

Cultivation of GMOs began way back in 1996 and has entered the 25th milestone year in 2020. There has been a continuous rise in the global area planted with GM crops. Twenty-six countries cultivated 191.7 million hectares of GM crops in the year 2018 which is a pointer to the worldwide adoption of transgenic technology (James 2018). Soybeans, maize, cotton, and canola are the major GM crops cultivated globally.[1] Several GM crops like mustard, tomato, cabbage, cauliflower, cotton, and brinjal are at different stages of field trials in India (Warrier and Pande 2016). Insect-resistant Bt cotton is the first GM crop to have been granted approval for commercial release in India in 2002. Insect-resistant Bt brinjal was on the verge of being given permission for commercial cultivation in 2010; however, following a series of public consultations, a moratorium was introduced on its release. Herbicide-tolerant GM Dhara Mustard Hybrid-11 (DMH-11) was also apparently lined up for approval for commercial cultivation. All these three cases got extensive media coverage in India. A glance at the chronology of the events lining the approval process reveals that the first application for small-scale trial of GM mustard was submitted in 2003. The biosafety dossier for the environmental release of DMH-11 was submitted in 2015, and the risk assessment and management documents were submitted in 2016. More than 18 years have passed. Approval for GM mustard is yet to see the light of the day, and Bt cotton continues to be the sole transgenic crop cultivated in India.

23.2.3 Possible Risks and Hazards from GMOs

The power of genetic modification techniques raises health, environmental, socio-economic, and ethical concerns. Health concerns include concerns of toxicity of the gene product and the potential of GM foods to induce allergy in humans, decreased nutritional value of foods, etc. Random insertion of genes may possibly lead to gene

[1] http://www.isaaa.org/resources/publications/briefs/54/executivesummary/default.asp

silencing or augmentation of gene expression of regulatory elements leading to the production of more toxins and allergens. Environmental concerns include potential damage to the environment, evolution of pest resistance, loss of biodiversity, and harmful effects on non-target organisms. The transfer of genes to non-target species is also a matter of concern. For instance, the transfer of gene encoding tolerance to herbicides from crops to weeds may result in superweeds. Social concerns include increasing control of agriculture by biotechnology corporations. The development of GM crops is a lengthy and costly process, and the patents are held by a handful of corporates. Farmers from developing countries are often unable to buy costly seeds widening the gap between the rich and the poor. Consumer concern has resulted in stringent regulation for GMOs. In recent years, several countries have adopted labeling policies for GM food. The never-ending and often intense debate on the application of recombinant DNA technology for food production has resulted in rigorous regulations on the detection, quantification, traceability, and labeling of foods derived from GMOs. The ensuing section describes the approach taken by major authorities across the globe to regulate GM and gene-edited crops.

23.3 The Regulatory Systems for Genetically Modified and Gene-Edited Organisms

To give consumers a choice, the freedom to choose between GM and non-GM, several countries have come up with labeling legislations for GM foods. Efforts have been made by regulators across the globe to keep pace with the advancements in the area of genome engineering technologies particularly in the area of healthcare and agriculture. New techniques such as CRISPR offer several benefits over conventional and GM crops with respect to accuracy, speed, cost, and regulatory requirements (Parisi et al. 2016). Employing these techniques, plant breeders can develop traits in crops that permit increases in yield, resistance to pests, adaptation to climate change, and pharmaceutical and industrial applications (Richroch 2019). However, regulatory policies regarding the cultivation of transgenic crops may hinder the large-scale adoption of these techniques (Bilichak et al. 2020). Thus, it offers great opportunities, but also creates regulatory challenges. Whether changes in legislation and policy are needed is being actively considered by several governments (Myskja and Mhyr 2020). A comparative evaluation of the cultivation and consumption of GMOs and their products in the USA, the European Union, Japan, Argentina, Australia, and Canada has revealed the distinctiveness of these countries' policy toward GMOs (Hamburger 2019).

