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Microbial Biotechnology in Food and Health

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Applied Biotechnology Reviews Series

Microbial Biotechnology in Food and Health

Edited by

Ramesh C. Ray
Centre for Food Biology and Environment Studies, Bhubaneswar,
Odisha,
India

Series Editor

Ramesh C. Ray

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Contributors

Mostafa Aghamirzaei Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran; Vice-chancellors for Food and Drug, Healthcare Network of Fardis, Alborz University of Medical Sciences, Fardis, Alborz Province, Islamic Republic of Iran

Saber Amiri Department of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, West Azerbaijan Province, Islamic Republic of Iran; Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran

Cristiani Baldo Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

Md Latiful Bari Center for Advanced Research in Sciences, University of Dhaka, Dhaka, Bangladesh

Sudhanshu S. Behera Department of Fisheries and Animal Resource Development, Directorate of Fisheries, Government of Odisha, Odisha, India; Centre for Food Biology and Environment Studies, Bhubaneswar, Odisha, India

Maria Antonia Pedrine Colabone Celligoi Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

Steve C.Z. Desobgo Department of Food Process and Quality Control, University Institute of Technology of the University of Ngaoundere, Cameroon

Suman Guleria Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

Reena Gupta Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

Pratima Khandelwal Teaching-Learning Centre, Global Academy of Technology, Bengaluru, Karnataka, India

Nasim Khorshidian Food Safety Research Center (Salt), Semnan University of Medical Sciences, Semnan, Iran

Sachin Kumar Biochemical Conversion Division, Sardar Swaran Singh National Institute of Bio-Energy, Kapurthala, Punjab, India

Yogita Lugani Enzyme Biotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India

Hajar Madahi Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran

Akshita Mehta Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

Swati S. Mishra Department of Biodiversity and Conservation of Natural Resources, Central University of Orissa, Koraput, Odisha, India

Amir M. Mortazavian Department of Food Technology, Faculty of Nutrition Sciences and Food Technology/National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Parisa Mostashari Nutrition and Food Sciences Research Center, Tehran Medical Sciences, Islamic Azad University, Tehran, Tehran Province, Islamic Republic of Iran

Nicole Caldas Pan Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

Sandeep K. Panda School of Biotechnology, KIIT University, Bhubaneswar, Odisha, India

Hanny Cristina Braga Pereira Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

Ramesh C. Ray Centre for Food Biology and Environment Studies, Bhubaneswar, Odisha, India

Mohammad Sarbazi Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran

Roji Sharma Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

Poonam Singh Organic Building Materials, CSIR-Central Building Research Institute, Roorkee, Uttarakhand, India

Balwinder Singh Sooch Enzyme Biotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India

Samira Tizchang Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran

R.S. Upendra Department of Biotechnology, School of Applied Science, REVA University, Bengaluru, Karnataka, India

Josiane Alessandra Vignoli Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

Hatice Kalkan Yıldırım Ege University, Department of Food Engineering, Izmir, Turkey

Mojtaba Yousefi Food Safety Research Center (Salt), Semnan University of Medical Sciences, Semnan, Iran

Sharmin Zaman Center for Advanced Research in Sciences, University of Dhaka, Dhaka, Bangladesh

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Microbial biotechnology in food and health: present and future food safety regulation

1

Md Latiful Bari, Sharmin Zaman
Center for Advanced Research in Sciences, University of Dhaka, Dhaka, Bangladesh

1.1 Introduction

Microbial biotechnology is defined as any technological application that uses microbiological systems, microbial organisms, or derivatives thereof, to make or modify products or processes for specific use (Okafor, 2016).Traditional biotechnological processes (such as classical mutagenesis and conjugation) and recombinant gene technology have been widely applied for the genetic improvement of the microbial cultures for use in food fermentations, despite food safety regulatory control, and consumers concern exist. To date, no commercial GM (genetically modified) microorganisms that would be consumed as living organisms exist. However, products of industrial GM producer organisms are widely used in food processing, and no major safety concerns have been raised against them. Production of enzymes and various food-processing ingredients such as monosodium glutamate, polyunsaturated fatty acids, and amino acids are produced using GM microbial cultures. Rennet which is widely used as a starter in cheese production across the globe is produced using GM bacteria. Thailand currently makes use of GM *Escherichia coli* as an inoculant in lysine production. Many industrially important enzymes such as α-amylase, gluco-amylase, lipase and pectinase, and biobased fine chemicals, such as lactic acid, amino acids, antibiotics, nucleic acid, and polysaccharides, are produced in China using GM starter cultures. Other developing countries which currently produce enzymes using recombinant microorganisms include Cuba, Brazil, India, and Argentina (FAO, 2010). In addition, ingredients derived from microbial fermentation or extracted from microalgae are steadily gaining ground in the food industries (Dufossé, 2018). Thickening or gelling agents (e.g., polysaccharides such as xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), lipids (polyunsaturated fatty acids—PUFAs, sterols), flavor compounds (gamma-decalactone, diacetyl, methyl ketones), vitamins, essential amino acids, pigments/colorants (carotenoids, azaphilones) (Dufossé et al., 2014; Venil et al., 2014), surfactants, and acidulants (lactic acid, citric acid) are currently produced using GM microorganisms (Yin et al., 2017; Kamzolovaet al., 2015).

Despite all these above-mentioned product developments, many advanced technological developments also occurred that may influence food safety in future on different scales (from global to molecular) and in different time frames (from decades

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to less than a minute). This necessitates development of new risk assessment approaches, taking the impact of different drivers of change into account, requiring active governmental policy setting and other drivers may decrease food safety risks. Recent molecular techniques developments make it possible to rapidly assemble information on the genome of various isolates of microbial species of concern. Such information can be used to develop new tracking and tracing methods, and to investigate the behavior of microorganisms under environmentally relevant stress conditions. These novel tools and its insight need to be applied to objectives for food safety strategies, as well as to models that predict microbial behavior (Havelaar et al., 2010). Monitoring of contamination in the food chain combined with surveillance of human illness and epidemiological investigations of outbreaks and sporadic cases continue to be important sources of information (EPA, 1997). In addition, the increasing complexity of the global food systems necessitates improved communication between all parties involved: scientists, risk assessors and risk managers, as well as consumers.

1.2 Microbial cell factories

Microbial cell factory is an approach to bioengineering which considers microbial cells as a production facility in which the optimization process largely depends on metabolic engineering. Many recent articles emphasize the power of microorganisms which are able to modify, to improve the properties of many food products or by-products; for example:

- Production from whey of peptides with bacterial antivirulence effects (Ali et al., 2019);
- Wheat, rice, corn, and amaranth flour proteins treated with microbial transglutaminase, followed by immunoreactivity testing of gluten-sensitized sera toward modified flours (Scarnato et al., 2019);
- Bioconversion of beet molasses to alpha-galactosidase and ethanol (Álvarez-Cao et al., 2019);
- Production of fructo-oligosaccharides from aguamiel, the sap from agave plants (Picazo et al., 2019); or
- Degradation of toxic steroidal glycoalkaloids from potato juice, a by-product of the potato industry, of the starch processing (Hennessy et al., 2018).

Furthermore, the scientists emphasize the crucial role of microorganisms currently playing and are likely to continue to play in future as microbial cell factories for the production of food grade components and biobased ingredients. This is due to the versatility in their metabolic pathways and biochemical profiles, amenability for easy large-scale cultivation, and a long history of production by well-investigated production strains. Efforts have been made to reduce the production costs of components produced by algal ponds and microbial fermentation, since synthetic ones or those extracted from natural plant sources can often be produced more economically. Fungi, yeasts, bacteria, and microalgae are considered as promising living organisms for sustainable, large-scale production of commodities such as food, feed, chemicals,

materials, and biofuels. Joseph et al. (2019) described the perspectives of bioproduction of the recombinant sweet protein thaumatin, as the most promising alternatives of sugar and artificial sweeteners. Recombinant DNA technology is used in the most favorable host known today, the methylotrophic yeast, *Pichia pastoris*. Sen et al. (2019) have provided comprehensive information on the application of microbial pigments in the food industry. In addition, Li et al., 2019 examined chemical modification of orange *Monascus* pigments and fine analyses of commercial red and yellow *Monascus* pigments present in Chinese market. Sarika et al. (2019) reported the biopreservative efficacy of *Lactobacillus rhamnosus* bacteriocin on stored fish fillets. Brizuela et al. (2018) used blend cultures of native *Lactobacillus plantarum* and *Oenococcus oeni* strains for inducing malolactic fermentation of Patagonian Malbec wine. In addition, the primary and secondary microbial metabolisms are also of crucial importance, as shown by Li et al. (2019), with small GTPases involved in *Monascus ruber*, a filamentous fungi used for more than a 1000 years in Asia for the production food ingredients, or as demonstrated by Sgobba et al. (2018) who genetically modified *Corynebacterium glutamicum* for utilization of alternative feedstocks such as pentose sugars or hexosamines, to produce the amino acids L-glutamate, L-lysine, and the carotenoid lycopene (Tilloy et al., 2014; Yu et al., 2014; Murashchenko et al., 2016; Semkiv et al., 2017). All the above-mentioned research work illustrates how microbial cell factories (native, engineered, or heterologous) are able to provide at an industrial scale biobased components for the food industry. It is impressive that a broad range of different compounds ranging from small organic acids to more complex secondary metabolites or polymers such as oligopeptides can now be produced using microbial cell factories (Dufossé and Fouillaud, 2019).

1.3 Understanding food safety principle

Food safety has been defined as "a reasonable certainty that no harm will result from intended uses under the anticipated conditions of consumption" (OECD, 1993). There are various definitions of foodborne diseases (FBD). The WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) report defines FBD as "a disease commonly transmitted through ingested food. FBDs comprise a broad group of illnesses, and may be caused by microbial pathogens, parasites, chemical contaminants and biotoxins" (WHO, 2015). FERG follows the Codex Alimentarius Commission (CAC) definition of food as "any substance, whether processed, semi-processed or raw, which is intended for human consumption, and includes drink, chewing gum and any substance which has been used in the manufacture, preparation or treatment of food but does not include cosmetics or tobacco or substances used only as drugs". According to the CAC, bottled and packaged water, as well as other drinks, are foods.

The agents responsible for FBD are called hazards. CAC defines a hazard as "a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect" (Codex, 2007). Hazards are divided into three categories: biological, chemical, and physical.

- The **biological hazards** are living organisms, including viruses, bacteria, protozoa, molds, and parasites, which have the ability to infect people or produce toxins injurious to health.
- **The chemical hazards** are substances intentionally produced by industry (such as pesticides) and natural chemicals (e.g., those produced by volcanic emissions or toxic metals) which are injurious to health.
- **The physical hazards** include fragments of different materials such as stones, metal, or glass. Physical hazards, except nanomaterials and radionuclides, do not transfer to human tissues, so impacts on human health are the result of physical trauma.

The biological hazards as broadly defined include agents causing food allergies and food intolerances (Taylor and Hefle, 2005). Food allergies are abnormal immunological responses to a particular food or food component, usually a naturally occurring protein. They include antibody-mediated allergies (e.g., acute reactions to milk or peanuts) and cell-mediated conditions (e.g., coeliac disease in response to gluten). Food intolerances, on the other hand, do not involve abnormal responses of the immune system. Important in low- and middle-income countries (LMICs) is lactose intolerance, which results from genetically inherited inability to metabolize milk sugar (lactose) due to lack of a specific enzyme (lactase).

Mycotoxins (chemical compounds produced by molds) and phycotoxins (chemical compounds produced by algae and accumulated in marine organisms) are sometimes considered to be biological hazards and sometimes to be chemical hazards.

Other food issues may have health implications but are not necessarily diseases transmitted by food. Food fraud is probably common in developing countries, especially for high value foods. It may have health impacts if the adulterant is harmful (e.g., addition of melamine to milk) or if adulteration lowers the nutritional quality of food (e.g., addition of water to milk). Food spoilage is caused by microbes, but these are mostly different from the microbes causing FBD so spoiled food may not be unsafe and vice versa. However, good hygienic practices can reduce both types of microbes. Antimicrobial residues very rarely cause adverse reactions in people consuming them. A more important human health impact is the use of antimicrobials in agriculture that contributes to antimicrobial resistance in pathogens, which can infect people resulting in diseases that do not respond to antibiotics (Marshall, and Levy, 2011).

In the context of food safety, risk is the probability of harm resulting from a given exposure to a hazard. Risk, by definition, has two elements: probability, which captures the likelihood of occurrence, and harm, which refers to the nature and extent of ill effects. Finally, the WHO defines a health system as "all the activities whose primary purpose is to promote, restore or maintain health" (WHO, 2015). By extrapolation, a food safety system can be considered as those activities whose primary purpose is to ensure food is safe to eat. As such, the food safety system includes actors whose main mandate is assuring food safety (e.g., food safety authorities) and actors who are concerned with food safety as one aspect of food (e.g., local government authorities, institutional providers of food, and workers at all stages of the "farm to fork" food production-to-consumption pathway).

1.4 Food safety assessment of microbial biotechnology derived food

Because microorganisms have been used for centuries to produce foods and food ingredients, considerable experience has been gained regarding the factors that need to be taken into account in ensuring that such substances are safe. The new molecular techniques for introducing and modifying heritable traits in microorganisms have greatly expanded the pool of genetic traits available for strain improvement. These new methods permit specific, well-defined sequences, including genes from plants and animals, to be introduced rapidly into a variety of microbes. The maintenance of scientific principles is important in ensuring that foods and food ingredients produced from microorganisms are safe for the intended use. Although there is a great diversity of biotechnology products, including foods and food ingredients, it is recognized that the nature of safety assessment evaluation of these substances may vary with respect to both the food or food ingredients and the proposed use of the product. The chemical nature of the substance to be produced and the amount that will be used in food are critical factors to be taken into account in a food safety assessment. Amino acids, organic acids, flavors, thickeners, antioxidants, preservatives, and enzymes are produced by fermentation used in food processing (FAO/WHO, 2001). They are also used for the production of food ingredients (e.g., single-cell protein). In these applications, viable organisms are not intended to be part of the finished food, but the metabolites to be used in the food and thus did not consider as potential hazard (Zhang et al., 2015). On the other hand, microbes are used as essential constituents of food in order to produce characteristic effects, such as acid and flavor (e.g., dairy starter cultures), or microbes intended for use as probiotics. These applications involved organism itself will become a component of food thus consider as potential hazard. Several potential hazards that could result from the use of GM microorganisms to produce food or food ingredients include (FAO/WHO, 2010):

- Microbes that produce toxic substances or are pathogenic;
- Introduced genetic material (e.g., vectors) that encodes harmful substances or that can be transferred to other microbes in which it produce harmful traits;
- The transfer of pathogenic microbes to marker genes that encode resistance to clinically important antibiotics;
- The production of unexpected products (or increased levels of normal products) of genes, effects on multiple genes (pleiotropy), or secondary effects that result in harmful substances in the finished food;
- Potential adverse consequences of modified microbes in the human gastrointestinal tract;
- Possible adverse effects (e.g., allergenicity) of new or altered proteins;
- Possible adverse nutritional changes in food;
- Possible adverse effects of changes in the structure and/or function of gene products.

These potential hazards are not new and can be controlled adequately using sound microbiological principles. The molecular biology techniques greatly enhanced the ability of scientists to address these concerns and ensure safety.

1.5 EU and US regulations of microbial biotechnology derived food

The United States and the European Union (EU) share a common desire to provide a safe food supply and credible regulatory systems. However, they have adopted two very different regulatory approaches to deal with the increasing numbers of biotechnology-derived food products coming to market.

1.5.1 United States

In the United States, any food ingredient of natural biological origin that has been widely consumed for its nutrient properties prior to January 1, 1958, without known detrimental effects, which is subject only to conventional processing as practiced prior to January 1, 1958, and for which no known safety hazard exists, will ordinarily be regarded as generally recognized as safe (GRAS). In 1977, FDA notified microorganisms and microbial-derived ingredients as GRAS food ingredients (42 FR 14491, Mar 15, 1977), and in May 29, 1992, FDA publishes its biotechnology policy in the Federal Register [51 FR 23309 (1986); 57 FR 22984 (1992)] in which FDA stated that it would focus on the safety of a food and not the process by which the food was developed (FDA, 2019). Under this policy, the food products of biotechnology are to be treated no differently than ordinary food, except in cases in which the genetic engineering process adds a new substance to the food that is not GRAS, or that is significantly different in structure, function, or amount than substances found ordinarily in food. Food additives derived from microorganisms have been listed in 21 CFR (Code of Federal Regulations) 172 and 173. Substances derived from microorganisms affirmed by FDA as GRAS and are listed in 21 CFR184; and substances derived from microorganisms for indirect uses affirmed by FDA as GRAS and are listed in 21 CFR186. In addition, foods that may contain or be derived from microorganisms are listed in 21 CFR Parts 131, 133, 136, and 137 (FDA, 2018). Any ingredient affirmed as GRAS in this part shall be used in accordance with current good manufacturing practice. For the purpose of this part, current good manufacturing practice includes the requirements that a direct human food ingredient be of appropriate food grade; that it can be prepared and handled as a food ingredient; and that the quantity of the ingredient added to food does not exceed the amount reasonably required to accomplish the intended physical, nutritional, or other technical effect in food. If the ingredient is affirmed as GRAS with specific limitation(s), it shall be used in food only within such limitation(s), (a) including the category of food(s), (b) the functional use(s) of the ingredient, and (c) the level(s) of use.

For example, the α-amylase enzyme preparation from *Bacillus stearothermophilus*. In this category of food,

a) α-Amylase enzyme preparation is obtained from the culture filtrate that results from a pure culture fermentation of a nonpathogenic and nontoxicogenic strain of *Bacillus stearothermophilus.* Its characterizing enzyme activity is [α]-amylase (1,4 [α-D glucan glucanohydrolase (E.C. 3.2.1.1).

b) The ingredient meets the general and additional requirements for enzyme preparations in the "Food Chemicals Codex," 3d ed. (1981), pp. 107−110, which is incorporated by reference in accordance with CFR.
c) The ingredient is used in food with no limitation other than current good manufacturing practices in accordance with CFR.

The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current good manufacturing practice conditions of use:

- The ingredient is used as an enzyme, as defined in CFR, in the hydrolysis of edible starch to produce maltodextrins and nutritive carbohydrate sweeteners.
- The ingredient is used at levels not to exceed current good manufacturing practices.

More recently, the use of biotechnology has led to new pesticide products that control a variety of pests. These biologically produced pesticides, which use the inherent pest-fighting abilities of many existing plants and microbes, have properties that distinguish them from those of conventional chemical pesticides. When these products have unique biological properties, they may also pose unique regulatory challenges; to address these challenges, the Environmental Protection Agency (EPA), the U.S. Department of Agriculture (USDA), and the Food and Drug Administration (FDA) have shared responsibility for regulating agricultural biotechnology in the United States. In particular, EPA regulates pesticides created through biotechnology as a part of its regulatory jurisdiction over all pesticides marketed and used in the United States. The federal government has a coordinated, risk-based system to ensure that new biotechnology products are safe for the environment and human and animal health and established as a formal policy in 1986 termed as "the Coordinated Framework for Regulation of Biotechnology". The Coordinated Framework is based upon existing laws designed to protect public health and the environment. The US government has written new regulations, policies, and guidance to apply these laws to biotechnology-derived products. Within USDA, the Animal and Plant Health Inspection Service (APHIS) is responsible for protecting agriculture from pests and diseases. Under the Plant Protection Act, USDA-APHIS has regulatory oversight over products of modern biotechnology that could pose such a risk. Accordingly, USDA-APHIS regulates organisms and products that are known or suspected to be plant pests or to pose a plant pest risk, including those that have been altered or produced through genetic engineering.

1.5.2 European Union

Microorganism derived from food known as "food cultures" (FC) used directly in food production are regarded as food ingredients in the EU, a category of food ingredients with a very long history of use in a great variety of food products. FC, like other food ingredients, must fulfill the requirements set out in the General Food Law (EU Regulation No. 178/2002). In Article 14 of General Food Law mentioned that food shall not be placed on the market if it is unsafe, and it is the food business operator's responsibility for ensuring food safety (EU, 2002). FC used for the fermentation of food is not subject to EU premarketing regulation, unless it is regarded as being novel to the EU market and its consumers (Patterson, 2000).

Novel food (NF) means food that was not consumed to a significant degree within the Community before May 14, 1997 and has to comply with the updated NF regulation EU 2015/2283 (EU, 1997; Schulz, and Schmit, 2015). Any foods and food ingredients which consist of microorganisms, fungi, or algae or any biological agents must comply with this regulation. An NF must undergo a premarket evaluation and authorization procedure including risk assessment by the EU Food Safety Authority (EFSA) and risk management by the EU Commission before it can be placed on the market. However, microorganisms-derived food containing live microorganisms has not be evaluated or authorized in the EU since 1997.

A gene modification of a microorganism used as FC is also included in NF category. However, the NF notification procedure has been abandoned with respect to the GM foods regulation and now has to comply with EU directive No 2001/18 (EU, 2001), EU regulation No. 1830/2003(EU, 2003), and Commission regulation No. 641/2004(EU, 2004a,b).

1.5.3 The EU principle of biotechnology derived food

The EU principles for biotechnology regulation introduces certain principles that should underlie all strategy for regulation of biotechnology, whether set at community level or, in accordance with the subsidiary principle, by the member states. All biotechnology regulation should be science-based and people-centered and should respect human life, dignity, ethical values, and the fundamental values of the rights. The following four principles are outlined for use in community legislation:

- Risk governance and product authorization
- Safeguarding the internal market
- Proportionality and consumer choice
- Predictability, modernization, and impact assessment

The principles are explained as products must have a favorable risk assessment prior to authorization; the precautionary principle should be used where there is scientific uncertainty. Regulation must be proportionate, coherent, efficient, feasible, and enforceable. It must also be regularly monitored, evaluated, and updated in line with scientific and technological progress. Consumers must be able to make informed choices about GM products. Since biotechnology is a global technology, with global impacts, regulation should also take into account the international context in which the community is operating. The relevant international agreements to which the community has consented are to be taken into account during the development of legislation. The policy and legislation on biotechnology depends on widespread understanding of the approach being taken and the principles on which it is based. In particular, concepts such as scientific uncertainty, absence of zero risk, the precautionary principle, and risk analysis and management should be explained to the public.

The basic legislative requirements (Directive 2000/54/EC) for biological agents are defined as: “microorganisms, including those which have been genetically modified, cell cultures and human endoparasites, which may be able to provoke any infection,

allergy or toxicity". These biological agents were classified into one of four risk groups:(Directive 2000/54/EC, Article 2.)

Group 1:	Any biological agents unlikely to cause human disease.
Group 2:	Biological agents that can cause human disease, or that may be a hazard to workers, but are unlikely to spread to the community and for which there is usually effective prophylaxis or treatment available.
Group 3:	Biological agents that can cause severe human disease and present a serious hazard to workers, that may present a risk of spreading to the community and for which there is usually effective prophylaxis or treatment available.
Group 4:	Biological agents that cause severe human disease and are a serious hazard to workers, that present a high risk of spreading to the community and for which there is usually no effective prophylaxis or treatment available.

Risks to workers health and safety from exposure to biological agents must be assessed and managed. Along with the classification of the biological agent, the assessment should take into account recommendations from a competent authority; information on diseases which may be contracted as a result of the work; and potential allergenic or oxygenic effects may occur. Thus, the results of risk assessments are to be made available to competent authorities and any accident or incident which may have resulted in the release of biological agents. To ensure public health, several regulatory authorities have been formed with significant responsibilities for the assessment of food derived from microorganisms as shown in Table 1.1.

1.5.4 EFSA and the QPS list

The qualified presumption of safety (QPS) list is the EFSA fast-track risk assessment tool that is used by EFSA panels when evaluating products with microorganisms that require a premarket authorization (e.g., feed additive cultures, cell factories producing enzymes/additives/vitamins, novel microorganisms, plant protection) (Ricci et al., 2017). The list covers all risk assessment for microorganisms for human, animal, and environmental use—"from farm to fork", and harmonizes the work of the EFSA panels, makes the approach more transparent, improves the consistency of the assessments, and makes better use of resources by focusing on those organisms that present the greatest risks or uncertainties.