23.3.1 The US Regulatory Framework on Genetically Modified Organisms

The USA was one of the first countries to come up with comprehensive regulations for biotechnology products. The United States Department of Agriculture (USDA),

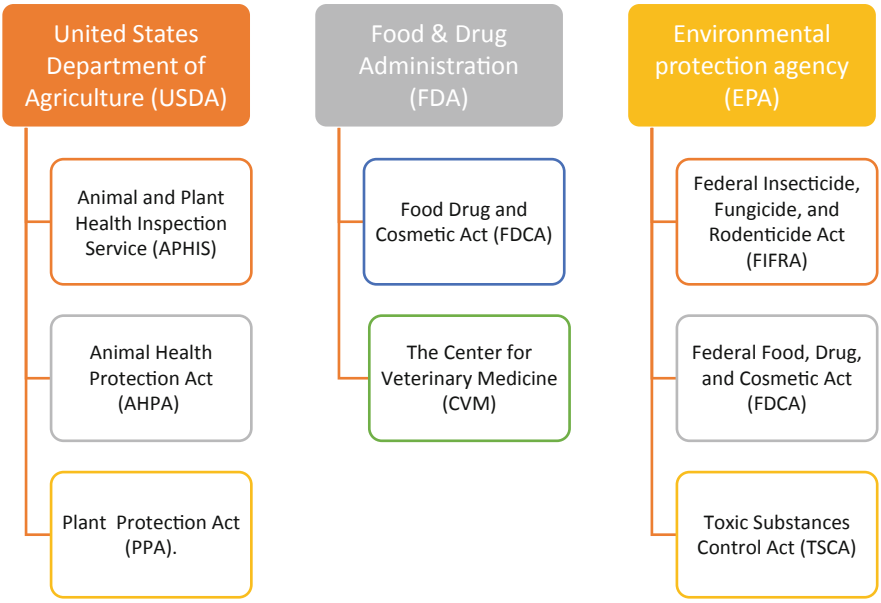

Fig. 23.1 Schematic representation of the US federal agencies and the acts under which biotechnology products are regulated

Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) are the three statutory agencies mandated with the responsibility of regulating products of biotechnology for their intended applications (Grossman 2019) (Fig. 23.1).

To facilitate oversight by the statutory agencies, the Coordinated framework for the regulation of biotechnology was brought out as early as 1986 (OSTP 1986). It was subsequently updated in 1992 and more recently in 2017. A striking feature common to all the versions of the Coordinated framework is that the basis of regulation of biotechnology products is not the process or the technique by which the product is made; rather, emphasis is on the characteristics, the type of application, and the environment into which it is introduced. The Coordinated framework 2017 which can be downloaded from the EPA website provides a rigorous regulatory framework that enables innovation and transparency for the regulation of products arising from newer scientific advancements in biotechnology and its applications (EPA 2017). It clarifies the roles and responsibilities of each of the federal agencies and further lays down mechanisms for cooperation and sharing of information among them. A brief description of the US statutory agencies is provided below. Additional information on US regulatory requirements can be obtained from relevant agency's website appended at the end of the chapter.

23.3.1.1 United States Department of Agriculture (USDA)

Biotechnology products that are likely to create risk to agricultural plant and animal health are regulated by the Animal and Plant Health Inspection Service (APHIS), within USDA under the regulatory provisions of the Animal Health Protection Act (AHPA) and the Plant Protection Act (PPA). The import, interstate movement, or release into the environment of certain genetically engineered organisms produced using plant organisms, DNA from plant pest organisms, or those which meets the definition of a plant pest are regulated by APHIS.[2]

23.3.1.2 Food and Drug Administration (FDA)

FDA follows a voluntary food safety and regulatory consultation process for foods derived from genetically engineered plant varieties for humans and animals and regulates the safety of such foods and food ingredients, under the regulatory provisions of the Federal Food, Drug, and Cosmetic Act (FDCA).[3] The Center for Veterinary Medicine (CVM) under FDA is mandated with the responsibility of evaluating the safety of food derived from genetically engineered animals under the new animal drug provisions of the FDCA.[4]

23.3.1.3 Environmental Protection Agency (EPA)

Biotechnology products are regulated by EPA under the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); Federal Food, Drug, and Cosmetic Act (FDCA); and Toxic Substances Control Act (TSCA). The sale, distribution, and use of all pesticides, including the ones produced using genetic engineering, are regulated under the FIFRA, whereas the presence of pesticide chemical residues in food is regulated under sect. 408 of the FDCA by EPA. Under the TSCA, manufacturers and importers of new organisms (intergeneric) are required to report to EPA.[5] Intergeneric microorganisms include those created by intentionally combining genetic material belonging to different taxonomic genera. EPA requires applicants to provide data that the pesticide will not cause any harmful effects on health and environment. There is also a provision for Experimental Use Permits, a permit that allows developers to gather data in support of an application during registering, while ensuring appropriate regulatory checks are in place to safeguard health and the environment.

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[2] https://www.epa.gov/sites/production/files/201701/documents/2017_coordinated_framework_update.pdf

[3] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096095.htm

[4] http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM113903.pdf

[5] https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/update-coordinated-framework-regulation-biotech

Table 23.2 Key EU legislations governing GMOs (https://ec.europa.eu/food/plant/gmo/legislation_en)

Legislation	Mandate
Directive 2001/18/EC	Deliberate release of GMOs into the environment
Regulation (EC) 1829/2003	Authorization procedure for GM food and feed
Regulation (EC) 1830/2003	Traceability and labeling of GMOs and GMO-derived food and feed products
Regulation (EC) 1946/2003	Transboundary movement of GMOs
Directive 2009/41/EC	Contained use of GMOs
Directive (EU) 2018/350	Environmental risk assessment of GMOs (Amending Directive 2001/18/EC)

23.3.1.4 Regulation of Genome-Edited Products in the USA

Regulatory systems need to evolve with technological advancements. Accordingly, APHIS has recently revised regulations pertaining to the import, movement between states, and release of certain genetically engineered organisms into the environment with the objective of easing the regulatory burden for producers of novel genetically engineered organisms that are not likely to create risks of plant pests (APHIS 2019). In the USA, products resulting from genome editing of modern forms of mutagenesis are exempted from regulatory oversight provided they are not plant pests (Lassoued et al. 2019). The first CRISPR genome-edited plant exempted from regulation in the USA was the common white button (Agaricus bisporus) mushroom that was genetically engineered to resist browning by targeting one of the gene encoding polyphenol oxidases (PPOs), an enzyme responsible for browning (Waltz 2016). USDA has approved drought-tolerant soybean variety developed with CRISPR and false flax (Camelina sativa) with enhanced omega-3 oil (Waltz 2018).

23.3.2 European Union (EU) Regulatory Framework on Genetically Modified Organisms

The EU has a robust regulatory framework for the cultivation of GMOs or for placing GM food and feed and derived products on to the market (Bruetschy 2019). It has established harmonized measures for the authorization and risk assessment of GMOs that are valid throughout the EU. However, individual member states can provisionally restrict the use and/or sale of GMOs by invoking the safeguard clause of Directive 2001/18/EC. Legislation for labeling (0.9% threshold) and traceability has been implemented to enable consumers to choose and to ensure the traceability of GMOs. The key EU GMO legislations are summarized in Table 23.2. Additionally, there are several rules, recommendations, and guidelines. The most significant aspect of the EU regulatory framework is that it is predominantly focused on the process, i.e., the technique used to create the genetically

modified crop. Contrastingly, the Coordinated Framework of the USA focuses on the nature of the biotechnology product as against the process employed to create it.