The EFSA and the Panel on Biological Hazards (BIOHAZ) to deliver a scientific opinion on the maintenance of the list of qualified presumption of safety (QPS) biological agents intentionally added to food or feed (EFSA, 2005). At EFSA, the BIOHAZ Panel assesses the following safety measures of biological agents also taking into account includes:

- The definition of the taxonomic unit (establishing identity of the group)
- The body of knowledge
- The possible safety concerns (pathogenicity)
- For some species, the end use

Table 1.1 Role of the main entities involved in the food derived from microbial cultures (FDMC) safety assessment in the European Union (EU).

EU Commission	The European Commission (EU) is responsible for creating the general food law and risk management (policy) of safety systems together with the member states.
EFSA	The European Food Safety Authority (EFSA) is an agency funded by the EU that operates independently of the European legislative and executive institutions and EU member States. EFSA is responsible for the risk assessment.
FEEDAP	The EFSA Panel on Additives and Products or Substances (FEEDAP) used in animal feed provides scientific advice on the safety and/or efficacy of microorganism-based additives and products or substances used in animal feed.
BIOHAZ	The EFSA panel on Biological Hazards (BIOHAZ) provides scientific advice on biological hazards in relation to food safety and microbiological criteria.
IDF	The International Dairy Federation (IDF) represents the global dairy sector and ensures that the best scientific expertise is used to support high quality milk and nutritious, safe, and sustainable dairy products.
EFFCA	The European Food and Feed Cultures Association cooperates both within the EU and globally, with a wide range of stakeholders in the area of microbial FDMC.

The scheme for assessing the suitability of microorganisms for QPS status is described in Fig. 1.1 (Barlow et al., 2007).

The introduction of a QPS list was made in 2007 based on the opinion of the EFSA Scientific Committee after a public hearing (Barlow et al., 2007), and between September 2017 and March 2018, the QPS notification list was updated with 46 microorganisms from applications for market authorization. From these, 28 biological agents already had QPS status, 15 were excluded of the QPS exercise from the previous QPS mandate (10 filamentous fungi and one bacteriophage) or from further evaluations within the current mandate (two notifications of *Streptomyces* spp. and one of *Escherichia coli*), and one was excluded where confirmatory data for the risk assessment of a plant protection product (PPP) were requested (*Pseudomonas* sp.). Three taxonomic units were (re)evaluated: *Paracoccus carotinifaciens* and *Paenibacillus lentus* had been previously evaluated in 2008 and 2014, respectively. Microorganisms, which are not on the QPS list, are not necessarily considered to be unsafe. However, it must have undergone the evaluation through a full safety assessment for the particular use. EFSA receives requests for the evaluation of FC species, and only the EU Commission can decide which microorganism applications require premarket approval and safety evaluation.

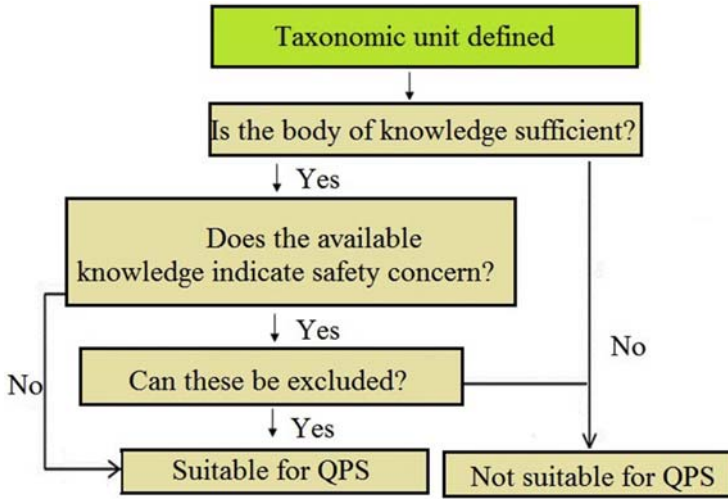

Figure 1.1 A generalized scheme for assessing the suitability for Qualified Presumption of Safety (QPS) status of a microorganism.

1.5.5 EFFCA and the IDF inventory

To fill the gap left by regulation, EFFCA (European Food and Feed Cultures Association) has proposed a definition of FC. The latest update from 2015 defines FC as "safe live bacteria, yeasts or molds used in food production, and they are in themselves a characteristic food ingredient. FC preparations are formulations, consisting of concentrates ($>10^8$ Colony Forming Units/g or mL) of one or more live and active microbial species and/or strains, including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage and to facilitate their application in the food production process". However, some cases were standardized but yet to be published (Bourdichon et al., 2012a; EFFCA, 2018).

To prevent the creation of uncertainty around the existing fermented dairy products, the EFFCA and International Dairy Federation (IDF) jointly produced "**Inventory of Microorganisms with a Documented History of Use in Food"**, the first inventory of FC, which was published in 2002 became a de facto reference for FC in practical use. In 2012, updated inventory of microorganisms used in food fermentations was published covering a wider range of food matrices (dairy, meat, fish, vegetables, cereals, beverages, and vinegar). In addition, a review and update of the taxonomy of the microorganisms used in food fermentations were made in order to bring the taxonomy in agreement with the current standard of nomenclature. The inventory was expanded from 113 species in 2002 to 264 species in 2012 (Bourdichon et al., 2012a). Since both microbial taxonomy and the role of MFC in foods evolve constantly through new scientific evidence, an action team has been established within a standing committee of the IDF to update, and thereby maintain the relevance of the newly published inventory (Bourdichon et al., 2012b,c).

1.6 Microbial genomics tool in food production, processing and preservation

Genomics, a new field of science, analyzes and compares the complete genome (genetic material of an organism) of organisms or a large number of genes in a simultaneous fashion. Now genomics is also entering food production and processing. Microorganisms play important roles in our foods. Microbial genomics can help us understand what microorganisms do and how they do it, in ways that were not previously possible, helping us to better understand how they can be manipulated for our benefit. Consumers' demand for fresh tasting products and convenience foods—such as ready-to-eat chilled multi-component products—is increasing rapidly. Therefore, processing methods for mild preservation and novel nonthermal preservation technologies are increasingly needed. However, a major limitation of classic food microbiology research is that the effect of preservation strategies on spoilage microorganisms can only be determined after a number of days and with separate determinations for each organism. This classic approach is very time-consuming since it consists the tasks that includes colony counting of surviving microorganisms or toxin production on plates. Hence, it is important to develop better knowledge and methods, enabling a quick and reliable prediction of the best processing conditions and a direct insight into the effectiveness of the conditions being applied.

Genomic technologies offer a new alternative to the classic approach in food processing and preservation. They allow quick identification of microorganisms present in the (raw) product, and they could help to directly measure the total response of the target spoilage microorganisms to the applied preservation methods. By making use of this applied microbial genomics, it might be possible one day to reduce the number of experiments needed to measure all relevant responses. The tools used for that are small chips containing the information of thousands of genes of food spoilage microorganisms, attached to a solid surface (like a glass slide) in a grid-like array. These so called "microarrays" could enable in the long run the outcome prediction of a preservation treatment and the definition of additional preservation steps if necessary. As a result, process control could be improved significantly, and the energy input into preservation processes could be reduced. Thus, giving hope to result in improving sensory properties and significant energy savings (due to tailor-made process conditions) and decreased product losses. For example, *Campylobacter jejuni*, a member of *Campylobacter* species, grouped under the name of thermophilic campylobacters, are "formidable" pathogens. Among them, *Campylobacter jejuni* is one of the world's most "successful" food poisoning bacteria and is probably responsible for more than twice as many cases of poisoning as *Salmonella*. Until recently, however, there has been relatively little research into why the bacterium is so virulent (Sheppard et al., 2010a,b). *C. jejuni* is thought to have 1700 genes. Genomics is being used to explore the activity of individual genes and to look at the variety of proteins produced by the organism when it faces different environmental challenges. The adaptability of *C. jejuni* derives from a powerful set of regulatory genes which enable it to change its metabolism rapidly depending on its environment, for example whether it is in

contaminated raw chicken, or residing in the human gut (Woodall et al., 2005). Applying microbial genomic research, a food sample can easily be tested for the presence of those genes, which would indicate *C. jejuni* contamination (Revez et al., 2014). This test is significantly faster than conventional techniques, and thus could be use in sterilization methods, which is less damaging to the food product. However, application of microbial genomics is not limited only to preservation technologies. In principle, all processes in which living (micro)organisms are involved are amenable to the concept. Genomics of food microbes generates valuable knowledge that can be used for metabolic engineering, improving cell factories, and development of novel preservation methods. Furthermore, pre- and probiotic studies, characterization of stress responses, studies of microbial ecology, and, last but not least, development of novel risk assessment procedures will be facilitated. Genomics technology can even be applied as measures for traceability from farm to table.

1.7 Microbial genomics tool to increase food safety

Since foodborne pathogens represent a serious threat to food safety and public health, the main challenge faced by food processors is ensuring a safe food supply include changing population demographics, changes in eating habits, alterations in the methods used to control microbial spoilage, and the development of novel products. The entire genome sequences of several foodborne pathogens have been determined, and the genomes of many more are currently being sequenced (Table 1.2**).** The analyses of these genome sequences and the results of subsequent experiments with "omic" technologies will significantly impact our ability to ensure food safety. Genomics technologies can:

- Aid in the development of pathogen detection and identification tools,
- Identify mechanisms used by pathogens to survive the various conditions encountered in the food-processing environment, e.g., low pH, preservatives, or salt (identify pathogen stress response mechanisms),
- Provide insights into the physiology and evolution of pathogens, and
- Identify systems important for pathogen survival in vivo during infection (virulence factors). (Begley and Hill, 2010).

Genomics and related technologies may help food processors develop effective preservation strategies by using a knowledge-based combination of preservation hurdles [predictive modeling, hazard analysis, and critical control points (HACCP)].

Whole genome sequencing (WGS) has been broadly used to provide detailed characterization of foodborne pathogens. These genomes for diverse species including *Salmonella, Escherichia coli, Listeria, Campylobacter,* and *Vibrio* have provided great insight into the genetic make-up of these pathogens. Numerous government agencies, industry, and academia have developed new applications in food safety using WGS approaches such as outbreak detection and characterization, source tracking, determining the root cause of a contamination event, profiling of virulence and pathogenicity attributes, antimicrobial resistance monitoring, quality assurance for

Table 1.2 Food-poisoning microorganisms and their genome sequence availability.

Bacterium	Relevant characteristics	Number of genome sequences available on NCBI genome database
Listeria monocytogenes	Gram positive	24
Yersinia enterocolitica	Gram negative	1
Aeromonas hydrophila	Gram negative, toxin producer	2
Clostridium botulinum	Gram positive, toxin producer, spore former	15
Bacillus subtilis	Gram positive, toxin producer, spore former	5
Bacillus licheniformis	Gram positive, toxin producer, spore former	2
Bacillus cereus	Gram positive, toxin producer	20
Salmonella	Gram negative	20
Vibrio parahaemolyticus	Gram negative, toxin producer	7
Escherichia coli	Gram negative	20
Staphylococcus aureus	Gram positive	20
Clostridium perfringens	Gram positive	9
Campylobacter jejuni	Gram negative	13

Adopted from Begley M.Á. and Hill C., Food safety: what can we learn from genomics?, Annual Review of Food Science and Technology 1, 2010, 341−361.

microbiology testing, as well as many others. The molecular food safety investigation is rapidly changing from the use of traditional molecular subtyping methods to WGS-based typing methods (Fig. 1.2)**.** This has brought about a transition period. This transition period is giving researchers the opportunity to address important issues such as standardization, quality control, methodology, and regulatory use (Ronholm et al., 2016).

Genome sequences are now available for many of the microbes that cause foodborne diseases. The information contained in pathogen genome sequences, together with the development of them and whole-genome DNA microarrays and improved proteomics techniques, might provide tools for the rapid detection and identification of such organisms, for assessing their biological diversity and understanding their ability to respond to stress. The genomic information also provides insight into the metabolic capacity and versatility of microbes; for example, specific metabolic pathways

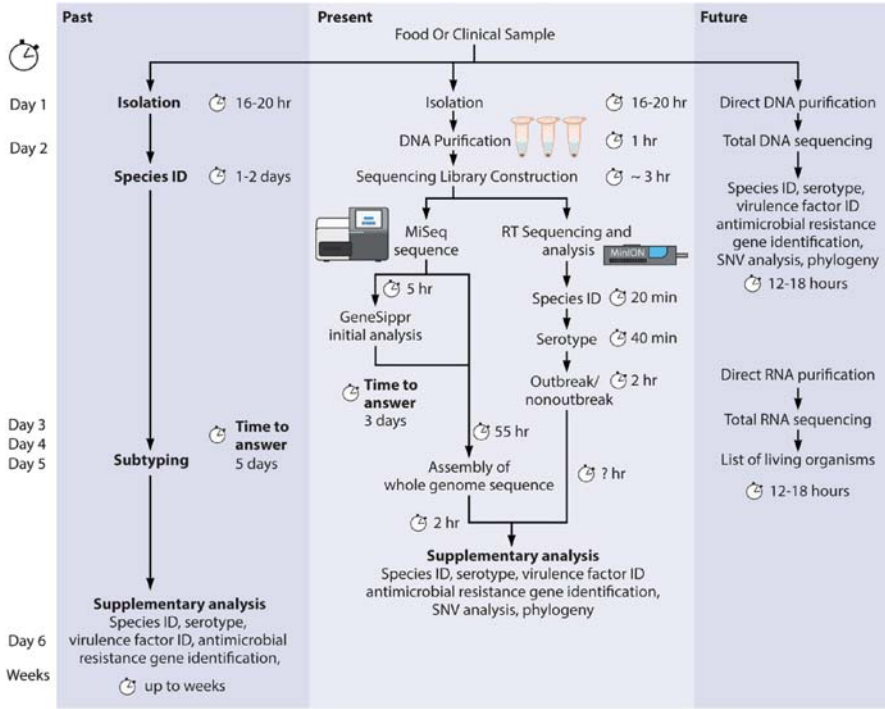

Figure 1.2 Past, present, and potential future workflows of pathogen detection by WGS and traditional techniques.
Adopted from Ronholm, J., Nasheri, N., Petronella, N., & Pagotto, F., 2016. Navigating microbiological food safety in the era of whole-genome sequencing. Clinical Microbiology Reviews 29 (4), 837−857.

might contribute to the growth and survival of pathogens in a range of niches, such as food-processing environments and the human host. New concepts are emerging about how pathogens function, both within foods and in interactions with the host. The future should bring the first practical benefits of genome sequencing to the field of microbial food safety, including strategies and tools for the identification and control of emerging pathogens.

1.8 Conclusion and future perspectives

The significance of microbial diversity within species is the genome sequences of a large collection of strains should be determined for more accurate conclusions to be drawn from in silico and comparative genome analyses. In addition, as a consequence of microbial diversity, it is important that conclusions on the physiological properties of a pathogen not be based on a single strain of a species. Large collections of strains should be included in all experiments. The functions of a large proportion of the genes identified in bacterial genomes are unknown and cannot be predicted from

computational and homology analyses. Future efforts should focus on the mutation of these loci and analysis of the phenotypes of the resultant mutants and expression studies should be performed in real life situations including actual food matrices and not just under standard laboratory conditions. However, the technical challenges associated with accurately measuring bacterial gene expression in complex matrices will need to overcome to realize this goal. However, the knowledge gained from experiments on bacterial stress responses will have to be incorporated into practical concepts that can be used for food preservation. The results from these experiments might allow the development of a knowledge-based combination rather than an empirical combination of appropriate preservation hurdles. Finally, new methods of foodborne pathogen detection need to be tailored to the practical needs of the food industry, despite significant improvement of microbial technology and efforts done by all parties involved.

Acronyms

APHIS	Animal and Plant Health Inspection Service
BIOHAZ	The EFSA panel on Biological Hazards
CAC	Codex Alimentarius Commission
EFFCA	The European Food and Feed Cultures Association
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
FB	Foodborne diseases
FDA	Food and Drug Administration of the US
FDMC	food derived from microbial cultures
FEEDAP	The EFSA Panel on Additives and Products or Substances
FERG	Foodborne Disease Burden Epidemiology Reference Group
GM	genetically modified
IDF	The International Dairy Federation
LMICs	low- and middle-income countries
OECD	Organization for Economic Co-operation and Development
QPS	The Qualified Presumption of Safety
USDA	The U.S. Department of Agriculture
WHO	World Health Organization

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Insights into the role of yeasts in alcoholic beverages

2

Hatice Kalkan Yıldırım
Ege University, Department of Food Engineering, Izmir, Turkey

2.1 Introduction

Alcoholic beverages provide a great variety of spirits such as wine, beer, whisky, brandy, vodka, gin, sake, cider, etc., which are produced by fermentation process from a wide range of raw materials, i.e., cereals, flowers, fruits, and vegetables. The international alcoholic beverages and spirit industry are quite extensive that produces different types and styles of spirits depending on the cultivars and geographical origins of the raw material and production processes adopted.

Most of the alcoholic fermentations are carried out as spontaneous or natural fermentation by indigenous yeast flora. These yeasts are originated from the surface of the raw materials (fruits, cereals, and vegetables). The production of alcoholic beverages by spontaneous fermentation causes yeasts to be exposed to different physical treatments prior to alcohol fermentations such as pressing, crushing, blending, malting, and mashing of the substrates. In case of using starter (pure- or coculture) during fermentation, the effects of such activities on yeasts are skipped.

Yeasts play a central role in the maintenance of food quality and safety in beverages and spirits. In production process, yeasts have the ability to tolerate ethanol concentration up to 15%−20% (v/v) as well as some yeasts such as genetically modified strains of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* are capable of fermenting sugar into ethanol rapidly and efficiently (Satyanarayana and Kunze, 2009; Alcázar et al., 2017). Yeasts also contribute to beverage flavor and aroma with yeast-derived products such as organic acids, aldehydes, ketones, alcohols, and esters. Furthermore, yeasts have been shown as sources of colorants, vitamins, antioxidants, and supplemental additive for their nutraceutical or health-promoting features (Jacques et al., 2003; Querol and Fleet, 2006; Walker and Walker, 2018).

By using the immobilized technology, acacia and chestnut honey brandies were successfully produced. The results demonstrated that immobilized cell technique caused formation of honey brandies with lower ester contents and significantly higher content of volatile organic compounds (Miličević et al., 2018). In another study, the effects of immobilized yeast on aromatic compounds of distillates produced from two fig varieties commonly grown in Croatia were determined. According to the results, using of immobilized cell technique led to the production of spirits with higher ethanol, lower ester contents, and high sensory quality as compared with no immobilized cell technique (Miličević et al., 2017).

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A mixture of yeasts strains (*Torulaspora delbrueckii* ITD-00014a and *S. cerevisiae* ITD-00185) was used during production of spirits from different agave species. The best results (sensory properties) were obtained by using inoculant constituted of 75% *S. cerevisiae* ITD-00185% and 25% *Torulaspora delbrueckii* ITD-00014a yeasts (Nuñez-Guerrero et al., 2016).

Ethyl carbamate (EC) is one of the harmful byproducts which occurs in alcohol drinks, such as wine, rum, whisky, saki, and other fruit wines as a result of a spontaneous reaction between ethanol and urea in the fermentation process. The relationship between arginase *CAR1* gene in *S. cerevisiae* EC1118 and EC generation in sugarcane juice fermentation was investigated. The results indicated that the content of EC can be controlled by regulating the expression of arginase *CAR1* gene. Urea and arginase exhibited expression of arginase *CAR1* gene, which resulted in an increase of EC content. Besides arginine and urea, the expression of arginase *CAR1* gene was also significantly influenced by the content of ethanol, salt, and culture temperature. Low fermentation temperature (below 25°C) and lower ethanol and salt content showed some benefits in terms of controlling the EC content by decreasing the expression of arginase (Yang et al., 2017). The removal of EC present in alcoholic beverages is one of the important points in spirits production. The yeast possessing EC degrading enzyme was identified as *Meyerozyma caribbica* (Amorim et al., 2016; Thongekkaew et al., 2018).

This review article discusses the overall aspects of yeasts in production of various alcoholic beverages such as beer, wine, raki, cognac, whisky, tequila, vodka, etc., with emphasis on yeast physiology and their effects on quality characteristics of spirits.

2.2 The role of yeasts in alcohol fermentation

Yeasts have been used for the manufacture of alcoholic beverages for many years. Most of the yeast species are capable of fermenting sugar to ethanol (Barnett, 2003). Glucose catabolism is able to occur by respiration (respiratory dissimilation of sugars) and by alcoholic fermentation or by a combination of these two pathways. These pathways are impacted upon several factors such as types of microbial strains, time and culture conditions, etc (Verduyn et al., 1992). During alcoholic fermentation (Fig. 2.1), the metabolic pathway of yeast is characterized by a series of biochemical reactions usually involving formation of several hundred flavor-active compounds along with ethanol.

Some of the yeast species (i.e., *S. cerevisiae*) generate distinctive flavor and aroma during fermentation. On the other hand, some others yeast species (i.e., https://microbeonline.com/candida-albicans-pathogenesis-diagnosis/) cause undesirable or unpleasant taste and smells. Some useful yeast species for fermentation technology are shown in Fig. 2.2 (Aktan and Kalkan Yıldırım, 2014).

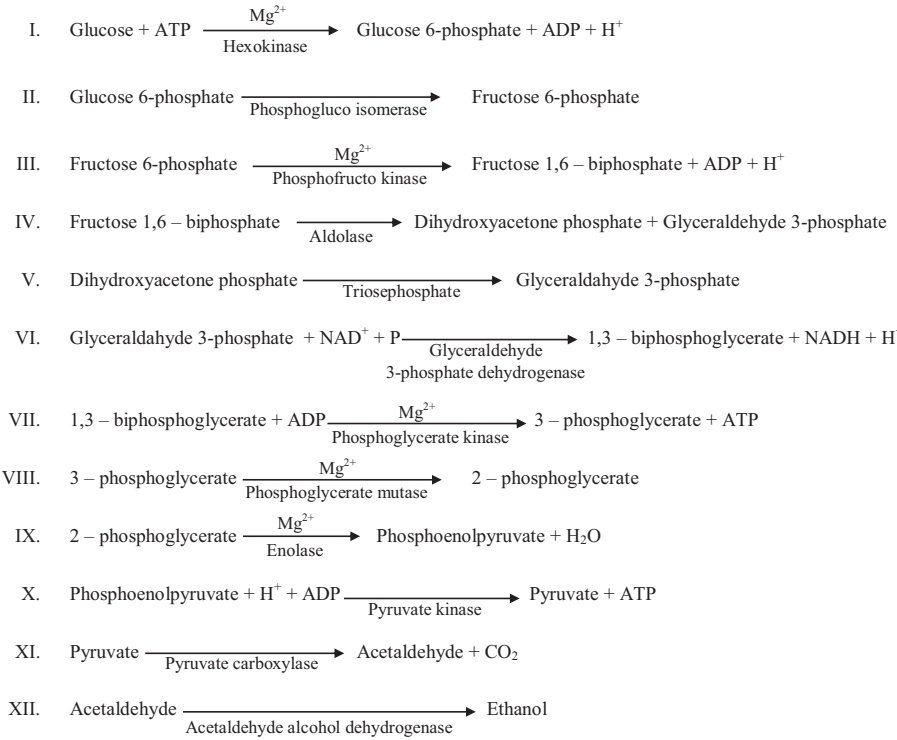

Figure 2.1 Alcohol fermentation steps.

2.2.1 Yeast description, physiology, and nutrition needs

2.2.1.1 Yeast description

Yeasts belong to the fungus group and reproduce by budding (or fission). Additionally reproduction types are:

- Segmentation-mitosis;
- Reproduction with spore;
- Sexual reproduction.