23.3.2.1 Regulation of Gene-Edited Products in EU

All genome-edited plants obtained by either ZFNs, TALENs, or CRISPR are subject to regulation in EU. In an interesting judgment, the Court of Justice of the European Union adjudicated that organisms developed from new techniques of mutagenesis would be subjected to EU regulations applicable to other GM organisms (Myskja and Myhr 2020). As per the ruling, all applications for approval of genome-edited crops will be governed by EU Directive 2001. This means that any genome-edited variety even if it does not contain foreign DNA would be regulated in the same manner as transgenic varieties. The inability of the currently available methods to differentiate certain genetic changes resulting from genome editing from those that are either produced naturally or using conventional techniques of breeding would impede the implementation of EU GMO legislations (Voigt and Münichsdorfer 2019). Consequently, implementation of the Court's ruling necessitates the development of strategies for the detection of gene-edited crops. Reforms in the EU GMO regulations will facilitate the use of new technologies for meeting societal challenges (Eriksson et al. 2020). Exemption from labeling requirements has been sought for foods and products developed using gene editing technology provided that they do not have foreign DNA (Eriksson et al. 2020a).

23.3.3 GMO Regulatory Framework in India

India is among the first countries to come up with robust biosafety guidelines and regulations for genetically engineered (GE) organisms (Warrier and Pande 2016). Regulations governing the manufacture, storage, and import/export of GE organisms and hazardous microorganisms were notified on the December 5, 1989, and came into force from October 1, 1993. Commonly referred as Rules 1989,[6] they were promulgated under the Environmental Protection Act (EPA) 1986. The Ministry of Environment, Forest and Climate Change (MoEF&CC) is the statutory agency mandated with granting approval for the environmental release and commercialization of GE plants in India under the provisions of Rules 1989. Manufacture and imports of GMOs whether for research or industrial applications are allowed only with the authorization of the GEAC. The main statutory authorities notified for handling of various aspects of GMOs are schematically represented in Fig. 23.2.

23.3.3.1 Genetic Engineering Appraisal Committee (GEAC)

The GEAC is an apex committee governed by MoEF&CC. The GEAC is tasked with the appraisal of activities and proposals employing the large-scale usage of GE

[6] https://ibkp.dbtindia.gov.in/DBT_Content_Test/CMS/Guidelines/20181115121526033_Rules-for-the-manufacture-use-import-export-and-storage-1989.pdf

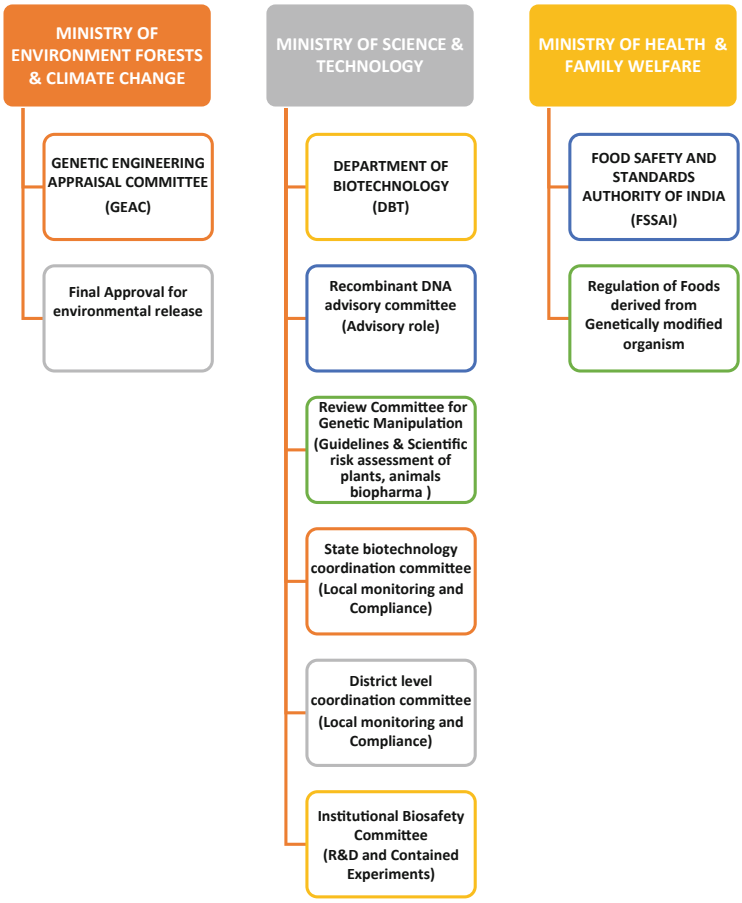

Fig. 23.2 Indian statutory bodies involved in the regulation of genetically modified and gene-edited organism