The traditional fermentation processes are carried out by a single species of yeasts, *S. cerevisiae*, hence for many, its name is synonymous with yeasts (Aktan and Kalkan Yıldırım, 2014). Several yeasts can develop true hypha similar to those of molds, whereas other species may form pseudohypha from elongated cells remaining attached together after budding. Instead of budding, some yeasts propagate by arthroconidia arising from cell division or splitting of hyphae. Sexual reproduction is known in less than half of yeast species; this may result in the generation of ascospores or basidiospores with or without preceding conjugation. Overall, yeasts represent a phylogenetically diverse group of fungi that can be classified either to *Ascomycetes* (e.g., *Saccharomyces, Candida*) or *Basidiomycetes* (e.g., *Filobasidiella, Rhodotorula*).

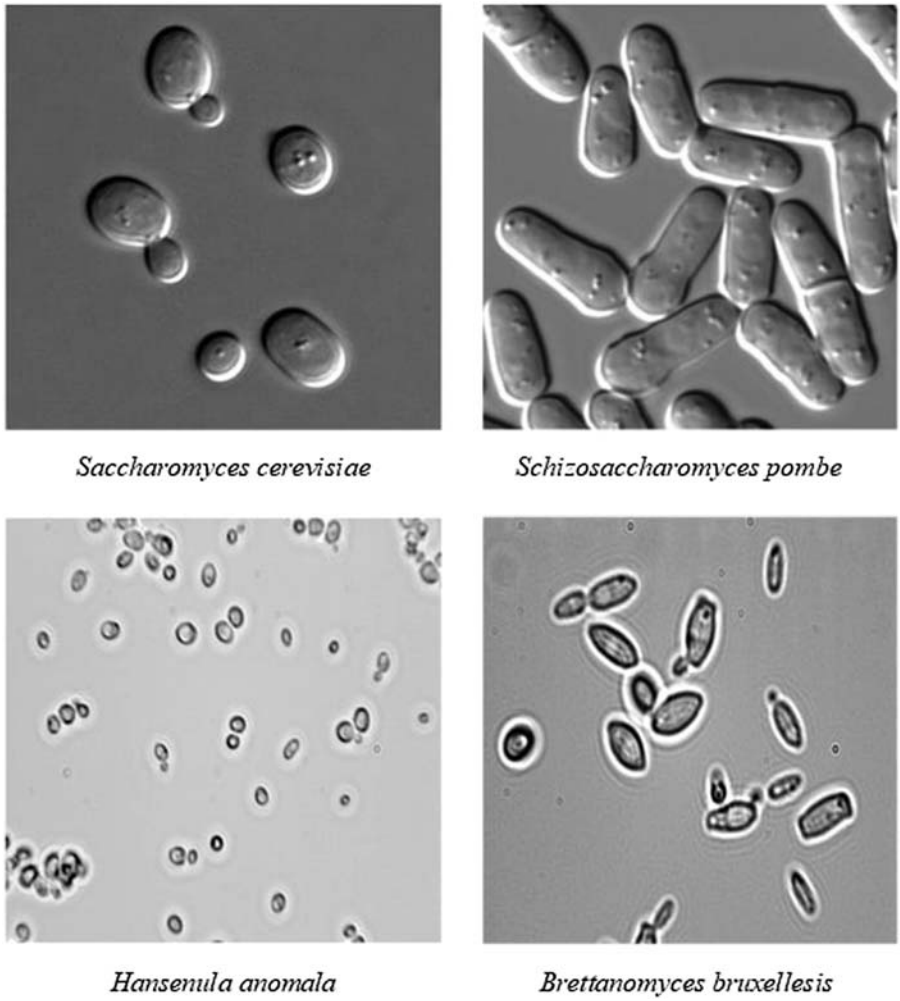

Figure 2.2 Various yeast strains.

Moreover, the genus of *Schizosaccharomyces* and few other species belong to neither of the above group and regarded as a separate group called *Archiascomycetes* (Satyanarayana and Kunze, 2009). The chemical composition of yeast cell is provided below in Table 2.1.

2.2.1.2 Yeast physiology

Yeast physiology may be defined as the understanding of the growth and metabolism of yeast cells. In general terms, it relates to how yeasts interact with their biotic and abiotic environment; in specific terms, yeast physiology relates to how yeast cells feed, metabolize, grow, reproduce, survive, and die.

Table 2.1 The chemical composition of yeast cell (75% water+25% dry matter).

Dry matters	Amount (in terms of percentage)
Carbohydrate	18%−44%
Protein	36%−60%
Nucleic acids	4%−8%
Lipids	4%−7%
Total inorganics	6%−10%
Phosphorus	1%−3%
Potassium	1%−3%
Sulfur	0.4%
Vitamins	Trace amounts

2.2.1.3 Yeast nutrition

Each yeast strain has its own demand of nutrition, and its metabolism directly and indirectly affects the chemical composition of the fermentation product (Furdikova et al., 2014). Understanding yeast nutritional requirements and acquisition strategies together with the regulation of nutrient transport is important not only for successful cultivation of yeasts in the laboratory but also for the optimization of industrial fermentation processes.

Majority of substances providing nutrition for the yeasts during fermentation originates from the raw material, stage of ripeness of raw materials, and processing technology. The most important is the availability of sufficient saccharides, utilizable nitrogen (for *S. cerevisiae* ~140 mg/L assimilable nitrogen concentration), and inorganic compounds, some supplements containing inorganic ammonium salts, vitamins, and growth factors (Furdikova et al., 2014). Yeasts acquire essential elements from their growth environment from simple food sources that need to be available at the macronutrient level ($\sim 10^{-3}$ M) in the case of C, H, O, N, P, K, Mg, and S or at the micronutrient level ($\sim 10^{-6}$ M) in the case of trace elements (Walker, 1998).

Using of pollen and brewer's yeasts at different concentrations during must fermentation of honey spirits, the importance of nitrogen supplements on fermentation kinetics had been demonstrated. It showed shortening of the fermentation time with no side effects on physicochemical parameters of honey spirit (da Silva et al., 2017). In a similar manner, Klosowski et al. emphasized the importance of source materials on formation of some spirits compounds such as carbonyl fractions and acetaldehyde concentrations (Kłosowski et al., 2017).

2.2.1.4 Yeast strains and genetic modification

Yeast species are usually classified into two groups: *Saccharomyces* and non-*Saccharomyces*. *Saccharomyces* species play an important role in fermentation with

S. cerevisiae as the dominant species. However, non-*Saccharomyces* yeasts involve different genera such as *Hanseniaspora, Issatchenkia, Pichia* and *Schizosaccharomyces, Brettanomyces, Zygosaccharomyces, Kluyveromyces*, *Candida,* and *Torulaspora* (Petruzzi et al., 2017).

Some strains of *Geotrichum* (S12, S13, TM, and TF) have an ability to decrease the content of higher alcohols and increase the ester contents in alcoholic drinks, caused mainly by cell adsorption and enzymatic reactions (Zhu et al., 2016). In another study, the influences of yeast strains on the physicochemical characteristics, methanol and acetaldehyde profiles, and volatile compounds of Korean rice distilled spirits were evaluated. The results demonstrated the importance of yeast strains overproduced volatile compounds of spirits (Kwak et al., 2015). The capacity of different yeasts (LMQA SNR 65:*S. cerevisiae*, LMQA BSG 7:*T. delbrueckii*) for industrial application especially for bioethanol production was demonstrated (Brexó et al., 2018).

Recombinant DNA techniques allow overcoming some problems and fulfilling the gap of knowledge regarding the yeast physiology. Current yeast strains have a number of limitations:

- Efficiency in fermenting malt/wort/must;
- Final conversion of carbohydrate to ethanol;
- Excessive yeast growth or insufficient growth;
- Being able to ferment all malt/must/wort sugars;
- Limitations in control over the flavors produced by yeasts;
- Variations of yeast flocculation properties;
- Usability of brewing yeast at high temperatures (cannot be used);
- Fermentation in case of vast quantity of carbohydrate;
- Tolerance to ethanol or osmotic pressure of yeasts;

Recombinant DNA methods have been used to introduce several new properties into fermented yeasts such as to degrade carbohydrates completely, modify processes which are responsible for flavor production and changes in yeast flocculation. In the meantime, yeasts are genetically modified by the transformation with an integration of plasmid vector. The recombinant yeasts plasmid could be maintained under nonselective growth conditions (Aktan and Kalkan Yıldırım, 2014; Hansen et al., 2016).

Recently, two new approaches have been considered: bioprospecting for novel strains and species, and genetic modification of known yeasts. The first approach includes isolation, screening, selection, and commercialization of special strains. The other approach is related to genetic manipulation of used culture. These approaches promise increasing the quality and diversity of fermented products. Additionally, new strains selected by bioprospecting can be further improved using synthetic biology techniques (Alperstein et al., 2020). Controlling microbial contamination in yeast fermentation by using different strategies has allowed higher efficiency of fermentation processes, and, hence, significantly affects the productivity of yeast (Seo et al., 2020).

2.3 The role of yeasts in beer production

Beer is one of the oldest known beverages in the world and is a staple low-alcohol product (Willaert, 2007). Beers have a broad diversity; they can be classified into ale and lager according to the yeast used and fermentation conditions. Ale beers are produced by ale yeasts that tend to float to the top of the vat at the end of the fermentation. In contrast, lager beers are produced by lager yeasts that sediment to the bottom of the vat at the end of fermentation. Ale beers are fermented between 15 and 26 °C temperatures whereas lager beers are fermented at cooler temperatures between 4 and 15 °C.

2.3.1 Step of beer production process

The stages of brewing have been shown in Fig. 2.3.

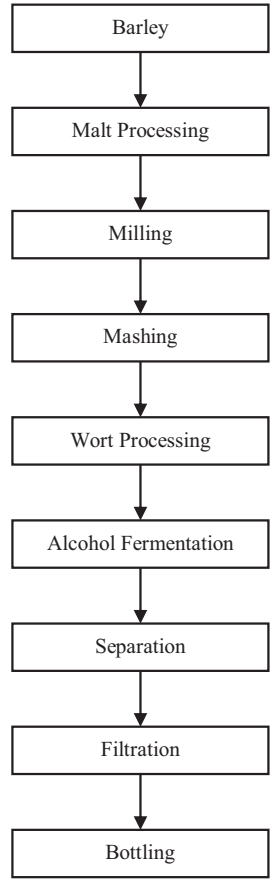

Figure 2.3 Steps in brewing.

2.3.2 Effects of yeast on beer quality

Yeasts play a crucial role in beer production and quality during fermentation. They not only convert wort carbohydrates into ethanol and CO_2, but also synthesize various key flavor compounds. Moreover, the yeast metabolism in beer fermentation leads to the formation of higher alcohol, esters, and vital compounds for aroma and taste of beer such as iso-amyl and iso-butyl alcohol and their acetate esters (Olaniran et al., 2017). One of the most important characteristics for brewers is the complete sugar utilization of the main wort sugars (glucose, fructose, sucrose, maltose, maltotriose) by yeast (Michel et al., 2016).

T. delbrueckii has been tested for the production of low-alcohol beer and enhancement of beer flavor profile (Canonico et al., 2016). *Saccharomyces ludwigii* and *Zygosaccharomyces rouxii* have been tested for production of low-alcohol beer. Differences in the concentration of both ethanol and flavor-active compounds were reported for both species. Some *S. ludwigii* strains produced a volatile composition associated with positive sensory descriptors, moderate concentrations of esters, and low concentration of off-flavors. In contrast, *Z. rouxii* produced higher concentrations of ethanol (>1.2% *v/v*), esters, higher alcohols, and diacetyl than *S. ludwigii* (De Francesco et al., 2015).

Flavor is an important factor in assessment of beer quality and for consumer acceptance. Flavor profile of beers can be influenced by many variables such as yeast strain, malt type, and fermentation parameters. Volatile compounds such as aldehydes, ketones, and nonvolatile components such as organic salts, sugars, amino acids, and organic/inorganic acids promote organoleptic features of beer. The flavor characteristics of malt are affected by processing parameters used in malt production. In studies conducted by researchers, synthesis levels of important higher alcohols and esters produced by different yeast strains during fermentation of wort produced from different colored malt types were investigated. The various Maillard reaction products (MRPs) such as melanoidins, furfural, and HMF (5-hydroxymethyl furfural) formed during production of dark malt were examined. Some of these products could have a negative effect on the growth of microorganisms. For example, melanoidins have been shown to inhibit the growth of bacteria due to chelate formation with some metal ions such as magnesium. Furfural and 5-hydroxymethyl furfural (HMF) have been shown to have a negative effect on yeast growth by inhibiting glycolytic enzymes and causing DNA damage (Dack et al., 2017).

Some yeast strains such as *S. cerevisiae* S288c produced a wide range of higher alcohols and esters compared to *S. pastorianus* L04. Furthermore, malt type-yeast strain interactions in respect of flavor improvement are required to gain better understanding of flavor synthesis that could assist in the development of new flavored products (Dack et al., 2017).

2.3.3 Yeast description, physiology, and nutrition needs

It is accepted that yeast is intimately linked to beer flavor and aroma. There is also a connection between yeast physiology and the synthesis of the higher alcohols and the volatile esters during beer production (Quain, 1988; Shen et al., 2003).

2.3.4 Yeast strains and genetic modification

Yeasts in breweries are divided into two main classes: bottom-fermenting and top-fermenting (Olaniran et al., 2017). While ale yeasts are usually *S. cerevisiae*, lager yeasts are mainly *S. pastorianus* or *S. carlsbergensis* in brewing. Genetic engineering techniques are used to alter the characteristics of yeast and barley in various ways that improve their performance in brewing. Different experimental approaches are usually correlated with the modification of brewer's yeast in order to produce beer with better features or new characteristics. Technical advances provide the construction of new strains of yeast with desired features.

There is a series of screening stages on yeast strain selection:

- Maltose utilization;
- Fermentation performance;
- Ethanol tolerance;
- Tolerance to compounds originating from hops;
- Amino acid utilization;
- Formation of phenolic off-flavors;

The important yeast species involved in the alcoholic fermentation of the beer are: *S. cerevisiae, Candida tropicalis, Kloeckera apiculata, Hansenula anomala, Torulaspora delbrueckii, Kluyveromyces marxianus,* and few non-*Saccharomyces* species (N'Guessan et al., 2010). Non-*Saccharomyces* yeast species that are also used in low-alcohol beer production include *Meyerozyma guilliermondii, Debaryomyces* spp., *Pichia* spp., *Wickerhamomyces anomalus, Brettanomyces anomalus, Brettanomyces custersii, Brettanomyces bruxellensis, Candida krusei, Cryptococcus kuetzingii,* and *Rhodotorula mucilaginosa* (Varela, 2016).

The shelf-life of beer is restricted because of its instability in the bottle. Haze-led instability in beer is mostly caused by colloidal particles formed by protein–polyphenol interactions. For this reason, beers are usually stabilized by removing at least one of these (protein or polyphenol) components. A genetically engineered *S. cerevisiae* strain was constructed with proline-rich QPF peptide using the C-terminal anchoring domain of α-agglutinin that could assist binding of polyphenols during fermentation to lower the polyphenol concentration (Cejnar et al., 2017).

2.3.5 Coculture application during alcohol fermentation

Beer has been produced by coculture of *T. delbrueckii* and *S. cerevisiae*. The study compared the volatile composition profiles of beer produced by mixed culture compared with the production by pure culture (*T. delbrueckii* and *S. cerevisiae*, separately). The cocultures improved the fruity/ester flavor and hop attributes as well as enhanced clarity and persistence, compact foam, and key quality traits of beer (Canonico et al., 2016).

In order to determine the contribution of non-*Saccharomyces* yeast on the sorghum beer quality, the application of *C. tropicalis* and *S. cerevisiae* as pure and coculture was studied (N'Guessan et al., 2010). The results indicated that *C. tropicalis* and

S. cerevisiae application (2:1 ratio) led to higher ethanol concentration than *S. cerevisiae* pure culture alone.

Producing beverages with diversified aroma profiles is one of the aim for most producers. The flavor profile of kiwi wine could be modified by coculture using *Saccharomyces bayanus* (Y5 or Y6) with *Torulaspora delbrueckii* Y7 that caused increase in phenethyl alcohol but decreased in octanoic acid and ethyl octanoate. Additionally, odor activity value of ethyl octanoate and ethyl hexanoate was increased by coculturing these yeasts. The decanal and terpinen-4-ol was enhanced by coculturing of different strains of *Saccharomyces* (Liu et al., 2020). Likewise, *Pichia* strains could be combined with normal beer yeasts for production of beer with increased values of esters (Iattici et al., 2020; Saerens and Swiegers, 2020).

2.4 The role of yeasts in wine production

Wine has been consumed by humans for thousands of years and is produced by crushing grapes and allowing the must to ferment using the organisms present on the grapes or by pure culture. The microbiology of wine involves two main phases: alcoholic fermentation and malolactic fermentations. The latter process relies on a heterogeneous microbiota composed of different indigenous microorganisms (e.g., yeast, bacteria, and filamentous fungi). The use of culture-independent methods to identify and quantify yeasts is an ideal tool for studying yeast species interactions. Most of them rely on the direct amplification of yeast DNA from wine by Polymerase Chain Reaction (PCR). Because of its specificity and sensitivity, one of the most promising PCR techniques in food control is the real-time quantitative PCR (QPCR) (Guillamón and Barrio, 2017). The steps in wine production process is shown by Fig. 2.4.

2.4.1 *Effects of* Saccharomyces *yeast on wine quality*

Wine production is based on spontaneous fermentations or inoculated must fermentations; in both cases, *Saccharomyces* plays a crucial role. *S. cerevisiae* strains predominate as the starter culture and ensure rapid and reliable grape juice fermentation, promising consistent and predictable wine quality.

2.4.2 *Non-*saccharomyces *yeast strains and genetic modification*

In winemaking, non-*Saccharomyces* yeast species contribute important organoleptic complexity (Ciani et al., 2010). One particular area of research that has grown immensely in the last couple of years is the role of non-*Saccharomyces* species in shaping wine sensory profile. Application of these yeasts in wine fermentation has resulted in production of different volatile compositions compared to wines fermented only with *S. cerevisiae*. This would generate wines with distinctive sensory profiles (Varela, 2016). *Candida zemplinina, Torulaspora delbrueckii, Hanseniaspora uvarum,*

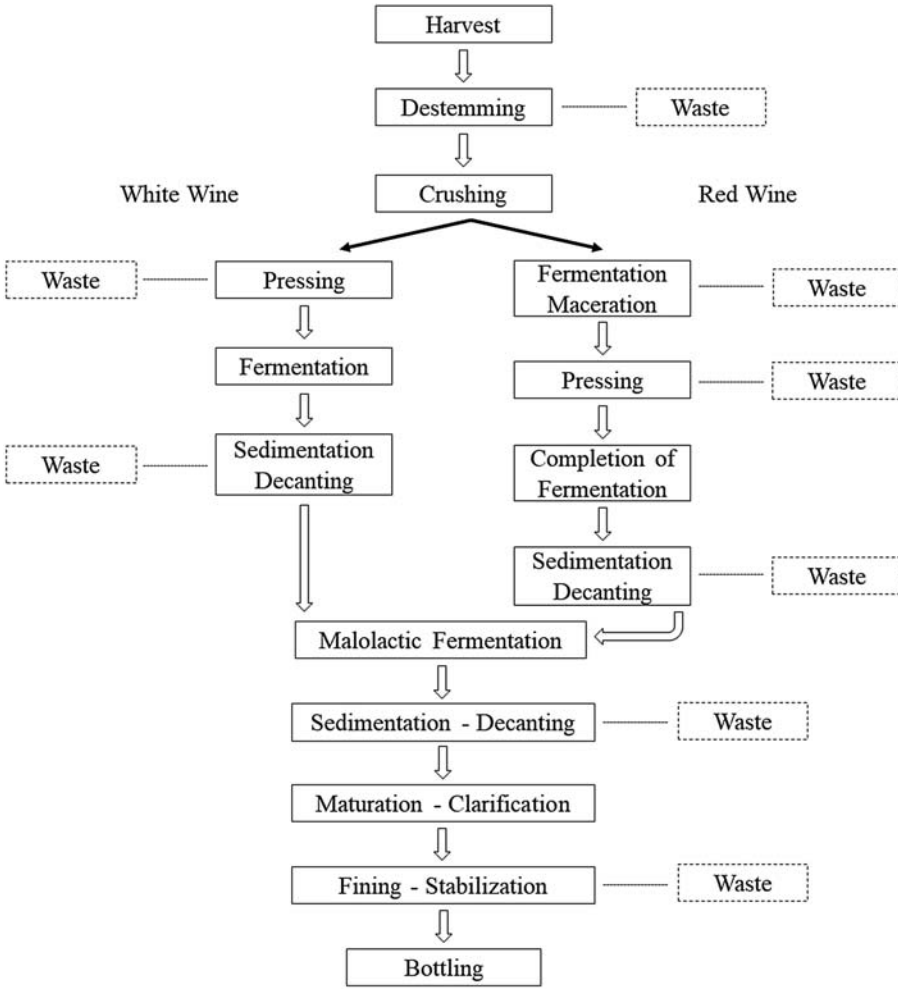

Figure 2.4 Red and white wine production process.

and *Metschnikowia pulcherrima* are used to conduct fermentations either in monoculture or in coculture with *S. cerevisiae* in wine researches (Moreira et al., 2005; Andorra et al., 2012).

Non-*Saccharomyces* species are particularly relevant in port wine production such that the fermentation is prematurely stopped, after the metabolism of only one-half of the available sugar, through fortification with aguardente. A study was carried out to isolate, identify, and characterize non-*Saccharomyces* species present in spontaneously fermenting port. From 500 non-*Saccharomyces* isolates, the three most abundant species, *Hanseniaspora uvarum, Lachancea thermotolerans*, and *Metschnikowia pulcherrima*, representing 89% of the isolates, exhibited particularly high diversity with high growth performance variability when exposed to typical stress conditions

associated with common enological parameters. Less abundant species included *Issatchenkia orientalis*, *Torulaspora delbrueckii, Hanseniaspora vineae, Hanseniaspora osmophila, Candida zemplinina, Rhodotorula mucilaginosa, Hanseniaspora guilliermondii, Issatchenkia occidentalis*, and *Zygosaccharomyces bisporus* (Mateus et al., 2020).

Using of new recombinant fusant yeasts based on *S. cerevisiae* and *Candida ethanolica* were constructed for production of high-quality (with enhanced aroma) low-alcohol cider (Wang et al., 2020). In a different study, *S. cerevisiae* and *Candida krusei* were fused by induction of electricity, and the obtained fusant yeast was successfully employed for cider production. The fusant yeasts developed offered a new approach to improve aroma and complexity of cider (Ye et al., 2013). Also, studies have been carried out by using fusant yeasts in wine production. By such technique, the properties of different cultures (i.e., *S. cerevisiae, Oenococcus oeni, Torulaspora delbrueckii*) were evaluated (Su et al., 2014).

2.4.3 Yeast description, physiology, and nutrition needs

Amino acids are essential components of must and wine. Moreover, the two major amino acids in must are L-arginine and L-proline, in addition to ammonium ions. Much of the ammonium, L-arginine, and other amino acids are utilized by yeasts during alcoholic fermentation (Mauricio and Ortega, 1997). *S. cerevisiae* can grow in a wide variety of nitrogen-containing substrates; the rate of consumption and the metabolism by these compounds are being dependent on the yeast strain, its physiological state, and the physicochemical properties of the wine. In addition, some amino acids are metabolic precursors of higher alcohols which make up a major group of wine aroma compounds. *S. cerevisiae* can use amino acids, either in the biosynthesis of proteins or as a nitrogen source. Amino acids are degraded by the cells and the nitrogen that they contain is liberated (usually, but not always, as ammonia) and used for the synthesis of other nitrogenous cell constituents. The carbon of the amino acid may also be used by the yeast for synthetic purposes (Beltran et al., 2004; Vilonova et al., 2007; Crepin et al., 2012).

2.4.4 Co-culture application during alcohol fermentation

In wine industry, coculture fermentations have been used for producing wine and sparkling wine. It leads to the sensorial complexity and showed very low production of so-called off-flavors such as phenols or sulfuric compounds. It produces strong fruity notes and is able to survive at high ethanol concentrations.

There has been increasing interest in the use of selected non-*Saccharomyces* yeasts in coculture with *S. cerevisiae*. Multistarter fermentation process is thought to simulate indigenous fermentation, thus increasing wine aroma complexity while avoiding the risks linked to natural fermentation (Sadoudi et al., 2012). Great differences have been shown in the metabolism of *S. cerevisiae* in single and in coculture with *Kloeckera apiculata* or *T. delbrueckii*. Moreira et al. (2005) reported an increase in the quantity of desirable compounds, such as higher alcohols and esters, when *S. cerevisiae*

cofermented with *Hanseniaspora uvarum.* In one study, *S. cerevisiae* was cocultured with *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine production. Significant higher levels of 2-phenyl ethyl acetate, 1-propanol, and 3 (methylthio) propionic acid were observed in case of using coculture with *H. guilliermondii*, whereas a higher content of total free fatty acids, pentanoic acid, ethyl hexanoate, 2-methyltetrahydrothiophen-3-one, and acetic acid-3 (methylthio) propyl ester were found in wine produced by the spontaneous fermentation. Usage of *Hanseniaspora* yeasts as costarter with *S. cerevisiae* led to production of wines with different chemical and aromatic profiles (Moreira et al., 2011). Maturano et al. used pure and mixed cultures of *S. cerevisiae*, *Hanseniaspora vinae,* and *Torulaspora delbrueckii* isolated from musts at different stages of spontaneous fermentation. *Saccharomyces* and non-*Saccharomyces* isolates produced a broad range of enzymes of enological interest throughout the fermentations, especially those related to hydrolysis of polymers present in grape juice. This contribute to hydrolysis of natural precursors and consequently affect the aromatic characteristics and quality of the wine (Maturano et al., 2012). Some non-*Saccharomyces* yeasts such as *Candida, Torulaspora, Kluyveromyces,* and *Metschnikowia* were also used in combination with *Saccharomyces* during alcohol fermentation. The overall effects of the non-*Saccharomyces* yeasts on fermentation and wine quality were strictly dependent on the *Saccharomyces*/non-*Saccharomyces* inoculum ratio that mimicked the differences of fermentation conditions (Comitini et al., 2011). A mixed culture of *T. delbrueckii* and *S. cerevisiae* was the best combination for improving the analytical profile of sweet wine considering volatile acidity and acetaldehyde production. Inoculating *S. cerevisiae* after fermentation by *T. delbrueckii* had less effect on volatile acidity and acetaldehyde production and resulted in stuck fermentation (Bely et al., 2008).

2.5 The role of yeasts in raki production

Turkish Rakı is a kind of traditional aniseed spirit produced in different regions of Turkey. Quantity and the repartition of the alcoholic fermentation compounds as fusel alcohols, esters, and aldehydes are responsible for the flavors and quality of the spirit (Yılmaztekin and Cabaroğlu, 2011). In the production of rakı, the first step is the production of suma.

2.5.1 Production of suma

Production of suma diagram can be seen below (Fig. 2.5).

2.5.2 Step of rakı production

The rakı production steps are shown in Fig. 2.6.

Aniseed spirits are produced by the distillation of pressed fermented grapes, dregs, and other raw materials, flavored with aniseed (*Pimpinella anisum* L), fennel

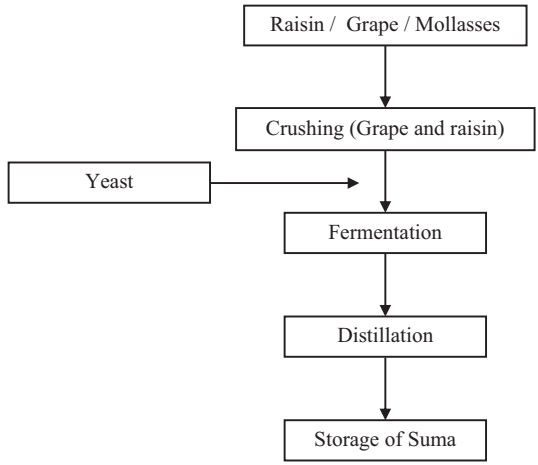

Figure 2.5 Production of suma.

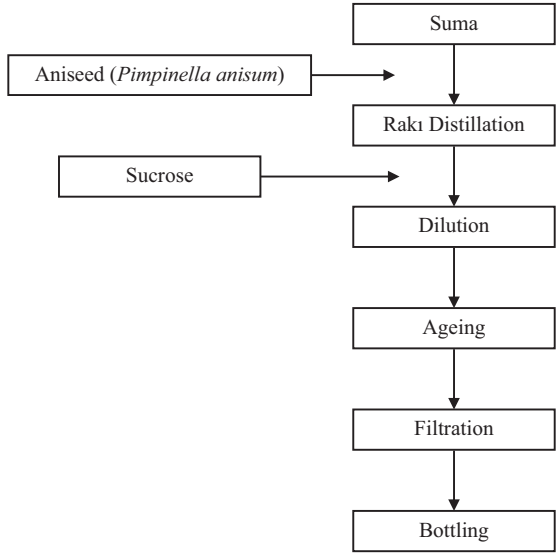

Figure 2.6 Rakı production process.

(*Foeniculum vulgare*), and/or some other plants. All round the Mediterranean basin, there are other similar aniseed spirit drinks such as pastis (France), anesone (Spain), sambuca (Italy), zebib (Egypt), and arak (Syria). Turkish rakı is a type of traditional aniseed spirit produced by double/triple distillation using aniseed (*P. anisum*). The first step in raki production is the production of suma. The main raw materials for suma production are raisins, molasses, and/or grape must (Anlı et al., 2007; Anlı and Bayram, 2010).

The essential oil from aniseed is extracted by steam distillation. Above 23°C, it is a colorless or faintly yellow liquid soluble in absolute ethanol but nearly insoluble in water (few mg/L) (Grillo, 2003). In Turkish rakı, the amount of water is sufficiently low, and ethanol remains solubilized in the water/ethanol mixture. But further addition of water induces the formation of an emulsion of ethanol droplets in a (water-ethanol) continuous medium.

Aniseed spirits are alcoholic drinks produced by using one of the following processes or a combination thereof:

- Maceration and/or distillation;
- Redistillation of the alcohol in the presence of the seeds or other parts of the plants specified above;
- Addition of natural distilled extracts of aniseed-flavored plants;

The minimum alcoholic strength of aniseed spirits is 15% (v/v). Only natural flavoring substances and preparations may be used during production of aniseed spirits. Other natural plant extracts or aromatic seed may also be used but the aniseed taste must remain predominant (Anonymous, 2005).

Rakı contains low molecular mass carbonyls (C1−C6), the byproducts of yeast fermentation, that is, intermediates in the formation of fusel oil. These may also result from alcohol oxidation at various stages of manufacture. Higher alcohols and esters produced during alcoholic fermentation are the major aroma compounds detectable in distilled alcoholic beverages. The major higher alcohols in Raki are: 2-propanol, n-propanol, 1-pentanol, 2-pentanol, 3-pentanol, n-butanol, 2-butanol, 3-methyl-1-butanol, 2-hexanol, and 2-heptanol (Anlı and Bayram, 2010; Güven, 2013).

Chemometric analysis of rakı products, followed by statistical analysis techniques, can be used to distinguish between traditional and flavored distilled alcoholic beverages. Distillation practices, which depend on company expertise and the preference of its consumer target, affect the organoleptic properties of the rakı product, especially with respect to the aldehyde and ethyl acetate content (Güven, 2013).

2.5.3 Rakı quality and chemical-sensory characteristics

The typical aroma of Turkish rakı comes from aniseed. In Turkish rakı production, the aniseed is always used directly in the second distillation. The composition of Turkish anise seeds were determined in a typical sample from Çesme/Izmir with gas chromatography (GC), and it was found that the major constituent of anise essential oil is transanethol (1-methoxy-4-(1-propenylbenzene) with a range covering 86.2%−89.0% of the total essential oils. The other components were methyl chavicol, cis-anethol, carvon, camphor, anisaldehyde, anisic acid, and anis ketone. The distillation of suma with and without aniseed does not affect the methanol and higher alcohol content of the final product (Anlı and Bayram, 2010).

2.6 The role of yeasts in cognac production

Cognac is one kind of specialty brandy well known all over the world which is produced in the French wine-growing region of Charentes, and this name can only be used for brandies from a defined region in France (Uselmann and Schieberle, 2015). Moreover, it must be produced according to the regulations of the AOC (Appellation d'origine control). Cognac has a number of categories, which reflect the time each one has spent in barrel. VS ("Very Special") cognacs must contain eaux-de-vie no younger than 2 years old; VSOPs ("Very Superior Old Pale") must be at least 4 years old; and for XO ("Extra Old"), as well as bottles labeled "Napoléon", "Extra" and "Hors d'âge", it is 6 years.

2.6.1 Step of cognac production process

Cognac production stages have been shown in Fig. 2.7.

2.6.2 The role of yeast for cognac quality

There are several in-depth studies on the control fermentation kinetics and the synthesis of volatile compounds produced during cognac fermentation. Grape must composition (acidity, nitrogen, sugars) and temperature, as parameters influencing both fermentation kinetics and analytical composition of cognac (higher alcohols, esters), have been thoroughly studied. According to the results and recommendations concerning the control of alcoholic fermentation, some important points are emphasized:

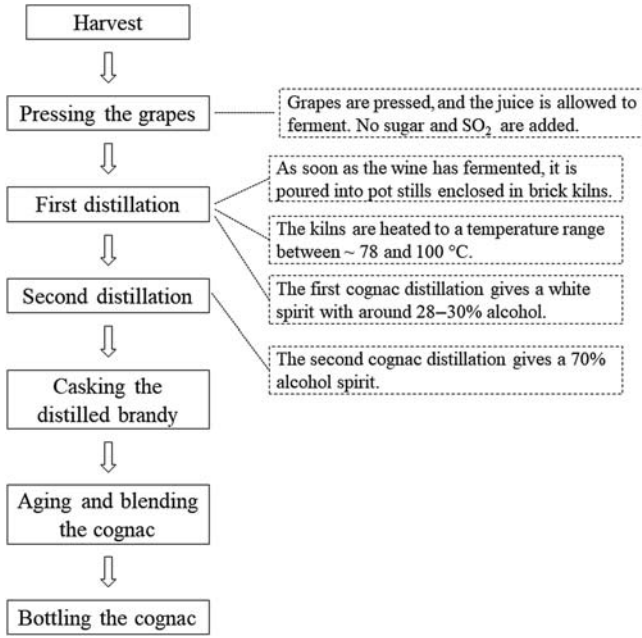

Figure 2.7 Cognac production process.

- Determination of harvest date, in order to optimize the equilibrium between sugars, acidity, and nitrogen;
- Management of must nitrogen content;
- Reduction of solid matter suspended in the must, temperature control;
- Inoculation with selected yeasts and selection of suitable yeast strain as crucial tools for fermentation control.

Amino acids concentration and volatile compounds are significantly influenced by the yeast strains in the fermentation. *S. cerevisiae* is the essential microorganism involved in the alcoholic fermentation and has several amino acid transport systems. The use of *S. cerevisiae* strains showed lower residual values of prooxidative amino acids such as phenylalanine, methionine, and leucine after the alcoholic fermentation (Fairbairn et al., 2017; Brice et al., 2018). The study indicated that yeast selection could be proposed as a tool for reducing oxidation. The results also confirmed that the production of oxidation aromas in wine and/or cognac was linked to the initial content of certain amino acids, and the selection of yeast strains that consumed a high quantity of these amino acids during alcoholic fermentation could be a useful tool for minimizing wine aroma degradation (Balboa- Lagunero et al., 2013). The (−)-*cis*- and (+)-*trans*-whisky lactones and (−)-*cis* and (+)-*trans* cognac lactones were isolated from different types of wood and identified as key flavors of aged alcoholic beverages such as whisky, brandy, wine, and cognac (Sabitha et al., 2007).

There are many reports devoted to the synthesis of (−)*cis* and (+)-*trans*-whisky lactones. Efficient synthesis of (−)-*cis* and (+)-*trans*-whisky lactones in 11 steps has been described (Jiang et al., 2010).

Using biotransformation procedures by yeast strains especially belonging to *S. cerevisiae*, cognac lactones could have been obtained in high level.

2.6.3 Yeast description, physiology, and nutrition needs

Yeasts are considerably larger in size than bacteria and in terms of quantity and economics. In terms of nutrition needs and growth, some requirements for yeast are given below:

- Water;
- A carbon source—fermentable carbohydrates as an energy source;
- Oxygen/lipids—if oxygen is present, membrane biosynthesis can be made by the yeast;
- A nitrogen source—for enzyme synthesis and growth;
- Vitamins—growth factor;
- Inorganic ions—essential for yeast metabolism.

2.6.4 Cognac flavor and sensory properties—yeast role

Various volatile compounds including higher alcohols, esters, aldehydes, acetyls, and fatty acids are formed by yeasts during the alcoholic fermentation. Distilling wine in the presence of yeasts involved in fermentation, lees leads to enrichment in fatty esters

such as ethyl octanoate (fruity, floral, pineapple, apple, and pear) and ethyl decanoate (fruity, pear, wine, etc.). The main factors influencing cognac quality are given below:

- Grape varieties;
- The location of vineyard;
- Used culture during fermentation;
- Climate;
- Distillation type;
- The oak used for the casks.

Brandy is defined as "an alcoholic distillate from the fermented juice, mash or wine of fruit, or from the residue thereof, produced at less than 1900 proof, in such a manner that the distillate possesses the taste, aroma, and characteristics generally attributed to the product" (Jacques et al., 2003). The famous saying in the cognac industry is that all cognacs are brandy but not all brandies are cognac. Cognac is a protected name and region, similar to champagne and sherry, and should not be regarded as generic "brandy", which is not tied to a specific country or area.

For the past three centuries, cognac has been almost recognized as the spirits that are distilled from grapes. It exhibits an abundance of qualities: fruit, subtle aromas, warmth, intensity, and above all complexity with the thousands of flavors (caramel, honey, nuts, vanilla, and flowers) from predominantly one grape variety and from the wood (smokiness flavor).

2.7 The role of yeasts in whisky production

Whisky is an alcoholic beverage with great worldwide acceptance and high commercial value, which is a distilled beverage, made of cereals by yeast and matured in wood barrels for certain period of time (Martins et al., 2017). Various types of whisky are produced in several countries in the world. The most popular ones have been produced in Scotland, Ireland, Canada, Japan, and USA. There are differences principally in proportion of cereals used as raw materials (malted barley) and also in type of still used for distillation.

During production of whisky, the objective of the fermenting mash with strains of *S. cerevisiae* is to convert mash sugars to ethanol and carbon dioxide. In the meantime, the strains produce quantitatively minor amounts of other organic compounds which contribute to the organoleptic qualities of the final distilled products. At the step of distillation, the alcohol strength is less than ~94% so that the distillate has the aroma and taste derived from the raw materials. The chemical interactions of compounds obtained by fermentation continued during aging (700 L casks for a minimum 3 years). So, even the aging process parameters are affected by metabolites produced by alcohol fermentation of different yeasts.

2.7.1 Steps of whisky production process

Whisky production process has been given by Fig. 2.8.

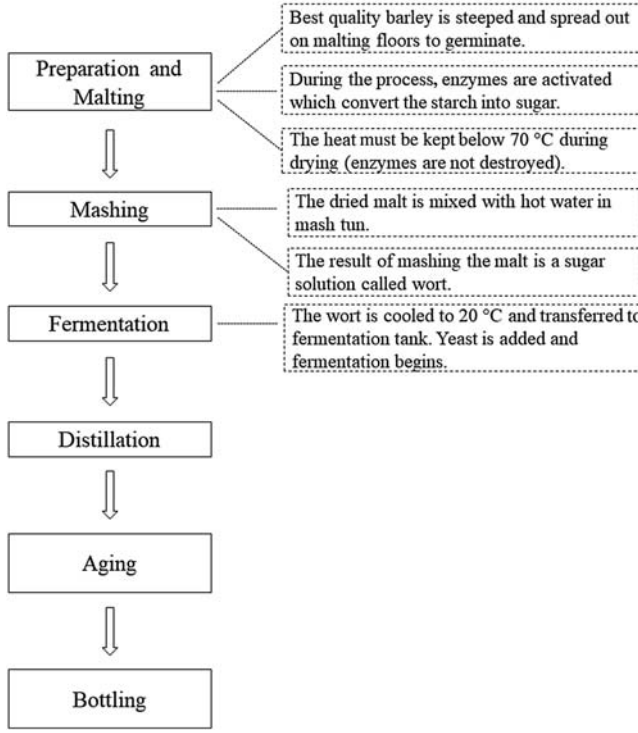

Figure 2.8 Whisky production steps.

2.7.2 The role of yeast in whisky quality

Considering the important role non-*Saccharomyces* species in sensory profile of distillates, there are undoubtedly enormous potentials in evaluating non-*Saccharomyces* yeasts for the production of spirits such as whisky. Esters produced during fermentation are important contributors to the flavor of whisky. Many types of esters are generated during wort fermentations. The mechanisms regulating ester metabolism is not characterized very well in most of the researches. However, ester levels produced during fermentation are influenced by a number of factors such as used yeast strain, fermentation conditions, and type of bioreactor (Aktan and Kalkan Yıldırım, 2014).

In a study done by Morimura et al. (1998), changes in the characteristics of yeast during storage (at 5 °C and 20 °C temperatures) and yeast properties at subsequent fermentations were examined. Cultivation of yeast is an important stage in whisky production in terms of synthesis of aromatic compounds by yeast. Intracellular sulfur compounds concentrations were detected during fermentation, and proper temperature for storage was determined. The results demonstrated that yeast stored at 5 °C exhibits higher viability and gives a higher ethanol production rate and has a higher intracellular level of sulfur content. Results also showed that storage at 5°C is suitable for maintaining the yeast activity and fermentation ability.

2.7.3 Yeast description, physiology, and nutrition needs

Malt wort consists of maltose, glucose, dextrins, and maltotriose. The utilization of wort sugars is one of the most important factors in terms of control of fermentation efficiency during the whisky process. The rate and extent of sugar utilization is controlled by environmental factors, the ability of yeast to transport sugars, and the rate of subsequent metabolism.

Yeasts could ferment maltose, glucose, fructose, and sucrose in malt during production of whisky. However, maltotriose as the second most abundant sugar of wort is not fermented. Consequently, this causes some losses in ethanol yield (Zastrow et al., 2001).

The requirements of *S. cerevisiae* strains used in distillery practice are to maintain very high viability. The other very important property of these strains is to tolerate concentrations of high ethanol (12%−15%; v/v) and the capacity to metabolize oligosaccharides such as maltotriose and maltotetraose in order to maximize the conversion of starch into ethanol and carbon dioxide (Jacques et al., 2003).

2.7.4 Yeast strains and genetic modification

There have been several molecular studies on yeast used for fermentations in whisky industry. From the viewpoint of yeast researchers, one of the key tools is the availability of *S. cerevisiae* genome sequence. Further, new methods have been developed to measure levels of gene expression of all genes in the cell at any time. Although cDNA microarray methodology is very informative, the results do not always reflect actual protein levels and enzyme activity as a result of posttranscriptional regulation and modifications. One of the main results is that the expression of glucoamylase may release fermentable sugars from dextrins, and, hence, final amount of ethanol could increase (Klaassen et al., 1996).

The maltotriose transporting efficiency varies between different *Saccharomyces* strains. So, several *Saccharomyces* strains, including those used in whisky production, were screened for growth on maltotriose in a conducted research. A study was performed to examine genetic aspects that influence the ability of different *Saccharomyces* yeasts to transport maltotriose. Even small variations in protein sequences of different *Saccharomyces* yeasts could lead to variation in utilization of available carbon sources in their immediate surroundings and changes in their functional characteristics (Zastrow et al., 2001; Salema-Oom et al., 2005; Vidgren et al., 2005; Smit et al., 2007).

Saccharomyces strains were identified and controlled in case they are able to grow efficiently on maltotriose as sole carbon source. The isolated AGT 1 alleles were sequenced, and their chromosomal locations were determined in the strains from which they were cloned. The data suggested that the genetic variation among the AGT1 loci−encoded transporters was the reason for the variation in maltotriose transport efficiency among different *Saccharomyces* strains. These studies allowed prospects for the development of yeast strains with improved maltose and maltotriose uptake capabilities that, in turn, could increase the overall fermentation efficiencies in the beer and whisky industries (Smit et al., 2007).

Enhanced fermentation of starch and its dextrin products is important for whisky industries. Most *Saccharomyces* strains ferment glucose and maltose and partially maltotriose, but these strains are not capable of utilizing dextrin. There are some special strains of *Saccharomyces*, (i.e., *S. diastaticus),* which produce extracellular glucoamylases that are capable of utilizing wort dextrins (Hill and Stewart, 2009).

Yeast and lactic acid bacteria produce aroma compounds such as lactone and convert hydroxyl fatty acids during whisky production. In a study regarding fatty acid hydroxylase characteristics and genomic sequence in *Lactobacillus sakei* strain, hydroxylase was used for cloning (Suzuki et al., 2016). Interested genetic region removed by restriction enzyme (*Sau3A; Sau3A* genes from *Staphylococcus aureus* 3A) was ligated in plasmid DNA and transformed to competent cells. Using SDS-PAGE analysis, the results demonstrated that fatty acid hydroxylase produced by recombinant cells was 46 kDa (optimum pH 6.0) (Suzuki et al., 2016).

2.7.5 Coculture application during alcohol fermentation

There are a few studies about coculture application in spirits. However, some researchers claimed that lactic acid bacteria and yeast produce aroma compounds (lactone) with a sweet fruit smell and could convert hydroxyl fatty acids during whisky production process (Suzuki et al., 2016).

2.8 The role of yeast in tequila, vodka and rum production

2.8.1 Step of tequila production

Tequila is an alcoholic beverage which is obtained from the distillation of fermented juice of the agave plant. There are two classifications of tequila based on agave content: 100% tequila, produced using only agave, and 51% tequila, which contains sugars from other sources such as cane and corn added in the fermentation step, at up to 49% by weight. It is important to note that only tequila 51% can be exported in bulk outside Mexico since 100% tequila must be State-bottled. Based on maturation, there are four classifications of tequila: silver, without maturation; gold, containing permitted additives and colors (generally caramel color); aged tequila matured at least 2 months in white oak tanks with a maximum capacity of 159 gallons or barrels; and extra-aged matured at least 12 months in white oak barrels. Tequila production process has been given by Fig. 2.9.

2.8.2 Steps of vodka production

Vodka is a neutral spirit distilled and treated with charcoal or other materials to be without distinctive character, aroma, taste, or color. Originally, it was made from potatoes. Although some eastern European vodkas are still made from potatoes and corn, most of the high-quality imports and all vodka made in the United States are distilled from cereal grains such as wheat.

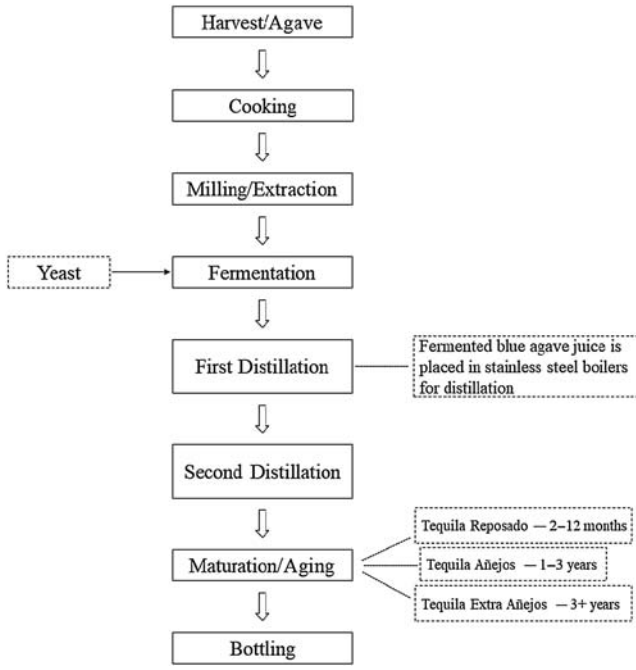

Figure 2.9 Tequila production process.