or hazardous microorganisms for research as well as industrial production under the provisions of the EPA 1986 and Rules 1989. It is responsible for monitoring the release of GE organisms and products into the environment and is empowered to take punitive action. The environmental risk assessment of genetically engineered plants is carried out as per the guidelines issued jointly by MOEF&CC and DBT in 2016.[7]

[7] https://ibkp.dbtindia.gov.in/DBT_Content_Test/CMS/Guidelines/20181115134800192_Environmental%20Risk%20Assessment%20of%20Genetically%20Engineered%20Plants-%20A%20Guide%20for%20Stakeholders,%202,016%20.pdf

23.3.3.2 Review Committee on Genetic Manipulation (RCGM)

The RCGM is a committee that is responsible for monitoring the biosafety aspect pertaining to activities that involve hazardous microorganisms and GE organism and cells and products thereof and lays down procedures restricting or prohibiting their manufacture, sale, import, and use. Functioning directly under DBT, the RCGM periodically brings out guidelines detailing regulatory procedures for activities entailing the use of GE organisms in research as well as industrial and environmental applications. Recombinant DNA safety guidelines and regulations were first brought about by DBT in 1990. The revised version was published in 1994. Guidelines for the evaluation of allergenicity and toxicity of transgenic plants, plant parts, and seeds were issued in 1998. To facilitate exchange (interstate and inter–institutional within India), import/export of GE organisms and products derived thereof for research purpose, simplified procedures/Guidelines were first issued in 2015 and revised recently in 2020.[8] The tools of recombinant DNA research have changed tremendously with the advent of the new gene editing tools necessitating the need for proper containment guidelines. The earlier recombinant DNA safety guidelines were therefore revised in 2017, and updated regulations and guidelines for research involving recombinant DNA and biocontainment were issued.[9] Since 2019, an online portal system, Indian Biosafety Knowledge Portal (IBKP), is in operation to facilitate online applications to RCGM. All the above guidelines and regulations can be downloaded from the official website of DBT appended at the end of the chapter.

23.3.3.3 Institutional Biosafety Committee (IBSC)

All institutions and organizations working with recombinant DNA are mandated to have IBSC that serves as the nodal point for the implementation of the biosafety guidelines at the institute level. It is responsible for reviewing the biosafety aspects of all experimental studies involving recombinant DNA technology. There are more than 500 IBSCs currently active in India (Ahuja 2018). The safety procedures that need to be adhered to while dealing with GE organisms and non-GE hazardous microorganisms have been detailed in a handbook issued by DBT in accordance with Rules, 1989. The IBSC plays a crucial role in ensuring adherence to these safety procedures. The handbook has been recently revised and released (IBSC 2020) and facilitates the effective monitoring of recombinant DNA activities carried out at the institute level.

23.3.3.4 District Level Biotechnology Committee and State Biotechnology Co-Ordination Committee

The onus of implementing the Rules 1989 at the district level and state level lies with the District Level Biotechnology Committee (DLC) and State Biotechnology

[8] Revised simplified procedures/guidelines on import, export and exchange of GE organisms and products thereof for R&D purpose, 2020. https://ibkp.dbtindia.gov.in/Content/Rules

[9] http://dbtindia.gov.in/sites/default/files/uploadfiles/Regulations_%26_Guidelines_for_Reocminant_DNA_Research_and_Biocontainment%2C2017.pdf

Co-ordination Committee (SBCC), respectively. The DLC has the authority to monitor and review the biosafety aspects in establishments involved in the usage and applications of GMOs/hazardous microorganisms as well as its release in the environment. The SBCC functions as a nodal agency and is authorized to evaluate damages, resulting from the release of GE organisms, and to take on-site remedial measures. They are empowered to take penal actions when statutory provisions are violated.