In practice, neutral spirit is a purified, odorless, tasteless, and colorless ethanol produced by distillation and rectification techniques that remove any significant amount of congeners. It is used in the production of beverages such as vodka and gin. Neutral spirit and brown spirits are two main product types from continuous distillation. Neutral spirit can be made from any feedstock but is usually made from grain or molasses. This spirit has very low odor and taste, and is used for nonaged products (known as white spirits) such as gin and vodka. It may also be used to blend with a highly flavored product and aged in wood barrels. This blended product usually has a lighter flavor than its pot or single column still counterpart.

Vodka production process is shown by Fig. 2.10.

2.8.3 Steps of rum production

Rum is an alcoholic spirit made from sugarcane or its derivatives (fermented juice of sugarcane, sugarcane syrup, sugarcane molasses, or other sugarcane byproducts), which produced at less than 190 proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to rum, and bottled at not less than 80 proof, and also includes mixtures solely of such distillates. Rum production process has been given by Fig. 2.11. Significant factors that affect the taste, quality, color, and viscosity of rum are:

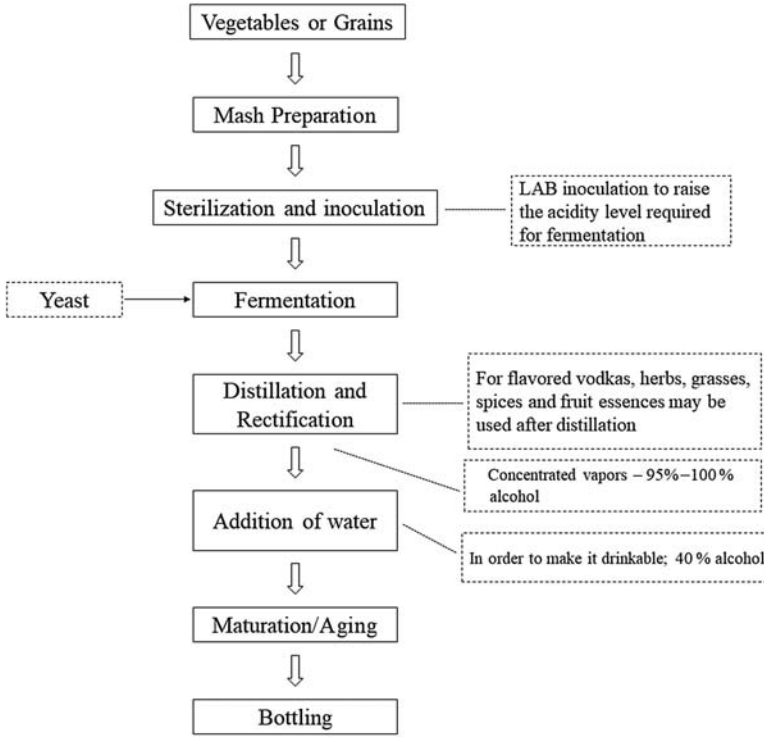

Figure 2.10 Vodka production process.

- ➢ Raw fermenting materials;
- ➢ Method of fermentation;
- ➢ Types of yeast used during fermentation;
- ➢ Method(s) of distillation;
- ➢ Process of maturing;
- ➢ Blending;
- ➢ Water quality used during production.

2.8.4 The role of yeast on tequila, rum, vodka quality

As one of the most important alternative for improving fermentation productivity of alcoholic beverages is to use a strain of yeast/coculture capable of both efficiently converting sugars into alcohol and producing the appropriate organoleptic compounds that impart pleasant aroma to the final product.

Yeasts also play an important role in the production of alcoholic beverages typical of Mexico especially tequila, which are obtained from different agave juices. The first part of this process is the transformation by yeast fermentation of agave must in an alcoholic aromatic product, which is then distilled yielding the typical agave beverage. As regards to tequila fermentation, a wide variety of yeasts are present at the beginning

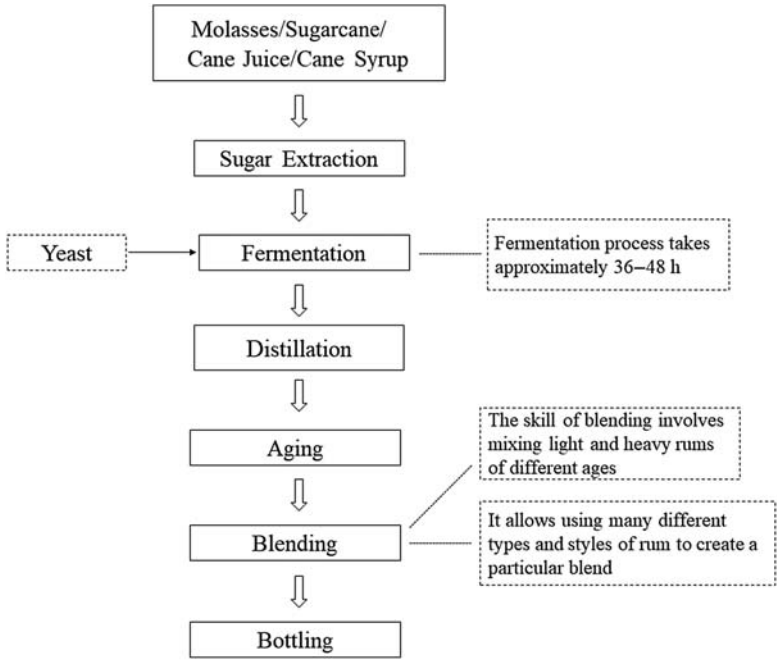

Figure 2.11 Rum production process.

of the fermentation. Among the fermenting yeasts, the most frequent isolates belong to the species *S. cerevisiae, Kigelia africana*, and *Candida krusei* (Jacques et al., 2003). Traditionally, industrial tequila production has used spontaneous fermentation or *S. cerevisiae* yeast strains. Potential of non-*Saccharomyces* strains for alcoholic fermentation has been investigated in a few studies. A study, conducted on *Saccharomyces* and non-*Saccharomyces* yeasts isolated from grape and agave musts, has revealed a correlation between strain technological aptitude and origin, explained as a specific adaptation to fermentation conditions, which probably determine different physiological and enological properties. Thus, the significant differences in β-glucosidase and β-xylosidase activities between *S. cerevisiae* agave and grape strains could indicate a certain specialization to metabolize different cellulosic materials from grape juice and agave plant (Fiore et al., 2005).

In another study, the performances of different non-*Saccharomyces* yeasts (*Kluyveromyces marxianus* and *Pichia kluyveri*) were compared to one *Saccharomyces* yeast strain in tequila fermentation under industrial conditions, and all yeasts were isolated from agave musts. According to the results, agave tequilana juice was fermented at an industrial level using two non-*Saccharomyces* yeasts (*Pichia kluyveri* and *K. marxianus*) with fermentation efficiency higher than 85%. *Pichia kluyveri* (GRO3) was more efficient for alcohol and ethyl lactate production than *S. cerevisiae* (AR5), while *K. marxianus* (GRO6) produced more iso-butanol and ethyl-acetate than *S. cerevisiae* (AR5). The level of volatile compounds at the end

of fermentation was compared with the tequila standard regulation. All volatile compounds were within the allowed range except for methanol, which was higher for *S. cerevisiae* (AR5) and *K. marxianus* (GRO6). The variations in methanol may have been caused by the *Agave tequilana* used for the tests, since this compound is not synthesized by these yeasts (Amaya-Delgado et al., 2013).

Some yeasts such as *S. cerevisiae* (CCMA 0187 and CCMA 0188), *Candida parapsilosis* (CCMA 0544) and *Pichia anomala* (CCMA 0193) were successfully used for reduction of vinasse obtained from the production of beverages such as tequila and cachaça (dos Reis et al., 2018). In a study related to cachaça (Brazilian spirit) production, the effects of autochthonous yeast species and commercial strains on product's chemical profile were determined. The results indicated that the overgrowth of some non-*Saccharomyces* species during fermentation can lead to higher concentration of contaminant compounds and could contribute to the distinctive chemical quality and flavor of cachaça (Portugal et al., 2017).

In another study done with selected yeast strains during cachaça production, products with homogeneous organoleptic characteristics were obtained (Barbosa et al., 2016). The microbial population dynamics in spontaneous fermentation during production of spirits like cachaça play an important role in formation of their chemical composition (Mendonça et al., 2016; Portugal et al., 2016).

Agave fermentation is performed with batch systems in tequila production. However, continuous cultures could be a good option in terms of technological alternative to increase productivity and efficiency of sugar to ethanol conversion. Fermentation of agave juice used as a culture medium with the addition of *S. cerevisiae* was carried out in order to prove the necessity of supplementing yeast extract and alleviate nutritional deficiencies of agave (Hernández-Cortés et al., 2016). The ethanol productivity and volatile compounds concentrations in continuous fermentations of agave juice supplemented with yeast extract were higher than in fermentation without supplementations.

Using of non-*Saccharomyces* yeasts (*K. marxianus* strain LEV-03-ITTG) for production of sensorial accepted products from *Agave americana* L. and panela honey such as "comiteco" spirit was demonstrated (Varela, 2016; Lara-Hidalgo et al., 2017).

S. cerevisiae is an efficient ethanol-producing microorganism. However, a concentration of high ethanol and other metabolites can affect yeast viability and decrease the ethanol yield. Many studies have focused on improving the fermentative efficiency, mostly through the genetic engineering of genes that have a direct impact on specific metabolic pathways.

In tequila production, some companies do not use specific strain of *S. cerevisiae* and instead allow natural fermentation to proceed. Others inoculate the wort with fresh packages of baker's yeast or commercial dried yeast to obtain initial populations of $20-50 \times 10^6$ cells/mL.

To achieve high yields and maintain a constant quality in tequila, some companies use yeast strains isolated from a natural fermentation of cooked agave juice. Nutrients are added, and special conditions such as a high sugar concentration or temperature are maintained. These isolated and selected yeast strains have been deposited in National Microbial Culture Collections. In recent study, during tequila fermentation process the *YNR034W-A* gene in the BY4741 laboratory strain was isolated, and a wild-type yeast

strain (AR5) was overexpressed. The expression profile of the *YNR034W-A* was investigated during growth and glucose treatment. Transformant derivatives of the AR5 strain showed an improved efficiency during fermentation of *Agave tequilana* Weber juice. It was suggested that the improved fermentative efficiency was the result of higher stress tolerance response in the *YNR034W-A* overexpressing transformant (Vargas-Maya et al., 2017).

Fermentative capabilities and volatile compounds produced by *Kloeckera africana/Hanseniaspora vineae* K1, *K. apiculate/H. uvarum* K2 and *S. cerevisiae* S1 and S2 yeast strains as a pure and mixed cultures during *Agave tequilana* juice fermentation were investigated (González-Robles et al., 2015). The results indicated that *Kloeckera/Hanseniaspora* strains have limited growth and sugar consumption as well as low ethanol yield and productivity in comparison to *S.cerevisiae* in pure and mixed culture applications. *S. cerevisiae* presented a similar behavior reaching high biomass production, completely consuming the sugar, leading to high ethanol production in case of using pure and mixed cultures. In the meantime, the presence of *S. cerevisiae* strains in the mixed cultures contributed to the generation of higher alcohols, acetaldehyde, and ethyl esters, whereas *Kloeckera/Hanseniaspora* strains stimulated the production of ethyl acetate and 2-phenyl ethyl acetate compounds.

Tequila fermentation has complex microbial process carried out by distinctive indigenous yeast species (classified into *Saccharomyces* and non-*Saccharomyces* species). Nowadays, it has been considered that using mixed starter cultures of several yeasts genera and species is beneficial to improve the sensorial characteristics of the final products (taste, odor, and aroma). Therefore, some researchers focused on a *S. cerevisiae/K. marxianus* yeast pair isolated from tequila and mescal fermentations (Lopez et al., 2014). Cultures of *Saccharomyces* and *Kluyveromyces* were evaluated as with pure/mixed combination and direct/indirect interaction during tequila production. The sugar consumption and ethanol production in both cases were similar (pure/mixed). Thus, the interaction phenomena between the two yeasts were different in direct and indirect contact, *Kluyveromyces* being always much more affected than *Saccharomyces*.

Application of pure culture (three strains of *S. cerevisiae*) during vodka production was evaluated as an alternative for higher production. The results showed that the ethanol concentration (39.7% v/v) fell within the legally stipulated range. The levels of copper and furfural remained undetectable, but the methanol (35.04 mg/100 mL) content and the levels of some secondary compounds (148.33 mg/100 mL) were both higher than the guidelines (20 and $\leq$ 50 mg/100 mL), respectively (Menezes et al., 2016).

During vodka production from potato hydrolysate, various *S. cerevisiae* strains were investigated by Menezes et al. (2016). For each isolate, efficiency of strains was evaluated. In addition, isolates were treated in fermentation medium containing potato hydrolysate supplemented with sucrose (17% w/w). The results demonstrated that using baker's yeast in the second treatment has higher substrate-to-product conversion and higher ethanol content as well as higher fermentation yield. Additionally, sucrose assisted in increasing the sugar content in the wort as well as increasing the yield of ethanol. Fermentation analysis showed that baker's yeast allowed higher overall efficiency (89%), ethanol yield (0.47 g ethanol/g total reducing sugars), and ethanol productivity (6.05 g/L/h).

2.9 Conclusion and future perspectives

Yeasts play a central role in the maintenance of high quality of alcoholic beverages.

- ❖ Yeasts contribute to beverage flavor and aroma in almost all alcoholic beverages (beer, wine, whisky, cognac, raki, gin, tequila, and rum);
- ❖ Application of recombinant DNA techniques in yeasts allows overcoming some problems related to efficiency in fermentation;
- ❖ Application of coculture fermentation could be useful for improving physicochemical and sensory properties of alcoholic beverages;
- ❖ Considering future consumer preferences of more sophisticated alcoholic beverages, new perspectives in studies are required including optimization of fermentation with different *Saccharomyces* and non-*Saccharomyces* species and genetically modified strains.

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The impact of biotechnology on dairy industry

3

Saber Amiri[1,2], *Mostafa Aghamirzaei*[2,3], *Parisa Mostashari*[4], *Mohammad Sarbazi*[2], *Samira Tizchang*[2], *Hajar Madahi*[2]

[1]Department of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, West Azerbaijan Province, Islamic Republic of Iran; [2]Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran; [3]Vice-chancellors for Food and Drug, Healthcare Network of Fardis, Alborz University of Medical Sciences, Fardis, Alborz Province, Islamic Republic of Iran; [4]Nutrition and Food Sciences Research Center, Tehran Medical Sciences, Islamic Azad University, Tehran, Tehran Province, Islamic Republic of Iran

3.1 Introduction

Probiotics have been defined as selected living microorganisms used as dietary supplements having potential for improving health or nutrition of man or animal following ingestion (Amiri et al., 2019). The important probiotic microorganisms associated with dairy products are *Lactobacillus, Bifidobacteria*, *Saccharomyces,* and *Streptococcus* species. Probiotic products increase health after consumption and contain microorganisms which are viable, specific, and effective on important systems of nutritional physiology. Fermented dairy products have long been applied as the fundamental vehicles for probiotic strains. Next, cheeses have been used for concatenation of probiotic microorganisms, but they may offer a number of advantages compared with fermented milks (Gomes et al., 2011; Minervini et al., 2012). Fermented milks have lower pH, less solid consistency, and relatively lower fat content compared with cheese and yogurt (Moghanjougi et al., 2020; Karimi et al., 2012). Different factors must be considered when using probiotics in fermented dairy products including that probiotics must be viable and present in high counts at the time of consumption to achieve the desired benefits.

Dairy fermentation process has relied on the activity of lactic acid bacteria (LAB), which play a crucial role in converting milk as raw material to fermented dairy products. In dairy industry, different industrial strains of LAB are used as probiotic starter cultures (Table 3.1). For example, *Lactococcus lactis* (mesophilic probiotic starter) is used for different types of dairy products such as cheese, butter, and buttermilk (Wouters et al., 2002; Broome et al., 2003; Giraffa et al., 2010); *Lactococcus lactis* subspecies *lactis* biovar diacetylactis is used in Gouda and Edam cheeses, sour cream, lactic butter, and buttermilk (Leroy and De Vuyst, 2004); *Streptococcus* and *Thermophilus* (thermophilic probiotic starter) are used for yogurt and different types of cheeses particularly hard and semi-hard high-cooked cheeses (Beresford et al.,

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Table 3.1 Different industrial strains of lactic acid bacteria that are used as probiotic starter cultures.

Species/subspecies	Application in probiotic dairy products	References
Lactococcus		
Lactococcus lactis subspecies *lactis*	Mesophilic probiotic starter used for different types of dairy products such as cheese, butter, and buttermilk.	Broome et al. (2003), Wouters et al. (2002)
Lactococcus. lactis subspecies *lactis* biovar *diacetylactis*	Used in Gouda and Edam cheeses, sour cream, lactic butter, and buttermilk.	Wood (2012), (Leroy & De Vuyst, 2004)
Lactococcus lactis subspecies *cremoris*	Mesophilic probiotic starter used for different types of dairy products such as cheese, butter, and buttermilk.	Weerkam et al. (1996)
Streptococcus		
Streptococcus thermophilus	Thermophilic probiotic starter used for yogurt and different types of cheese particularly hard and semi-hard high-cooked cheeses.	Broome et al. (2003), Beresford et al. (2001)
Lactobacillus		
Lactobacillus acidophilus, *Lactobacillus delbrueckii* subspecies *bulgaricus,* *Lactobacillus delbrueckii* subspecies *lactis*	Mesophilic probiotic starter used for different types of dairy products such as cheese, butter, and buttermilk.	Briggiler-Marcó et al. (2007), Slaterry et al. (2010) Broome et al. (2003),Giraffa et al. (2010)
Lactobacillus helveticus	Used in Gouda and Edam cheeses, sour cream, lactic butter, and buttermilk.	Broome et al. (2003), Griffiths and Tellez (2013)
Lactobacillus casei	Probiotic milk and starter added during the ripening of cheese.	Briggs (2003)
Lactobacillus plantarum	Starter added during the ripening of cheese.	Leroy and De Vuyst (2004)
Lactobacillus rhamnosus	Probiotic starter added during the ripening of cheese.	Coppola et al. (2005)
Leuconostoc		
Leuconostoc mesenteroides subspecies *cremoris*	Mesophilic starter culture used for Edam, Gouda and fresh cheeses, lactic butter, and sour cream.	Weerkam et al. (1996), Slaterry et al. (2010)

2001; Broome et al., 2003); *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* (mesophilic probiotic starter) are used for different types of dairy products such as cheese, butter, and buttermilk; *Lactobacillus casei*, *Lactobacillus rhamnosus,* and *Lactobacillus plantarum* are used in probiotic milk and starter added during the ripening of cheese (Briggs, 2003; Leroy and De Vuyst, 2004; Coppola et al., 2005); and *Leuconostoc mesenteroides* subspecies *cremoris* (mesophilic starter culture) is used for Edam, Gouda and fresh cheeses, lactic butter, and sour cream.

3.2 Probiotic microorganisms used in fermented milk products

In the following sections, the different probiotic organisms used in dairy products are discussed.

3.2.1 *Lactobacillus acidophilus*

Lb. acidophilus ferments glucose, maltose, fructose, saccharose, galactose, and trehalose, but does not create ammonia from arginine and does not metabolize mannitol (Ozbas, 2004). This strain shows antimicrobial effect because of the formation of antibiotic materials (lactocidin, acidolin, lactocin B, and acidophilin) and organic acids. It is necessary to mention that *Lb. acidophilus* has a resistance to bile acid and antibiotic effect on intestinal pathogens such as fecal *Escherichia coli* strains (Uzun, 2006; Ahmed et al., 2010; Rezazadeh-Bari et al., 2019). Ozden (2008) and Kilic (2008) reported that ingested *Lb. acidophilus* can adhere in intestines and live in gastric juice for 2 days.

3.2.2 *Lactobacillus casei*

Lb. casei grows in the presence of folic acid, niacin, and capantothenate. It can utilize sorbate and sorbitol but its fermentation rate with saccharose and maltose is low. Also, it shows powerful proteolytic effect after lysis and does not have ability to form gas (Ernas and Karagozlu, 2003; Wu et al., 2009). Because of its probiotic traits, it can be used in different fermented milks such as cheese to improve flavor and texture. *Lb. casei* is used as an adjunct culture to improve sensorial properties and/or develop new products such as Actimel, Gefilus, Vifit, and Yakult, traditional fermented milk products like kefir, ve Lactic acid bacteria Zeer, cheese such as parmesan and provolone (Yerlikaya, 2014; Stefanovic et al., 2017).

3.2.3 *Lactobacillus gasseri*

Lb. gasseri has beneficial effects in gastrointestinal system to decrease fecal mutagenic enzymes because of its probiotic activity. Also, this strain can attach to intestines and has a role in macrophage stimulation and bacteriocin production. Due to its probiotic activity, it is used in the manufacture of fermented milk products (Uzuner, 2012).

3.2.4 Lactobacillus rhamnosus

Because of its probiotic activity, *Lb. rhamnosus* GG is known as the most common organism in fermented milk products used for children consumption. Some of the probiotic characteristics of *Lb. rhamnosus* GG include being natural to human intestinal flora, adhesion to gastrointestinal path, and resistance to low pH values (Canbulat and Ozcan, 2007). Because Sherwood Gorbach and Barry Goldin discovered this strain, it has the suffix "GG". It is known as one of the most common microorganism in probiotic productions. Because of its favorable effects on children's health, it can be used in products for children widely (Yerlikaya, 2014; Maleki et al., 2020).

3.2.5 Lactobacillus helveticus

Lactobacillus helvetics is a strain of LAB that is Generally Recognized as Safe (GRAS) and is commonly found in dairy products, fermented foods, and probiotic supplements. Several *in vitro* studies demonstrated that members of this multifunctional LAB strain generally have high extracellular proteinase and other enzymatic activities that could affect human health such as the modification of gut microbiota, inhibition of pathogens, modulation of the host immune system, improvement of food quality, and production of bioactive peptides from food molecules (Bian et al., 2016; Zhou et al., 2019).

3.2.6 Enterococcus faecium/Enterococcus faecalis

E. faecium and *E. faecalis* have probiotic traits so that they can be used in fermented milk products and other probiotic supplements to reduce the cholesterol absorption from digestive system (Erginkaya et al., 2007). Although, they have biotechnological traits such as ability to form bacteriocin and usage in fermented milk products, there are circumstantial evidences to consider them as foodborne pathogens as certain *E. faecalis* strains can cause clinical infections. It is necessary to mention that *E. faecalis* is found in animal and human feces, as well as on plants and thus, its usage as a sanitation indicator is decreased (Kaleli and Durlu-Ozkaya, 2000; Foulquie Moreno et al., 2006; Bhardwaj et al., 2008; Fernandez et al., 2015).

3.2.7 Streptococcus thermophilus

S. thermophilus is an elliptical or circular, gram-positive bacterium. Though many streptococcal genera have pathogenic traits, *S. thermophilus* is known as "GRAS". This microorganism is usually used in combination with other starter cultures for the production of cheese (Italian and Swiss types), yogurt, and other dairy products. Also *S. thermophilus* is known as the second most significant industrial dairy starter after *La. lactis* (Fernandez et al., 2015). *S. thermophilus* shows symbiotic relationship with *Lb. bulgaricus* in yogurt production. In the beginning, *S. thermophilus* has activity in the milk fermented to produce yogurt, consumes oxygen, and increases the

acidity of the milk slightly. *Lb. bulgaricus* grows in this media rapidly and produce valine that is necessary for growth of *S. thermophiles* (Iyer et al., 2010).

3.2.8 *Propionibacterium* (PAB) species

In the early decades of the 19th century, this group of bacteria was isolated from Emmental cheese and named them *Bacillus acidi-propionici* and *Bacterium acidi-propionici*. Classical PAB species known as "dairy-propionibacteria" have important roles in maturation of cheese. In dairy products, *Propionibacterium acidipropionici, P. ferudenreichii,* and *P. jensenii* are present prior to the other species (Cousin et al., 2011). It is necessary to mention that *P. freudenreichii* subsp. *freudenreichii* can be considered as a potential probiotic microorganism, a view based on (a) the formation of propionic acid, (b) bacteriocins, (c) synthesis of vitamin B_2, (d) better exploitation of fodder, (e) growth stimulation of other beneficial bacteria, and (f) survival during gastric digestion. Also *P. freudenreichii* subsp. *shermanii* contributes to the propionic fermentation by converting lactic acid to propionate, acetate, and CO_2, that the latter is important for the "eye" formation in Swiss-type cheese (Fernandez et al., 2015).

3.2.9 *Saccharomyces cerevisiae boulardii*

S. boulardii can form ascospore and grows in standard yeast medium. Also, it has the ability to ferment carbohydrates and can inhibit pathogen's growth. In the 1960s, lyophilized commercial preparation of *S. boulardii* was investigated and used as a treatment for diarrhea in France till now. In addition, lyophilized *S. boulardii* is used clinically in South America, Europe, and Africa. Clinical studies have shown that *S. boulardii* has antitoxic, antimicrobial, enzymatic, and metabolic activities (Billoo et al., 2006; Szajewska, 2012).

3.2.10 *Bifidobacterium* spp.

Thirty different species of Bifidobacteria isolated from humans, animals, insects, and the environment have been recognized. Six species *(Bifidobacterium lactis, B. breve, B. bifidum, B. infantis, B. adolescentis,* and *B. longurn)* are important in the dairy production, primarily for the production of probiotic dairy products (Fernandez et al., 2015). These bacteria grew in popularity considering low acid formation during their shelf life and higher consumption of L (+) lactic acid compared to D (−) lactic acid. Among the many probiotic properties that have been referred to this species are (i) anticarcinogenic activity, (ii) folic acid synthesis, (iii) improvement of food nutritional value, and (iv) the induction of immunoglobulin production (Martinez et al., 2013). Within different probiotic microorganisms, *B. lactis* has been investigated mostly for its useful effects on human health. It is selected for fermented milk products due to the oxygen and acid tolerance in comparison to other species (Janer et al., 2004; Elizaquivel et al., 2011; Akalin et al., 2012).

3.2.11 The genus *Leuconostoc*

In this genus, the organisms that are associated with dairy starter cultures are *Leuconostoc mesenteroides* subsp. *cremoris* (previously known as *Leu. cremoris* or *Leu. citrovorum), Leu. mesenteroides* subsp. *dextrunicum,* and in some instances, *Leu. lactis, Leu. mesenteroides,* and *Leu. pseudomesenteroides* can produce CO_2 that is important for the "eye" formation in some types of cheeses. These microorganisms are usually used in multiple or mixed-strain cheese and other dairy starter cultures that include flavor producers (Fernandez et al., 2015).

3.2.12 The genus *Pediococcus*

The genus *Pediococcus* spp. is consisted of *P. pentosaceus, P. damnosus, P. acidilactici, P. dextrincus, P. parvulus, P. halophilus, P. inopinatus,* and *P. urinaeequi.* These are the main species which have role in probiotic production, fermentation process, and pediocin production. *Pediococcus* spp. can be found in cheese but their accurate role is not completely understood (Porto et al., 2017).

3.2.13 Molds

Molds are usually used in the cheese industry for the production of some semisoft cheese types. Their main role is to improve the aroma and flavor and to modify the shape and texture of the curd. However, some adventitious molds (*Penicillium, Fusarium,* and *Aspergillus*) can synthesize mycotoxins and cause health risks (Benedict et al., 2016). The molds can be divided into two types, taking into consideration their color and growth characteristics, namely the white and blue molds. The white mold, which grows externally on the cheese (e.g., Brie and Camembert) is known as *Penicillium camemherti;* however, in some previously dairy textbooks or commercial culture suppliers, the following *Penicillium* species have been reported *(P caseicolunz, P caseicola, P candidurn, and P. alhunz),* but these are considered biotypes of, or synonyms for, *P. camemberti*. The blue mold, *P. roqueforti* (other synonyms for this blue mold may include *P. gorgonzolae* and *P. stilton*), grows in the cheese internally, and examples of "blue cheeses" are Roquefort, Mycella, Danish Blue, Blue Stilton, and Gorgonzola. Although the current classification of *P. roqueforti* is into two varieties (i.e., *P. roqueforti* var. *roqueforti,* which is used in cheese making, and the ubiquitous patulin-producing variety known as *P. roqueforti* var. *carneum*), it should be reclassified into three species (*P. paneum, P. carneum* and *P. roqueforti*) on the basis of molecular genetics and biochemical profiles (Banjara et al., 2015; Hameed, 2016; Hallen-Adams and Suhr, 2017).

It should be noted that other types of molds which have very limited application, or are traditionally used in some parts of the world, are *Mucor rasmusen,* used in Norway for the manufacture of ripened skimmed milk cheese, and *Aspergillus oryzae,* used in Japan for the production of soya milk cheese varieties. For example, *Geotrichum candidum* grows on the surface of the milk to form the white velvet layer on Viili, which is a cultured milk product from Finland (Tamime and Robinson, 2007).

3.2.14 Yeasts

In general, the presence of yeasts in dairy products is considered as contamination and has negative effects. Also some yeast species such as *Candida tropicalis, C. krusei, C. albicans,* and *C. glabrata* have pathogenic traits. Candidiasis and trichosporonosis can be caused by some pathogenic yeast species that spread widely (Kasahara et al., 2014; Banjara et al., 2015). However, as mentioned elsewhere, certain species of yeast are added to cheese milk to improve the flavor and appearance. Also in the dairy industry, the addition of yeasts to milk, besides the LAB, results in a yeast-lactic fermentation. This type of fermentation is limited to the production of Koumiss and Kefir dairy drinks. It was suggested by many researchers in the past that *Candida kefyr* (old name *Candida kefir)* and *Lb. kefir* are the only organisms that are intimately associated with Kefir grains, but other researchers have included more yeast species, acetic acid bacteria, and LAB (Gao and Li, 2016).

Capsular polysaccharide forming *Lb. kefiranofaciens* from Kefir and fermented milks had a resistance to syneresis and a ropy consistency. Again, the microflora of Koumiss is not defined completely, but contains mostly lactobacilli, lactose-fermenting *(Torula koumiss, Saccharomyces lactis)* and nonlactose-fermenting *(Saccharomyces cartilaginosus)* yeasts, and the noncarbohydrate-fermenting yeast *(Mycoderma* spp.) (Koroleva, 1991; Oberman and Libudzisz, 1998).

3.3 Nutritional benefits of fermented dairy products

The following section describes some of the proposed health benefits of consuming fermented probiotic dairy products.

3.3.1 Relief of lactose intolerance

The disability of adults to digest lactose (milk sugar) is widespread in the world. Panesar et al. (2006) represented that consumption of lactose by those lacking sufficient levels of lactase produced in the small intestine can effect in symptoms of diarrhea, bloating, abdominal pain, and flatulence. Milk with cells of *Lb. acidophilus* aids digestion of lactose by these persons. It is necessary to mention that lactose intolerance signs when consuming fermented dairy products proved to have fewer symptoms than consuming the same amount of milk. Yogurt was found to be helpful in the digestion of lactose because the LAB used to make it, produce lactase, and digest the lactose (Gilliland, 1985). In yogurt, lactose utilization proceeds as long as the external pH allows metabolic activity of the LAB. When a lot of lactic acid is produced, so much so that the pH has reached 4.0 in yogurt and 4.5 in buttermilk, the yogurt (*Lb. bulgaricus*) or buttermilk bacteria (*Lactococcus lactis*) cease their metabolic activity and lactose is no further utilized. However, by rerouting metabolism of the LAB toward production of more pH-neutral components, further conversion of lactose will become possible. In this case, sugar metabolism of *Lac. lactis* is converted to synthesize L-alanine instead of lactic acid by cloning alanine dehydrogenase into this microorganism

(Panesar et al., 2009), by cloning alanine dehydrogenase into this microorganism, opens interesting possibilities for an effective removal of lactose. Not only will the fermented dairy product be free of lactose, but its sweetness and general taste will be enhanced by the formation of L-alanine amino acid.

3.3.2 Gastrointestinal tract infection

Gastrointestinal infections including diarrhea result from a change in the gut microflora caused by an aggressive pathogen. It is necessary to mention that viable LAB interfere with the colonization and subsequent proliferation of food-borne pathogens, thus preventing the manifestation of infection (Gandhi, 2000). *Lb. bulgaricus, Lb. acidophilus, S. thermophilus,* and *B. bifidum* have been involved to this effect. The useful and helpful effects of LAB and cultured dairy products have also been attributed to their ability to suppress the growth of pathogens either directly or through production of antibacterial substances. Antibiotics have been reported to kill normal bacteria as well, often resulting in disorder of the bacterial flora, leading to diarrhea and other intestinal problems (Gandhi, 2000; Panesar et al., 2009).

Replenishing the flora with normal bacteria during and after antibiotic therapy appears to minimize destructive effects of antibiotic use. Probiotics have been reported effective in prevention of various gastrointestinal infections. Also, there are a number of reports of benefits for sufferers of rotavirus infection, traveler's diarrhea, and antibiotic-induced diarrhea (Panesar et al., 2009).

3.3.3 Anticarcinogenic effect

It has been mentioned that fermented dairy products can protect against certain types of cancers. Consumption of yogurt, Gouda and Camembert cheese, buttermilk, and Iranian doogh protect against breast cancer. Clinical studies have shown that LAB exert anticarcinogenic effect either by prevention of cancer initiation or by suppression of initiated cancer. Anticarcinogenic effects of yogurt and dairy products fermented with *Lb. acidophilus* have been reported in mice (Shoukat, 2020). Different potential mechanisms by which LAB exert antitumor effects have been suggested such as changes in carcinogenesis, cellular uptake of mutagenic compounds, decreasing the mutagenicity of chemical mutagens, and suppression of tumors by recovered immune response (Ahmad et al., 2018).

3.3.4 Strengthening the immune system

The immune system provides the main defense against microbial pathogens that have entered human body. The immunostimulatory effects of yogurt are believed to be a consequence of its bacterial components (Gandhi, 2000). Clinical studies have shown effects of LAB on heightening levels of certain immunoreactive factors. It is necessary to mention that milk components such as whey protein, calcium, vitamins, and trace elements are capable of influencing the immune system. However, it should be mentioned that cytokine production, phagocytic activity, antibody production, T-cell

production, etc., are enhanced with yogurt consumption, and other dairy products contain LAB (Saini and Minj, 2020).

3.3.5 Reduce serum cholesterol

Some studies indicate that fermented dairy products have hypocholesteraemic effect. It is mentioned that intake of large quantities of fermented milk furnish factors that impair the synthesis of cholesterol. Also, it is necessary to mention that *Lb. acidophilus* has exhibited the ability to lower serum cholesterol levels. This promotes the potential wholesome aspects of dairy products fermented with *Lb. acidophilus* since hypercholesterolemia is considered to be one of the main factors contributing to cardiovascular disease. However, some probiotics strains may demonstrate this property while other strains do not (Song et al., 2015).

3.3.6 Avoid constipation

Constipation is common problem in people consuming the fiberless diet and also in elderly people. Some researches with *Lactobacillus* preparation and fermented milks have been published on this aspect (Panesar et al., 2009). They reported that constipation was reduced using *Lb. acidophilus* NCDO 1748, *Lb. casei* Shirota, and *Lactobacillus* GG.

3.3.7 Antihypertensive activity

Casein hydrolysate, extracted from an extracellular proteinase from *Lb. helveticus* (CP790), has been reported to show antihypertensive activity in mice. Two antihypertensive peptides which have been purified from fermented milk contain *Lb. helveticus* and *Saccharomyces cerevisiae*. These peptides inhibit angiotensin-converting enzyme that converts angiotensinogen I to angiotensinogen II, which is a potent vasoconstrictor (Beltrán-Barrientos et al. 2016; Ahtesh et al., 2018).

3.3.8 Antiallergenic properties

Probiotics may be able to prevent allergic reactions in individuals at high risk of allergies, such as food allergies. Probiotic microorganisms (both bacteria and molds) help to reinforce the barrier function of the intestinal wall, thereby possibly prevent the absorption of some antigens (Kirjavainen et al., 2003).

3.3.9 Vitamins metabolism

Milk contains vitamins of water or fat-soluble type. When probiotic starter cultures are growing in milk for fermentation, some vitamins may be utilized by the bacteria, reducing their concentration. On the other hand, some vitamins may be synthesized also by LAB, leading to increased content in fermented dairy products. It is necessary to mention that increase or decrease in the amount of vitamins greatly depends on the

strain of starter. However, generally it is reported that probiotic bacteria in yogurt produce folic acid, niacin, and vitamin B6. For example, the yogurt bacterium *Streptococcus thermophilus* produces the B_5 vitamin (folic acid), which is subsequently used, for growth, by the other yogurt bacterium *Lb. bulgaricus*. Also, *Lac. lactis* has the ability to produce B_5 vitamin. B_5 vitamin or folic acid seems to be specifically involved in the biosynthesis of purines and pyrimidines. Also, propionibacteria are known to synthesize vitamin B12 in cheese (Walther and Schmid, 2017).

3.3.10 Production of bacteriocins

Lactic acid bacteria are exerting antagonistic effect against a number of organisms, due to production of several antimicrobial substances. These include organic acids such as lactic acid and acetic acid, hydrogen peroxide, diacetyl, reduced pH and EH, and a number of bacteriocins. It should be noted that bacteriocins are the proteins produced by the bacteria that are inhibitory to closely related species. However, some of the bacteriocins of LAB showed wide spectrum activities. The exact mechanism for synthesis and other characteristics of some bacteriocins are still not clear. It is necessary to express that nisin is the only one, which is fully characterized and used as food preservative such as Iranian doogh; other bacteriocins produced by LAB are acidophilin, lactocidin, brevicin, helveticin, etc (Juturu and Wu, 2018; Abbasiliasi et al., 2017).

3.3.11 Production of nutraceuticals

Nutraceuticals are components that, through specific physiological actions, contribute to man's health. Some nutraceuticals from bacterial origin have been added to foods. Through strain selection and process optimization, the activity of LAB can be modified to increase the content of nutraceuticals in fermented dairy products. For example, fermented milks can be produced with lactic acid bacterial strains that produce high amounts of low-calorie polyols so as to reduce the sugar content. Also, the use of oligosaccharide-producing LAB that produce sugar polymers with a controlled structure and chain length (and hence molecular mass) may yield fermented dairy products with health applications. Health benefits of such oligosaccharides are attributed to their low-calorie character, their fiber like nature, and their bifidogenic effect. However, the proteolytic system of LAB can contribute to the liberation of health enhancing bioactive peptides from dairy products. This proteolytic system may improve absorption in the intestinal tract, stimulate the immune system, exert antihypertensive or antithrombotic effects, display antimicrobial activity, or function as carriers for minerals (Guan et al., 2019; Vibhuti et al., 2018).

The starter cultures used in the dairy industry are composed of selected strains of LAB, which were originally, present as part of the contaminating microflora of milk (Chamba, 2008; Pot and Tsakalidou, 2009). The main use of starter cultures is for production of lactic acid from lactose (milk sugar), which in most cases causes or assists in the coagulation of milk protein by lowering its pH value.

However, several starter microorganisms are added specifically for their ability to produce flavor compounds such as diacetyl. Also, starter microorganisms can also

influence flavor and texture of cultured and/or aged products through the breakdown of proteins, fats, and other milk constituents in addition to the pH effect. A decreased pH of cultured products can be inhibitory to certain spoilage such as *E. coli*, *Candida albicans,* and *Clostridium difficile*, although inhibition is also associated with other byproducts such as H_2O_2 (Chamba, 2008).

Viscosity of stirred milk is due to interaction between the exopolysaccharides (EPS) and the casein-matrix and/or absorption of ropy strains to the protein matrix. LAB producing EPS have potential applications as viscosity enhancers, texturizers, (Amiri et al., 2019; Panesar et al., 2006) and emulsifiers (Gilliland, 1985; Vera et al., 2018). Masud et al. (1991) presented that modification of texture properties of fermented milk by EPSleads to a higher viscosity and a lower degree of syneresis compared with products produced without EPS producing cultures. It is necessary to mention that nordic fermented milks contain EPS produced by *Lac. lactis* subsp. *cremoris* as a homofermentative LAB on milk (Panesar et al., 2009). Nasiri Boosjin et al. (2016) showed that best organoleptic properties were achieved in the product prepared with 2% mesophilic and thermophilic starter cultures and 2% probiotic (*Lb. acidophilus* and *B. bifidum*).

Rezazadeh et al. (2013) reported that the use the starters EPS to improve the texture of Iranian white cheese can be effective. Salem et al. (2005) reported that apparent viscosity values of probiotic ice cream mixes at a shear rate of 48.6/sec revealed that the addition of fermented milk with different cultures led to a slight increase in mix viscosity. It could be noticed that among all bio-ice cream mixes (containing *Lb. gasseri*, *Lb. rhamnosus*, *Lb. reuteri*, *Lb. acidophilus,* or *B. bifidum*), *Lb. reuteri* showed the highest viscosity, whereas the one with *Lb. acidophilus* demonstrated the lowest values. The higher viscosity values of probiotic ice cream could be related to slight protein precipitation due to the higher acidity (or lower pH values) of mixes compared to excess mix viscosity, decreased whipping rate, and a less stable mix.

3.4 Biopreservation of dairy products

Food supply and preservation were considered ever since early humans to the present day. Food must be stored appropriately after production; otherwise it will be spoiled. One of the methods for food preservation is by the use of food additives. Biopreservation using natural preservatives in addition to improving food safety and increasing its shelf-life, as a technological effect, has a positive effect on consumer health promotion. The term "Biopreservation" refers to application of natural methods to control/enhance safety of foods by useful microbes or their antibacterial products (Zeuthen and Bøgh-Sørensen, 2003).

3.4.1 Fundamental of biopreservation (basics of biopreservation)

Due to complex ecosystems of microbes, they need to compete for nutrients to survive. Also the microorganisms have developed diverse mechanisms; one of these conventional and practical strategies for defending a population's territory is releasing

Table 3.2 The classification of bacteriocins that are produced by lactic acid bacteria.

Classification	Molecular weight (Da)	Types and chemical properties	Thermal stability	Examples
Class I or Lantibiotics	Less than 5000	Type A: Linear	Stable	Nisin Carnocin U149
		Type B: Globular		Lactocin S Lactacin 481
Class II or Antilisterial peptides	More than 5000 and Less than 10,000	Type A: Including one peptide	Stable	Lactococcin MMF2 Sakacin G
		Type B: Including two linear peptides		Lactococcin G Lacticin F
Class III or Bacteriolysins	More than 30,000	Large peptide	Unstable	Helveticin J Helveticin V-1829 Acidofilin Lactacins A and B
Class IV or Complex bacteriocins	More than 30,000	Mixture of protein, lipid, and carbohydrate	Unstable	Plantaricin S Leuconocin S Lactocin 27 Pediocin SJ1

antimicrobial substances that inhibit the competitor's growth. LAB produce antimicrobial peptides as metabolites that have great potential for food biopreservation. These bacteria are generally regarded as safe and are considered as factories for production of natural antimicrobial metabolites. Organic acids (such as lactic acid, acetic acid, propionic acid, butyric acid, and etc.), antagonistic compounds (like hydrogen peroxide, carbon dioxide, fatty acids, ethanol, and diacetyl), antifungal metabolites (like hydroxyphenyl lactic acid, cyclic dipeptides, phenyllactic acid, and 3-hydroxy fatty acids), and bacteriocins (such as nisin, lacticins, pediocins, and enterocins) are antimicrobial metabolites synthesized by LAB (Gálvez et al., 2014).

3.4.2 Bacteriocins

Bacteriocins are a diverse group of extracellular bioactive peptides secreted by bacteria that are synthesized in ribosomes and have antimicrobial activity against other bacteria. In other words, they have antibacterial or bacteriostatic effects on bacteria or other bacteria. Bacteriocins include peptides or proteins with variable biochemical properties in terms of molecular weight, mechanism of action, and activity spectrum

(Ansari et al., 2018; Barbosa et al., 2018). These peptides are heat-resistant. The synthesis of bacteriocins is controlled by a plasmid gene, and they are produced to compete with other microbial flora (Caballero et al., 2015; Fuquay et al., 2011; McNeil et al., 2013). According to literatures, bacteriocins can be divided into four classes. Table 3.2 shows the classifications of bacteriocins that are produced by LAB (Gálvez et al., 2014; Balciunas et al., 2013; Reis et al., 2012; Güllüce et al., 2013).

There are three ways to use bacteriocins in foods by improving their safety purpose: (1) Adding pure or semi-pure bacteriocin as an additive in food composition, (2) the use of a compound of food that has already been fermented with a bacteriocin producer strain, and (3) the use of a bacteriocin producer as an adjunct or/and starter culturing in fermented foods for *in situ* production of bacteriocin. The growth of the starter producing bacteriocins in an environment such as milk, whey, lean dry milk, dextrose, or other plant derivatives is an approach for using these compounds (Fig. 3.1).

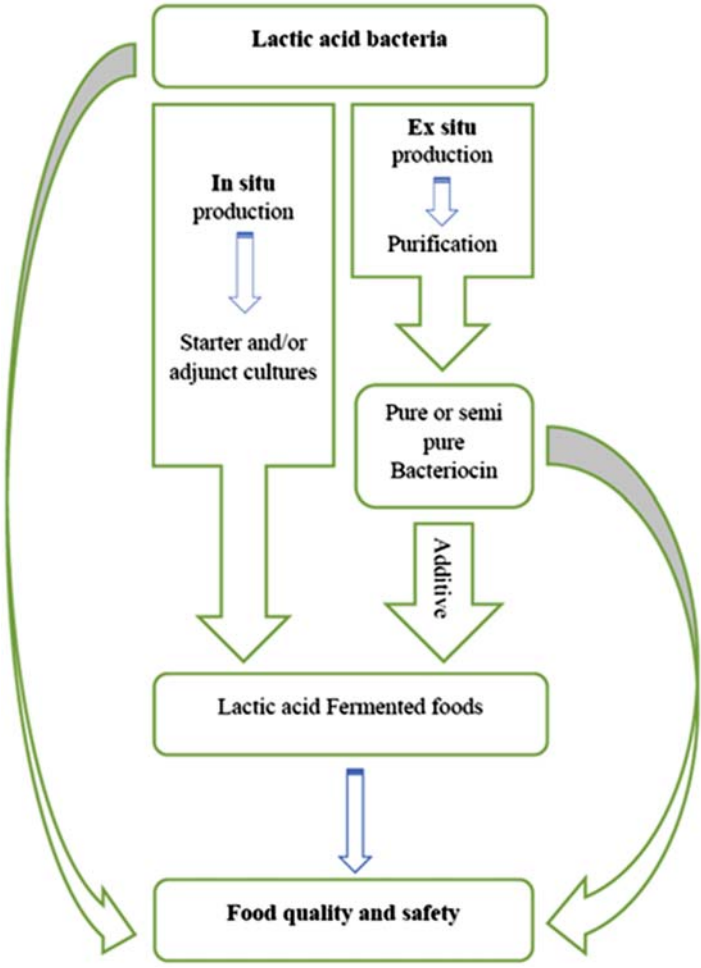

Figure 3.1 Application of lactic acid bacteria producing bacteriocins.

3.4.3 Ex situ *production of bacteriocins*

The *ex situ* production involves the addition of pure and semi-pure bacteriocins as a food preservative (its use depends on regulatory approval) or the use of a food which is previously fermented with one or more bacterial species of LAB as food additives (Abo-Amer, 2011; Bhat and Paliyath, 2012; Beshkova and Frengova, 2012; Lacroix, 2010; McNeil et al., 2013; Gálvez et al., 2014).