23.3.3.5 Food Safety and Standards Authority of India

The Food Safety and Standards Authority of India (FSSAI), a statutory body under the administrative control of the Ministry of Health and Family Welfare, was established in 2008 to operationalize the Food Safety and Standards (FSS) Act 2006 that was notified in the gazette of India on August 24, 2006. Food Safety and Standards Rules, 2011, were subsequently notified through a Gazette Notification dated May 5, 2011.[10] The primary objective of FSSAI is to ensure the availability of safe and wholesome food for human consumption. Food Safety Officers and the Food Safety Commissioners help in the enforcement of the FSS Act (www.fsssai.gov.in). The Commissioner of Food Safety is responsible for the efficient and effective implementation of the FSS Act in the state or union territory as the case may be. The mandate of FSSAI includes framing of standards for articles of food, regulating all aspects of their manufacture, storage, distribution, sale, and import. The process of development of a standard is driven by the principle of food safety and involves risk assessment. It involves deliberations by the Scientific Panels (SPs) constituted for product categories and laying down the standard(s), which are validated by the Scientific Committee (SC) and finally approved by the Food Authority before the process of issue of draft notification(s) of the standards/regulation is initiated for inviting public comments, followed by a final notification. Currently, there are 21 SPs notified by FSSAI, and one of them is the SP on genetically modified organisms and foods. According to sect. 22 of FSS Act 2006, no person shall manufacture, distribute, sell, or import articles of food that are genetically modified, without prior central government authorization (www.fssai.gov.in). FSSAI is presently in the process of framing draft guidelines for the labeling of genetically modified food items and is considering 1% as the labeling threshold.[11] An interesting development has taken in the meanwhile. In order to make sure that only food crops that are not genetically modified are imported into India, FSSAI recently issued a directive mandating "Non-GM cum GM Free" certificate for imported food consignment.[12] The apex regulator has identified 24 crops imported in India to mandatorily declare "Non-GM cum GM Free" certificate from January 1, 2021. The crops listed by FSSAI include tomato, alfalfa, eggplant, potato, apple, squash, melon, papaya, pineapple, plum, Argentine canola, Polish canola, bean,

[10] https://fssai.gov.in/upload/uploadfiles/files/FSS_Gazete_Rules_2011.pdf

[11] https://fssai.gov.in/upload/media/FSSAI_News_Labelling_BusinessStan_04_02_2019.pdf

[12] https://fssai.gov.in/upload/advisories/2020/08/5f3fb6de8a4f1Order_GM_Food_21_08_2020.pdf

chicory, cowpea, flax seed, wheat, maize, rice, safflower, soybean, sugar beet, sugarcane, and sweet pepper. As per the directive, food importers are required to submit a detailed certificate declaring that the product is non-GM origin and does not contain genetically modified organisms (GMO) and also not genetically modified.

23.3.3.6 Regulation of Genome Editing Technologies in India

Keeping pace with global advancements and with a view to lay the way forward for the development and sustainable use of technology involving gene editing in India, DBT has recently brought out draft guidelines that details acts and procedures applicable for genome editing (DBT 2020). Risk assessment of genome-edited organisms and products derived thereof and institutional mechanism for regulatory oversight is also incorporated in the guidelines. As per Rules 1989, gene technology refers to the application of the gene techniques called genetic engineering and includes self-cloning, deletion, as well as cell hybridization. This definition, being broad in scope, encompasses genome editing, process, as well as product. The degree of regulatory scrutiny will be decided by (1) the type of process or tools used for gene editing (e.g., SDN/ODM), (2) the extent of the genetic change brought about by editing, (3) the characteristics of gene-edited organisms, (4) unintentional changes if any, and (5) the intended use of the gene-edited organism or the products derived from them (DBT 2020). A systematic and structured approach for the risk analysis of gene-edited organisms/product is proposed that takes into consideration the trait that has been introduced, the type of genome editing tools employed, and the complexity of changes created in the organism or product. The draft guidelines have been put in the public domain seeking comments and suggestions from stakeholders. The regulation of organisms and products resulting from new and emerging technologies will be on a case-by-case basis based on Rules, 1989 (Chimata and Bharathi 2019). The National Academy of Agricultural Sciences, New Delhi, recently in 2020 issued a policy brief pertaining to the regulatory framework for genome-edited plants.[13] Two important recommendations that have been made are that i) products of genome editing using SDN-1 and SDN-2 approaches should be exempted from regulations and risk assessment as they do not carry any vector DNA and are similar to the products of spontaneous or induced mutations, as genome editing is a precise and targeted mutagenesis tool, and ii) the foods resulting from the SDN-1 and SDN-2 categories should not be treated as "genetically engineered or genetically modified food" under the FSS Act, 2006. FSSAI is presently laying down draft regulations for foods derived from GMOs. It is hoped that the apex regulator takes into consideration the regulatory changes happening globally in GM policies and includes provisions for regulating food derived from gene-edited organisms in the regulations.