Cheese whey is a major contributing factor to the production of cheese (95%−85% of milk volume), which includes the greenish gelatinized liquid from the milk after casein deposition. Important nutrients in cheese whey are lactose (5.0%−5.5% w/v), proteins (0.8%−0.8% w/v), fat (0.5%−0.5% w/v), minerals (10%−8% of dry matter), and vitamins of group B (Raiszadeh-Jahromi et al., 2020; Amado et al., 2016; Parashar et al., 2016). Lactose and protein are the major components of whey that make up 75% and 10%, respectively, in total solids. Whey protein is separated by ultrafiltration. The remaining liquid, whey trim, is mainly made up of lactose, salts, nonprotein nitrogen and water. Worldwide whey and whey products production in 2014 was estimated at 5.6 million tons, the main product of which was whey powder. In 2013, the total market for whey protein powder in the world was appraised at $ 8.9 billion and was projected to reach US $ 11.7 billion by 2017. Therefore, it is very important to find new ways to use whey protein powder. The current practices in the dairy industry in relation to whey protein powder include disposing of it as waste, dispersal of agricultural land, the sale of dry whey powder, and mixing it into animal feed (Kumar et al., 2012; Parashar et al., 2016). Currently, dairy factories around the world are looking for alternative approaches for the cost-effective utilization of whey powder. The use of whey powder is used as a direct source of lactose in some dairy factories. However, this requires extensive processing, including demineralization and dewatering. One promising way for direct use of whey-based lactose is to produce bio-based products. Several attempts have previously been made to use whey as a fermentation substrate for ethanol production, several of which are in the industrial scale in New Zealand, the United States, and Denmark. However, the low ethanol productivity due to low lactose and high processing cost (such as reverse osmosis for whey condensation) limits the use of whey premium as substrata for ethanol production (Schirru et al., 2014; Parashar et al., 2016).

Halami and Chandrashekar (2005) investigated the ability of *Pediococcus acidilactici* C20 to synthesize pediocin C20 on whey permeate. The results showed that the biosynthesis of pediocin C20 in the lactose-based medium was 1.0−1.5 fold higher than the glucose-based synthetic medium. Bertrand et al. (2001) investigated production of nisin-Z in pH-controlled batch fermentation by immobilized *Lac. lactis* subsp. *lactis* biovar. *diacetylactis* UL719 on gel beads of *k*-carrageenan/locust bean gum supplemented with whey permeate. A high production of nisin-Z (8200 IU/mL) was determined after 1h fermentation which was higher than maximum production of nisin-Z reported in literature for pH-uncontrolled batch fermentation by free-cell (850 IU/ml/h) and immobilized-cell (1760 IU/ml/h) by the same strain and fermentation conditions.

3.4.4 In situ *production of bacteriocins*

Bacteriocin production may be more important when the bacteria that produce it are added to foods as a starters or protective adjunct cultures (Bhat and Paliyath, 2012; Lacroix, 2010; McNeil et al., 2013). Devi et al. (2014) investigated the synthesis of bacteriocins by *Lb. plantarum* Acr2 and *E. faecium* NCIM 5423 in soymilk fermentation. *E. faecium* NCIM 5423 and *Lb. plantarum* Acr2 were adequate for producing bacteriocin after 6 h. Scanning electron microscope indicated the inhibitory effect of bacteriocin on *Listeria monocytogenes*. The coculture of *Lb. plantarum* Acr2 and *E. faecium* NCIM 5423 had reduced the *L. monocytogenes* count during 24 h by *in situ* production of bacteriocin in fermented soymilk.

3.5 Modification of milk yield through biotechnology

Biotechnology has many applications in the livestock industry, such as genetic modification of livestock, increasing the quality and quantity of livestock products, milk and meat quality control tools, changing and producing enzymes and hormones, and ultimately increasing productivity and reducing costs. Manipulating the endogenous milk proteins or adding new proteins to the milk is a suggested change for the milk. Elevated levels of human lactoferrin as well as high levels of β- and *k*-casein in milk fat are the result of cloned transgenic cows (Brophy et al., 2003; Batish and Grover, 2003).

3.5.1 *Modification in milk composition*

There are several strategies by which genetics can be used to alter milk composition. One such strategy is to correlate the differences in DNA (genes) between cows with specific production traits (e.g., milk composition) and then using the information in breeding programs to increase the number of cows with the more desirable genes, and thereby increasing the frequency of the genes in the dairy population. When correlation between specific alleles of the genes and milk production or composition is established, it will be possible to incorporate the information as selection indices into dairy breeding programs (Jimenez-Floresand Richardson, 1988; Dalgleish et al., 1989).

3.5.2 *Modification in protein*

Jimenez-Flores and Richardson (1988) reported that by controlling gene dosage and the regulatory regions of casein gene modification, functionality of the milk protein system can be modified by producing 20%−30% additional or novel caseins. Brophy et al. (2003) studied the functional properties of milk by alteration of casein ratios (beta:kappa caseins) and thereby micelle structure. The studies showed that reducing the size of the micelle led to an increase in casein content (Dalgleish et al., 1989), and

improved heat stability and cheese making properties of milk (Jimenez-Flores and Richardson, 1988; Singh and Creamer, 1992).

An increase of more than 50 percent in the stiffness of cheese was accessible by increasing casein. The results of β-casein modification included three main aspects: plasmin isolation and thus preventing the bitter taste in cheese, removal of the incision site for chymosin, and increasing the hydrophilicity of β-casein as a result of adding glycosylation sites (Batish and Grover, 2003). In the bovine species á S_2-casein could increase the nutritional value of casein on one hand due to its phosphorescence and cysteine, and on the other hand, it stabilized the micelle. Sabikhi (2004) reported that genetic engineering was able to increase the total milk protein by 13%−15% and the total milk casein by 17%−35%, but no such increase could be observed in the milk of nontransgenic cows.

3.5.3 Modification in fat

Studies have shown that C14: 0 and C16: 0 fatty acids are higher in milk, while unsaturated fatty acids (MUFA) and unsaturated fats (PUFA) are in lower concentrations (Kennelly et al., 1999). The quality of dairy products in various respects is affected by changes in milk fat and, of course, its organoleptic and rheological properties (Mortensen, 1983). According to Sabikhi (2004), the flavor and physical properties of dairy products are affected by the fatty acids that make up milk fat. Perhaps the healthiest type of milk for human nutrition contain $<10\%$ poly unsaturated fatty acids, $<8\%$ saturated fatty acids, and $>82\%$ monosaturated fatty acids such as Conjugated linoleic acid (CLA) and omega-fats (Batish and Grover, 2003; Sabikhi, 2007).

3.5.4 Modification in lactose

The most important sugar in milk is lactose. The enzyme lactase (also known as lactase-phlorizin hydrolase (LPH)) is part of the β-galactosidase family of enzymes that is essential for the digestion of lactose in the human body. This enzyme breaks down lactose into simpler sugars glucose and galactose, and then these sugars can be absorbed in the human body. Milk is one of the main sources of calcium, and in patients with lactose intolerance, not getting enough calcium is a significant problem, which is more acute in the elderly (Corazza et al., 1995). Due to the importance of lactose intolerance or sensitivity, low lactose milk is produced by biotechnological enzymes.

3.6 Biotechnology of flavor formation in fermented dairy products

Based on “Component Balance Theory”, the flavor of various dairy products is produced by the accurate balance and concentrations of a wide range of sapid and aromatic compounds (Raak et al., 2017).

3.6.1 Aroma of fluid milk

The weak flavor of milk is caused by low concentrations of numerous odorants (Bendall, 2001). Thermal processing of milk such as high or short-time pasteurization and ultrahigh-temperature (UHT) processing (typically at 728C for 15s, 135−1508C for 3−5s, respectively) are commonly employed methods to achieve microbial safety and shelf-life stability. In high temperatures, both flavor and color are affected and also, lipid oxidation products are increased. In addition, it can induce a strong "cooked" off-aroma and chalky taste in milk (Shipe, 1980; Zabbia et al., 2012). The "cooked" off-aroma is due to the production of volatile sulfur compounds, aldehydes and methyl ketones (Zabbia et al., 2012).

Czerny and Schieberle (2007) reported the influence of the packaging material on the odorant profiles of UHT milk. Volatile compounds that contribute particularly to UHT milk are lipid oxidation products (mainly 2-alkanones: C5, 7, 9, 11) and various heterocyclic compounds such as pyrazines, furans, lactones, and other products of nonenzymatic browning/Maillard reactions [e.g., maltol, 4-hydroxy-2,5- dimethyl-3(2H)-furanone (HDMF), 2,5-dimethylpyrazine and o-aminoacetophenone]. Sweetened condensed milk has a similar volatile flavor profile (Shimoda et al., 2001; Naude et al., 2009).

3.6.2 Aroma of fat-enriched milk products

The butter aroma is derived primarily from the volatile compounds existing in the fat fraction (Mallia et al., 2008), whereas flavor of cream is due mostly to the contributions from the aqueous phase of milk and fat globule membrane (Bolling et al., 2005). Pionnier and Hugelshofer (2006) analyzed cream that had been subjected to various processes (pasteurization, sterilization, and UHT) by Gas Chromatography−Olfactometry. They recognized 32 aroma compounds, which included ketones, acids, lactones, and sulfur compounds. Peterson and Reineccius (2003a) using AEDA (aroma extract dilution analysis) studied key aroma compounds (ketones and aldehydes) in sweet-cream butter such as hydrogen sulfide, acetaldehyde, dimethyl sulfide, diacetyl, hexanal, 2-methylbutanal, 3-methylbutanal, butanoic acid, dimethyl trisulphide, hexanoic acid, δ-hexalactone, nonanal, δ-octalactone, and y-dodecalactone. Likewise, the major aroma compounds found in heated butter were methional, (E)-2-nonenal, 1-hexen-3-one, 1-octen-3-one, δ-octalactone, δ-decalactone, hydroxyl methylfurfural, and skatole as intense odorants. In addition, Peterson and Reineccius (2003b) reported hydrogen sulfide, methanethiol, acetaldehyde, diacetyl, 2-heptanone, dimethyl trisulphide, nonanal, butanoic acid, 3-methylbutanoic acid, δ-hexalactone, and hexanoic acid in heated butter (Peterson and Reineccius, 2003b).

3.6.3 Aroma of dried milk products

Milk powder as a raw material is used widely in many food formulations. Thus, flavors and off-flavors of milk powders could develop in the final products (Kalyankar et al., 2015). Hydrocarbons, aldehydes, ketones, alcohols, fatty acids, esters, furans, phenolic

compounds, lactones, and nitrogenous compounds are the main compounds, which are formed over 99.5% of the total volatiles recovered. It was reported that the levels of flavor compounds in the commercial milk powder were very low, and their compositions were very complicated (Karagul-Yuceer et al., 2001, 2003a; 2003b). Among them, free fatty acids and lactones that were present at relatively high levels, were considered as basic contributors to the flavor of milk. In addition, aldehydes, aromatic hydrocarbons, and some heterocyclic compounds, like indoles or thiazole, seem to impart flavor of milk powder. However, high heat-treated nonfat dried milk had a potent aroma intensity caused by HDMF, butanoic acid, methional, oaminoacetophenone, trans-4,5-epoxy-(E)-2-decenal, sotolon, and vanillin (Karagul-Yuceer et al., 2001, 2003a; 2003b).

3.6.4 Aroma of fermented milk products

Diacetyl and acetaldehyde are the main odorants in yogurt, and other products that are produced by LAB used as starter cultures. Many other fermented types of milk are produced around the world involving LAB and/or in some cases fairly complex sets of microflora in which a host of volatile compounds play a critical role in the flavor. Fat content has an important effect on flavor release that is determined to some extent by the physical chemistry of the compound concerned. Münster cheese produced in the United States, using *Streptococcus thermophilus* as culture and no surface smear, was described by descriptive sensory analysis as cooked/milky, whey, milk fat/lactone, and sour and salty (Singh et al., 2003a, 2003b).The use of dynamic headspace dilution analysis (DHDA) methodology, formerly described by Cadwallader and Baek (1998), showed that the most aromatic compounds in the headspace of Müenster were 2, 3-butanedione, dimethyl sulfide, dimethyl disulphide, 2/3-methylbutanal, and 2-acetyl-2-thiazoline (Singh et al., 2003b; Cadwallader and Simgh, 2009).

3.7 Taste compounds in milk and dairy products

In food systems such as milk and dairy products, study of taste compounds in isolation or in matrices devoid of contribution from volatile aroma compounds is difficult because of the complex nature, in terms of both number of food constituents and their competing/synergistic effects on taste and/or aroma. Compounds which contribute to the taste of milk and dairy products can originate from three possible sources:

(i) Innately found in milk, e.g., lactose,
(ii) Added/produced during the manufacturing process (e.g., NaCl, lactic acid), and
(iii) Produced by many biochemical reactions happening during fermentation.

Major details available on LAB in the literature on taste compounds are summarized below. The main taste compounds in milk are lactose (approximately 0.3 times as sweet as sucrose) and the soluble salts, which cause a sweet and salty, taste, respectively. The sweet taste dominates, while salty taste is prevalent if the Na/lactose ratio is

high, as in the case of mastitis milk. The casein nearly masks the sweet taste of lactose in milk (Walstra et al., 1999). Lactose-hydrolyzed milk and whey have a sweeter taste than common pasteurized milk. A chalky taste is observed in high heat treated or UHT treated milks. This is probably the result of precipitation of colloidal calcium phosphate. Sodium chloride is a main contributor to the taste of cheeses. The main acid in fermented dairy products is lactic acid. The concentration of lactic acid, and also the pH, varies extremely with: i) the type of fermented dairy product, ii) initial production by the starter culture, iii) extent of loss in whey, and iv) its metabolism by the nonstarter microflora.

Several other acids, e.g., acetic, propanoic, and C4−10, also contribute to sour/soapy taste but they contribute frequently toward the aroma. Several characteristic tastes (sour, sweet, salty) compounds of Emmental and Swiss cheeses are acetic acid, propionic acid, lactic acid, succinic acid, and glutamic acid, each in free form and/or as ammonium, sodium, potassium, magnesium, and calcium salts, as well as corresponding chlorides and phosphates (Warmke et al., 1996). Magnesium and calcium propionate mostly cause the sweetish note in the taste profile of Emmental and Swiss cheeses. Casein is hydrolyzed to varying degrees depending on the fermented dairy products, resulting in the production of peptides and free amino acids. The precise role of the intermediate to small molecular weight peptides is not clear, but they are commonly accepted to play a significant role in the background taste of cheese (Fox et al., 1994). Numerous peptides have been identified in several types of cheeses as bitter. Peptides with a Q value (average hydrophobicity) $>$ 1400 cal/mol/residue and a molecular weight up to 6000 Da (molecules $>$6000 Da are possible to be too large to interact with the taste receptors) taste bitter, and no bitterness occurs when Q is also classified as potentially bitter in the Q value model proposed by Ney (1981). Different small peptides were detected in Comté cheese. Cyclic dipeptides were explained as bitter (Roudot-Algaron et al., 1993, 1994), and dipeptides with a gamma-glutamyl residue were found to be sour (e.g., y-Glu-Tyr), apart from y-Glu-Phe explained to have a complex taste, which was brothy and slightly sour, salty, and metallic (RoudotAlgaron et al., 1994). Also, Drake et al. (2007) studied compounds responsible for the umami taste in Cheddar and Swiss cheeses, and high- and low-intensity umami-tasting cheeses were selected by trained sensory panel. Their results indicated that the compounds include monosodium glutamate (MSG), disodium-5′-inosine monophosphate, disodium-5′-guanosine monophosphate, sodium chloride and lactic acid.

3.8 Conclusions

Studies have shown that probiotics are generally associated with reduced risks of type 2 diabetes, metabolic syndrome, and heart diseases, and improved weight management. Biopreservation (such as lactic, acetic, formic, propionic, butyric, hydroxylphenyllactic acid, and phenyllactic acid), antagonistic compounds (like carbon dioxide, ethanol, hydrogen peroxide, fatty acids, acetoin, diacetyl, reuterin, and

reutericyclin), antifungal compounds (like propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides, phenyllactic acid, and 3-hydroxy fatty acids), and bacteriocins (such as nisin, pediocins, lacticins) using natural preservatives in addition to improving food safety and increasing its shelf-life, as a technological effect, also has a positive effect on consumer health promotion. The application of many modifications (such as recombinant DNA techniques, cell culture, and monoclonal antibody (hybridoma) methods) to the dairy industry has already generated a number of products for improving milk production, animal health, and food processing, and will continue to do so. The general chemical/biochemical pathways, i.e., (1) heat-induced changes, (2) lipid oxidation, (3) glycolysis, (4) lipolysis, and (5) proteolysis create flavor in fermented dairy products.

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Detoxification properties of microorganisms in foods

4

Mojtaba Yousefi [1], *Nasim Khorshidian* [1], *Amir M. Mortazavian* [2]
[1]Food Safety Research Center (Salt), Semnan University of Medical Sciences, Semnan, Iran; [2]Department of Food Technology, Faculty of Nutrition Sciences and Food Technology/ National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran

4.1 Introduction

Today, the production of healthy and safe food is a key element in the food industry. Unlike the food industry's efforts to produce safe food, it is likely that food can be either contaminated during the process or prepared from contaminated raw materials (Zoghi et al., 2014). Therefore, food consumers can be environmentally exposed to both intentional and unintentional additives and pollutants which can have adverse effects on health over time. Environmental contaminants such as heavy metals, pesticides, and mycotoxins can be released into water and food production chain as well as formation of unwanted harmful chemical compounds during processing, resulting in various adverse health effects and chronic toxicity especially cancer (Khorshidian et al., 2016). Cancer is a very serious and complicated disease created by out of control and irregular growth of cell (Cho and Finocchiaro, 2009); its prevalence is notably increasing throughout the world. Except for genetic defects which contribute from 5% to 10% of cancer incidences, the rest (90%–95%) can be limited by changing lifestyle, increasing physical activity, avoiding smoking, and utilizing nutritionally balanced diet together with the foods free from contaminants (Davoodi et al., 2013). Application of good agricultural practices, avoiding use of pesticides in agricultural products, monitoring storage condition of raw materials, and control of environmental conditions in processing of foods have been recommended, as preventive ways of toxicants generation. Furthermore, alternative detoxification approaches using microorganisms such as lactic acid bacteria (LAB), probiotics, or yeasts have been investigated extensively (Ibrahim et al., 2006; Yousefi et al., 2017; Chiocchetti et al., 2018). In this chapter, decontamination of several toxicants in food by different microorganisms and the underlying mechanisms are discussed.

4.2 Detoxification of different toxicants in food products

Fig. 4.1 illustrates the factors affecting the binding ability and detoxification of toxicants by microorganisms.

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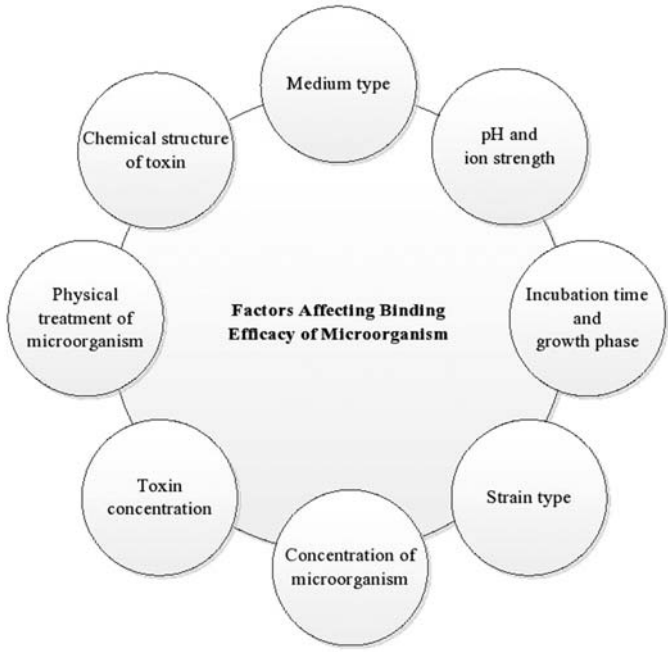

Figure 4.1 Factors affecting binding ability of microorganisms.

4.2.1 Process-induced food toxicants

4.2.1.1 Heterocyclic aromatic amines and polycyclic aromatic hydrocarbons

Heterocyclic aromatic amines (HAAs) are a group of chemical compounds that possess at least one heterocyclic ring and are classified as possible human carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 1978, 1993, 1994). These compounds are formed in muscle foods which are cooked at high temperatures (150−250°C), and it is assumed that "Maillard reactions" has a substantial role in the formation of aminoimidazoarenes (AIAs) (Busquets et al., 2004; Sugimura et al., 2004). Epidemiological studies have revealed that there is a correlation between HAAs intake and induction of several cancers in body such as colon, rectum, breast, pancreas, lung, prostate, stomach, and esophagus (Glade, 1999). In order to mitigate the risk of cancers induced by HAAs, using lower temperatures, shortening the time of cooking, and avoiding direct exposure to a naked flame are recommended (Cheng et al., 2006).

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds that contain two or more fused aromatic rings consisting carbon and hydrogen atoms. PAHs are formed as a consequence of incomplete combustion of fossil fuels, and since they are air pollutants, the soil and ground water can be contaminated and, therefore, they can enter into the food chain (Mottier et al., 2000; Yousefi et al., 2018; Amirdivani et al., 2019). In recent years, some investigations have been performed considering the potential inhibitory activity of probiotics toward HAAs and PAHs formed

in foods. In a study, detoxification of IQ (2-amino-3-methylimidazo [4,5-f] quinolone), MelQx (2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline), or PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine) at levels of 5−25 μg/mL in MRS (De Man, Rogosa and Sharpe) broth and modified MRS broth by *Lactobacillus casei* DN 114001 was assessed. It was found that growth and survival of the bacteria was not influenced during 24 and 168 h incubation in MRS broth and modified MRS broth, respectively. After 24 h incubation in MRS broth, IQ, and PhIP were decreased by 98%−99% while a reduction of 27% was observed in MelQx level. In modified MRS broth, a lower reduction was obtained due to lower cell density, and it was dependent on the growth phase of bacteria. A decrease of 49%−54% was obtained in IQ amount in the stationary phase of growth (after 24 h of cultivation), whereas the MelQx level was decreased 11.2% in the logarithmic (till 24 h), stationary and early death phase of growth which was an indicative of the ability of dead cells to absorb carcinogens (Nowak and Libudzisz, 2009).

Duangjitcharoen et al. (2014) studied degradation of PhIP and IQ (50 μg/mL) by three strains of *Lactobacillus (Lb.) plantarum*. A sudden binding of mutagens to the bacteria was observed at the beginning of the test. *Lb. plantarum* CM4 had the highest binding ability toward PhIP (46.32% after 24 h) and IQ (85.34% after 144 h).

Detoxifying effect of three LAB (*Bifidobacterium bifidium, Streptococcus thermophilus,* and *Lb. bulgaricus*) on 16 PAHs compounds (0.25 μg of each compound/mL media) was evaluated in MRS medium during different incubation periods (2−72 h) at 37°C. Furthermore, a yogurt by mixture of buffalo's and cow's milk inoculated with yogurt starter containing 0.02 μg/mL of each PAH was prepared and degradation of PAH compounds was also determined during 3 h. It was noted that PAH reduction was dependent on bacterial species and incubation period. During the incubation periods, the reduction percentage of PAHs was in the range of 46.6%−92.9%, 51.8%−94.9% and 77.7%−92.4% by *B. bifidium, S. thermophilus,* and *Lb. bulgaricus,* respectively. It is worth mentioning that the highest PAHs removal by *B. bifidium* and *S. thermophilus* was 92.6% and 96.0% after incubation for 10 and 12 h, respectively. However, the highest reduction by *Lb. bulgaricus* was 92.4% after 48 h incubation. At the end of incubation time (72 h), the PAHs removal ability was as follows: *Lb. bulgaricus* (91.5%), *S. thermophilus* (87.7%), and *B. bifidium* (46.6%). In yogurt samples, degradation of PAHs was not remarkable during 3 h incubation, and a reduction of 3.46% was obtained in the final product. It was mentioned that the removal of PAHs was pertained to several factors such as the type of microorganism, the interaction between microorganisms, the microbial concentration, the composition of the medium, and the microbial growth conditions of temperature and pH (Abou-Arab, 1997; Abou-Arab et al., 2010).