[13] Policy Brief: http://naasindia.org/Policy%20Briefs/PB7-Regulaory%20Framework.pdf

23.4 Challenges in the Identification and Quantification of Gene-Edited Organisms

Products derived from GMOs are subjected to mandatory labeling in several countries, and a plethora of approved methods for identifying and quantifying GMOs in food and agricultural products are available. The development of methods for crops and products derived from the gene-edited organisms, however, is in the nascent stage. How are we going to identify and quantify genome-edited plants? Differentiating genetic alterations, e.g., single nucleotide polymorphism (SNPs) created by genome editing from those that are either naturally produced or via conventional breeding practices, is challenging (Voigt and Münichsdorfer 2019). Concerns have been voiced to reconsider the implementation of the labeling requirements for many gene-edited products in view of the lack of molecular traceability methods for gene-edited products. Identifying and quantifying genome-edited plants will not be easy if they are to be regulated. Validation and harmonization of quantitative methods will be needed to address compliance with regulations for products derived from gene-edited organisms. A remarkable advancement pertaining to the detection of gene-edited organisms was reported recently in September 2020 (Chhalliyil 2020). Real-time PCR was used for identifying and quantifying herbicide-tolerant canola which is the first genome-edited crop commercialized. Tolerance to sulfonylurea and imidazolinone herbicides in canola was achieved by gene editing a single base pair in the canola AHAS1C gene. However, it remains to be seen whether this development would aid in resolving concerns expressed about the practicability of extending the regulatory requirements for GMOs developed using established techniques to products resulting from gene editing technology. It is anticipated that strategies for monitoring genome-edited plant products would be in place soon to meet the regulatory requirements. Regulators and scientific fraternity would be watching these exciting developments with keen interest.

23.5 Conclusion

Genetically modified food is one of the most debated subjects of the twenty-first century. The debate about the regulation of gene-edited crops is much more complex. Though several academic and research institutions worldwide are working on a plethora of novel traits, most results from genetic engineering have not been commercialized. We are in the 25th year of cultivation of GM crops; however, consumers are still wary of accepting it due to a variety of reasons. The rapidly growing use of genome editing has raised several health and safety concerns. It remains to be seen whether genome-edited crops will be accepted by consumers. Recombinant DNA technology and gene editing technology have the technical potential to overcome food production challenges, to mitigate biotic and abiotic stresses, and to develop nutritiously superior foods. The array of molecular tools and knowledge available today make it possible to design new kind of foods. To a large

extent, harmonized guidelines are available to assess new GM varieties; however, regulatory framework for GMOs needs to be strengthened. Regulators, academia, innovators, and all other stakeholders need to take stock of existing regulations and respond to the advances made by the newer technologies by devising new policies to regulate GMOs. As we have seen, the regulatory policies differ across countries, and there is a need for the harmonization of regulations for products arising from newer plant breeding technologies. Once regulations are put in place for genome-edited organisms, its implementation will become a daunting challenge for regulators and laboratories. A harmonized strategy for the detection of genome-edited organisms and products derived therefrom is very much the need of the hour.

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