Yousefi et al. (2019) studied the ability of *Lb. acidophilus* LA-5, *B. lactis* BB-12, *Lb. delbrueckii* sp. *bulgaricus* PTCC 1737, and *S. thermophilus* PTCC 1738 in removing four PAHs, namely, benzo (a) pyrene (BaP), benz (a) anthracene (BaA), chrysene (Chr), and benzo (b) fluoranthene (BbF) from contaminated phosphate buffer saline (PBS). They found that all the tested strains were able to remove four PAHs; however, *Lb. acidophilus* LA-5 showed the highest ability in PAHs removing, indicating that this reduction was strain specific. They also reported that in the almost all assays, removing of PAHs was as follows: BaP > Chr > BaA > BaF, which showed toxin specific of this

phenomenon. Furthermore, they figured out that cell viability was not required for the binding ability, and even acid-treated, heat-treated, and ultrasonic-treated bacterial cells showed more binding ability and they concluded that this action was not biodegradation and might be carried out through physical phenomenon by cell wall.

Rajendran and Ohta (1998) investigated the binding ability of LAB isolated from rice and wheat *miso* to eight different HCAs (Heterocyclic Amines). An efficient binding of isolated bacteria to Trp-P-1, Trp-P-2, MeAaC, and PhIP was observed. It was expressed that except one strain, the others bounded to Trp-P-1 and Trp-P-2 more than 85%. MeAaC was bounded to bacteria more than 90% while the extent of binding in the case of Glu-P-1, IQ, and MeIQ was relatively low. The hydrophobic interactions (Van der Waals) were important factors in the binding of mutagens. More hydrophobic compounds such as AαC and DiMeIQx were bound more efficiently than IQ and PhIP. Furthermore, the tryptophan pyrolysates were more hydrophobic than the quinolines, quinoxalines, and PhIP and were removed better than other HAA compounds. For subsequent studies, two isolates were selected and distinguished as *Pediococcus acidilactici* and named as *P. acidilactici* 1 and *P. acidilactici* 2 and cell wall fractions, heat-treated cells and cytoplasmic contents of the bacteria were assessed for their binding ability to HCA compounds. Except cytoplasmic content, pure cell wall and peptidoglycan fraction in both strains showed higher binding ability compared to the bacterial cells. It was observed that heat and enzymatic treatment of the cells was not effective on the binding capacity of the bacteria. It was elucidated that binding capacity of the bacterial cell wall and cells as a whole was not influenced by the damage; therefore, extracellular substances or structures had no function in this procedure. By acetylation of HCA compounds, binding ability disappeared which was explained by the substitution of the amino group by the acetyl group and indicating the role of the amino group in the binding ability. The proposed mechanism of binding activity was the reaction of peptidoglycan with amino group of mutagen compounds.

Absorption of Trp-P-1 and MeIQx to *Lb. delbrueckii* ssp. *bulgaricus* 2038 and *S. thermophilus* 1131 in distilled water, buffer solutions, and intestine was studied. Binding percentage of *Lb. delbrueckii* ssp. *bulgaricus* 2038 to Trp-P-1 and MeIQx were 94.1% and 60.8%, respectively, and in the case *S. thermophilus* 1131, reduction of 83.2% and 32.2% were obtained. Besides, removal of mutagen compounds was pH dependent. The highest removal of *S. thermophilus* 1131 to Trp-P-1 occurred in the range of 4−8, but *Lb. delbrueckii* ssp. *bulgaricus* 2038 removed Trp-P-1 and MeIQx at pH 7. The results of HCA absorption in the small intestine of rats by loop test showed that *S. thermophilus* was more efficient in Trp-P-1 removal than *Lb. delbrueckii* ssp. *bulgaricus* 2038 due to the similarity in the pH of absorption of Trp-P-1 in the small intestine (Terahara et al., 1998). In a work, detoxification of B[a] P and sodium azide by goat probiotics (*Lb. reuteri* DDL 19, *Lb. alimentarius* DDL 48, *Enterococcus faecium* DDE 39, and *B. bifidum* DDBA) at concentrations of 1×10^6, 1×10^8, and 1×10^{11} cfu (colony forming units)/ml was reported. A mixture of probiotics was more effective (74% binding) than any individual strains at the same cell concentration. Also, the B[a] P- probiotic complex was stable after washing with dimethyl sulfoxide (Apás et al., 2014).

Some studies investigated the antigenotoxic activity of probiotics and LAB in the presence of mutagens. In a study, the effect of feeding rats with *Lb. rhamnosus*

IMC501 before PhIP administration on the probiotic abundance in feces, fecal enzymatic activity, and DNA damage in the colon and liver cells was specified. It was stated that after 5 days of probiotic administration, the number of lactobacilli increased in the feces, and activity of β-glucuronidase and β-N-acetyl-glucosaminidase (high activity in patients with colorectal cancer) decreased 63% and 26%, respectively. It was also reported that the extent of DNA damage in colon cells was significantly decreased, whereas no genotoxic effect was recognized in liver cells (Dominici et al., 2014). In consistent with this study, genotoxicity of fecal water and the activity of two enzymes (β-glucuronidase and β-glucosidase) in human feces after incubation with 50 μg/mL IQ and three probiotic strains including *Lb. casei* ŁOCK 0900, *Lb. casei* ŁOCK 0908, and *Lb. paracasei* ŁOCK0919 illustrated a reduction in β-glucuronidase and β-glucosidase activity in feces (Nowak et al., 2012). It was assumed that probiotics could adhere to colonocytes and restrict absorption of mutagens to the intestine (Zhang and Ohta, 1991) or decrease their bioavailability (Lidbeck et al., 1992).

In another study by Klewicka et al. (2012), beetroot juice was fermented with *Lactobacillus brevis* 0944 and *Lb. paracasei* 0920, and its protective effect was evaluated against aberrant crypt foci (ACF) formation in rat colon in the presence of PhIP (10 μg/day). The fermented beetroot juice decreased the number of ACF, malondialdehyde in the liver, and cytotoxic and genotoxic effects of fecal water in PhIP-treated rats. In another experiment, a mixture of three HCAs- IQ, MeIQ, and PhIP was given to male rats for a 7-week period with a cumulative dose of 250 mg of the HCA per kg body weight. The effect of four different diets including supplemented with 20% water, 30% nonfermented milk, 30% fermented milk with *Bifidobacterium animalis* DN-173010, and 30% fermented milk with *S. thermophilus* DN-001 on ACF induction was determined. It was noted that consumption of milk, particularly fermented milk, significantly reduced the number of ACFs in rats. The inhibition degree was 66% in milk-supplemented diet, 96% in milk fermented with *B. animalis,* and 93% in milk fermented with *S. thermophilus* (Tavan et al., 2002). Zsivkovits et al. (2003) examined the effect of four *Lactobacillus* strains including of *Lb. bulgaricus* 291, *S. thermophilus* F4, *S. thermophilus* V3 and *Bifidobacterium longum* BB536 on DNA damage induced by HCAs which were generally found in fried beef (beef mix), and chicken mix in the liver and colon of female rats were examined. It was indicated that all the strains prevented damage caused by beef mix after giving of 1×10^{10} cells/animal, while in the case of chicken mix, the effect was not significant. It was also found out that the impact was considerable at 1×10^{7} cells/animal and even when was given 12 h before beef mix. Hence, consumption of probiotic dairy products several hours before cooked and fried meats would be beneficial considering the reduction of DNA damage.

4.2.1.2 Acrylamide

The International Agency for Research on Cancer has classified acrylamide as a probable human carcinogen following detection of this compound in different heat-treated carbohydrate-rich foods such as potato chips and crisps, coffee and bread by Swedish Food Administration (IARC, 1994). Acrylamide is an electrophile molecule which can react with nucleophilic groups such as amines and carboxylates, those are commonly

found in biological molecules such as DNA (Semla et al., 2017). Some strategies proposed for alleviation of acrylamide in food products include: reduction of precursors in raw materials (Mestdagh et al., 2008; Pedreschi et al., 2008), adjusting the process parameters such as temperature and pH as well as adding amino acid and salts (Lindsay and Jang, 2005; Ciesarová et al., 2006), postprocessing approaches like evaporation and polymerization (Banchero et al., 2013; Friedman and Levin, 2008), and recently, application of specific strains of LAB.

The ability of 14 strains of LAB to metabolize acrylamide (5 and 10 μg/mL) in vitro after 0, 4, and 12 h incubation at 37°C in different pH (3, 5, and 8) was investigated. It was noted that the degree of binding ability to acrylamide was dependent on pH, acrylamide concentration, and type of strain. Binding to acrylamide varied with respect to incubation time. It was proposed that binding was a rapid process and occurred passively on the bacterial surface (Serrano-Niño et al., 2014). Zhang and Ohta (1991) reported higher binding of pyrolyzed mutagen at pH 6-7 in consistent with the study performed by Hernandez-Mendoza et al. (2009) that implied *Lb. reuteri* NRRL 14171 and *Lb. casei* Shirota bound more effectively at pH 7 than pH 8. It was announced that the probable mechanism of pH influence on binding ability was due to competition between toxic compounds and protons to attach to the negatively charged binding sites (Huang et al., 1991). In another study by Serrano-Niño et al. (2015), the interaction of acrylamide and aflatoxin B_1 with teichoic acids (TAs) in the cell wall of 14 LAB strains was studied. It was suggested that there was a relation between components of TA and percentage of bound acrylamide. Lower levels of glucose, D-alanine, or TA caused a higher degree of binding of acrylamide to the cell wall of bacteria. H-bonds might develop between carbonyl oxygen and the amino group between adjacent acrylamide and D-Alanine directly attached to position D-4 (L-2) of ribitol. Moreover, the amine group of D-alanine might react with acrylamide units by means of a Michael addition reaction (Zamora et al., 2010). Also, hydrogen bonds might occur between carbonyl (C=O) oxygen of both AFB_1 and acrylamide, and the hydroxyl groups of either glucose residues or glycerol phosphate substituent attached to the poly (ribitol phosphate) chain. Accordingly, Zhang et al. (2017) pointed out that peptidoglycan from *Lb. plantarum* 1.0065 had the highest ability to bind acrylamide, and a positive relation between carbohydrate and alanine content of peptidoglycan and acrylamide binding percentage was observed. Moreover, the C−O (carboxyl, polysaccharides, and arene), C=O amide, and N−H amines groups of PGN were involved in AA binding.

4.2.1.3 Nitrosamine

N-nitroso compounds (NOCs) can cause tumor growth in humans, and their occurrence in food is a worrying issue. IARC has classified a number of nitrosamines as probably (Group 2A) or possibly (Group 2B) carcinogenic to humans (IARC, 1978). They are formed through interaction of secondary or tertiary amines with nitrosating substances, and their presence in food is a result of different processes during production, storage, cooking, and in some circumstances through migration from packaging materials (Sen et al., 1993). Various methods can be used to mitigate nitrosamine formation in foods including reduction of nitrite level in curing salt, utilization

of nitrite substituents, using ascorbic acid as an inhibitor agent, utilization of lower temperatures, and indirect heating (Habermeyer et al., 2009). There are also few studies that investigated the binding ability of probiotics or LAB to these compounds.

In a study by Grill et al. (1995), the effect of NDMA (N-nitroso-dimethylamine), NPIP (N-nitroso-piperidine), and NPYR (N-nitroso-pyrrolidine) on growth of six bifidobacteria strains (*Bifidobacterium breve* ATCC 15698, *B. infantis* ATCC 25962, *B. longum* ATCC 15707, *B. longum* ATCC 15708, *B. longum* BB536, and *B. animalis* ATCC 25,527) during 24 h in TYP medium was studied. It was implied that nitrosamines (2−200 μg/mL) had no influence on the growth of bifidobacteria, and only *B. longum* BB536 was able to degrade nitrosamines. At the level of 2 μg/mL, 20%, 16%, and 10% degradation for NPYR, NDMA, and NPIP were detected, respectively. At 20 μg/mL, 0.5%−1% decrease, and in the case of 200 μg/mL, no antimutagenic activity was observed. The inhibitory effect of bifidobacteria was ascribed to an intracellular enzymatic activity.

Nowak et al. (2012) evaluated the ability of five probiotic *Lactobacillus* strains (*Lb. rhamnosus* LOCK 0900, *Lb. rhamnosus* LOCK 0908, *Lb. casei* LOCK0919, *Lb. casei* DN114001, and *Lb. brevis* 0945) to bind and degrade NDMA under different culture conditions (24 h in MRS, 168 h in modified MRS N, and 168 h in phosphate buffer). It was observed that all strains were capable of NDMA reduction in MRS from 2 μg/mL to 0.40−0.92 μg/mL after 24 h whereas at the level of 20 μg/mL, NDMA was decreased to 6 μg/mL by only two strains including *Lb. rhamnosus* 0908 and *Lb. casei* DN 114001, and at the highest level (100 μg/mL), no binding was observed. In MRS N medium after 168 h, NDMA was weakly bound to bacteria, and it was dependent on growth phase and strain of bacteria. During logarithmic phase, NDMA was reduced to 0.3−0.8 μg/mL that was increased in stationary phase to the initial level of 10 μg/mL, and again in death phase, 0.6−0.9 μg/mL reduction was obtained in the case of *Lb. rhamnosus* 0900, *Lb. casei* DN 114001, and *Lb. brevis* 0945 while for *Lb. rhamnosus* 0908 and *Lb. casei* 0919, NDMA level remained unchanged. In phosphate buffer, *Lb. rhamnosus* 0900 decreased NDMA concentration from 2 to 1.45 μg/mL. At the concentration of 20 μg/mL, three strains had the capability of NDMA reduction, but this ability was not observed at the level of 100 μg/mL. The lower degree of NDMA reduction in MRS N compared to MRS was explained by the lower counts of bacteria and higher pH level. It was also announced that the most efficient strain in lowering the concentration and genotoxicity of NDMA was *Lb. brevis* 0945.

In a study of Duangjitcharoen et al. (2014), three strains of *Lb. plantarum* were studied in respect of their ability to degrade two nitroso compounds, including diphenylnitrosamine (DPN) and 1-nitrosopyrrolidine (NPR). Among the tested bacteria, *Lb. plantarum* CM4 had the highest binding ability of DPN (1−100 μg/mL) in a dose-response manner, and the highest degradation activity was observed at the concentration of 100 μg/mL that yielded 11.10 μ/mol nitrite/mL during 20 h of incubation time.

4.2.2 Pesticides residues

Pesticides play an important role in agriculture and food production by controlling the insects, weeds, plant pathogens, and microbial contaminations (Trinder et al., 2015).

Although pesticides are useful substances in some aspects, they can pose health risks to animals and humans. Pesticides often enter the gastrointestinal tract following oral ingestion of contaminated food or water (Damalas and Eleftherohorinos, 2011). It is well-established that bacteria can breakdown pesticides in environmentally contaminated soil and water in a process known as bioremediation (Yuan et al., 2011; Barman et al., 2014), but studies related to degradation activity in food are still scarce, and organophosphate pesticides are the most investigated ones in the literature. Organophosphate pesticides degradation occurs through the enzyme phosphatase produced by LAB. The enzyme converts organophosphate pesticides to dialkyl phosphate and aryl alcohol in the presence of water.

In a study by Zhang et al. (2014), skimmed milk was contaminated with five organophosphate pesticides (diazinon, methyl parathion, chlorpyrifos, fenitrothion, and malathion), and then the milk was fermented with 10 isolated LAB (*Lb. plantarum* 1.0317, *Lb. plantarum* 1.0624, *Lb. plantarum* 1.0315, *Lb. plantarum* 1.066, *Lb. brevis* 1.0209, *Lb. helveticus* 1.0203, *Lb. helveticus* 1.9204, *Lb. lactis* 4.0611, *Lb. bulgaricus* L6, and *S. thermophilus* 3.0503) and four strain combinations. The isolated and combined species showed different capacities regarding the degradation of pesticides. Among the 10 species investigated, *Lb. brevis* 1.0209 displayed the greatest acceleration of pesticide degradation, resulting in the increase of 225.4%. The authors declared that degradation of pesticides in milk would increase by increasing the phosphatase level. Zhao and Wang (2012) added seven organophosphate pesticide to milk samples and inoculated each with a strain of *Lactobacillus* spp., including *Lb. bulgaricus*, *Lb. paracasei,* and *Lb. plantarum*. A decreasing trend was observed in the level of pesticides due to degradation by bacteria. After 24 h incubation, the reduction level was in the range of 20.9% (methyl parathion, incubated with *Lb. paracasei*) to 46.9% (malathion, incubated with *Lb. plantarum*). The greatest degradation activity was observed in *Lb. bulgaricus* and *Lb. plantarum*. This was in accordance with the study of Bo et al. (2011), in which seven organophosphate pesticides (1.5 mg/kg) were added to bovine milk, and the milk was used to produce yogurt. The results indicated that degradation of pesticides was increased by one or both of the starter cultures, except in the case of malathion.

Degradation of two organophosphate pesticides (chlorpyrifos and phorate at 0.36 mg/kg) added to corn by *Lb. plantarum* 1.0315, *Lb. plantarum* 1.0624, *Lb. plantarum* 1.0622, and their combination at ambient temperature during 10 weeks was studied. It was indicated that the range of reduction level of chlorpyrifos and phorate in the treated samples was from 24.9 (phorate, incubated by *Lb. plantarum* 1.0624) to 33.4% (phorate, incubated by *Lb. plantarum* 1.0622 and *Lb. plantarum* 1.0315). The strain combination was more effective in organophosphate pesticides dissipation than single strain (Zhang et al., 2016).

In a work by Uygun et al. (2008), the dissipation of four organophosphate pesticides in wheat samples stored at ambient temperature for 5 month was investigated. During the storage period, the dissipation levels of insecticides in wheat were 76% (methyl and pirimiphos), 84% (chlorpyrifos methyl), 86% (fenitrothion), and 86% (malathion). In a work by Abou-Arab (1997), the degradation level of total dichlorodiphenyl-trichloroethane was 10.8%, 11.8%, and 4.8% by *Streptococci*, *Lactobacilli,* and yeasts isolated from Ras cheese, respectively, during 10 days incubation. Table 4.1 represents detoxification of process-induced toxicants and pesticides by various bacteria in different conditions.

Table 4.1 Binding of various process-induced food toxicants and pesticide residues to different microorganisms.

Microorganism type	Type of toxicant	Conditions	Consequence	Reference
Lactobacillus casei Shirota *Lb. reuteri* northern regional research laboratory 14171 *Lb. johnsonii* (ATCC) 3200 *Lb. acidophilus* ATCC 4796 *Lb. fermentum* ATCC 11976 *Lb. rhamnosus* ATCC 13075 *Lb. helveticus* ATCC 27558 *Lb. casei* ATCC 334 (L334) *Lb. casei* L9 (L9) *Lb. casei* L30 (L30) *Lb. casei* 12A (12A) *Lb. casei* 21/1 (L21/1) *Lb. casei* 7R1 *Lb. casei* (DPC) 3968)	Acrylamide (5 and 10 μg/mL)	0, 4, and 12 h incubation at 37°C at pH 3, 5, and 8.	11.89%−29.13% binding was observed at 0 h. After 4 h, 29.13% binding was obtained and after 12 h, the maximum binding was 24.95%. By enhancement of acrylamide concentration from 5 to 10 μg/mL, the binding ability of strains was decreased considerably, and in this case, LR showed the highest binding ability among the examined strains. The maximum binding at pH 8 was observed in the strain L334, while at pH 3, a substantial reduction in binding ability occurred.	Serrano-Niño et al. (2014)
Bifidobacterium breve ATCC 15698 *B. infantis* ATCC 25962 *B. longum* ATCC 15707 *B. longum* ATCC 15708 *B. longum* BB536 *B. animalis* ATCC 25527	NDMA, NPIP and NPYR (2−200 μg/mL)	24 h incubation in TYP medium.	At the level of 2 μg/mL, 20%, 16%, and 10% degradation for NPYR, NDMA, and NPIP were detected, respectively. At 20 μg/mL, 0.5%−1% decrease and in the case of 200 μg/mL, no antimutagenic activity was observed.	Grill et al. (1995)

Continued

Table 4.1 Binding of various process-induced food toxicants and pesticide residues to different microorganisms.—cont'd

Microorganism type	Type of toxicant	Conditions	Consequence	Reference
Lb. casei DN 114001	IQ, MelQx or PhIP (5−25 μg/mL)	Incubation for 24 h in MRS broth and 168 h in modified MRS broth.	98%−99% decrease in IQ and PhIP and 27% in MelQx in MRS broth.	Nowak et al. (2012)
Lb. reuteri DDL 19, *Lb. alimentarius* DDL 48, *Enterococcus faecium* DDE 39, and *B. bifidum* DDBA	B[a] P (5 and 10 μg/mL)	Incubation at 37°C for 2 h.	74% binding was observed by a mixture of probiotics.	Apás et al. (2014)
Lactobacillus plantarum 1.0317, *Lb. plantarum* 1.0624, *Lb. plantarum* 1.0315, *Lb. plantarum* 1.066, *Lb. brevis* 1.0209, *Lb. helveticus* 1.0203, *Lb. helveticus* 1.9204, *Lactobacillus lactis* 4.0611, *Lb. bulgaricus* L6, and *Staphylococcus thermophilus* 3.0503	Diazinon (0.6 mg/kg) methyl parathion, chlorpyrifos, fenitrothion, and malathion (1.2 mg/kg)	Incubation at 42°C for 24 h in skimmed milk.	*Lb. brevis* 1.0209 displayed the greatest acceleration of pesticide degradation, resulting in the increase of 225.4%.	Zhang et al. (2014)
Lb. bulgaricus, *Lb. paracasei,* and *Lb. plantarum*	Monocrotophos and phorate (0.5 mg/kg) dimethoate, fenthion, malathion, methyl parathion, and trichlorphon (1.2 mg/kg)	Incubation at 42°C for 24 h in skimmed milk culture.	The reduction level was in the range of 20.9% (methyl parathion, incubated with *Lb. paracasei*) to 46.9% (malathion, incubated with *Lb. plantarum*). The greatest degradation activity was observed in *Lb. bulgaricus* and *Lb. plantarum*.	Zhao and Wang (2012)

Commercial directed vat set (DVS)	Monocrotophos, phorate dimethoate, fenthion, malathion, methyl parathion, and trichlorphon (1.5 mg/kg)	Incubation at 42°C for 8 h during yogurt processing.	Degradation of pesticides was increased by one or both of the starter cultures, except in the case of malathion. The decrease in the level of seven pesticides in yogurt samples with fermentation time of 4 h would range from 9.2 (phorate) to 17.1% (dimethoate).	Bo et al.(2011)
Lb. plantarum 1.0315, *Lb. plantarum* 1.0624, *Lb. plantarum* 1.0622, and their combination	Chlorpyrifos and phorate at 0.36 mg/kg	Whole corn silage was inoculated with 1 × 106 cfu/g and fermented at 20°C for 10 weeks.	24.9% dissipation for phorate incubated by *Lb. plantarum* 1.0624. 33.4% for phorate incubated by combination of *Lb. plantarum* 1.0622 and *Lb. plantarum* 1.0315	Zhang et al. (2016)

4.2.3 Mycotoxins

Various types of fungi and yeasts can grow in different foodstuffs resulting in food spoilage and significant economic losses. Moreover, mycotoxins as secondary metabolites are also produced by fungi during food spoilage and safety of the foodstuffs could be endangered by these toxic substances (Blagojev et al., 2012). Generally, aflatoxins, fumonisins, ochratoxins, patulin (PAT), trichothecenes, and zearalenone (ZEA) can be named as the most important classes of mycotoxins that might exist in various foodstuffs (Dalié et al., 2010; Ahlberg et al., 2015). Due to their carcinogenic, teratogenic, mutagenic, hepatotoxic, and immunotoxic properties of mycotoxin, it seems that there is a health risk and an increasing concern about the presence of these compounds in foods (Hussein and Brasel, 2001; Dalié et al., 2010). Therefore, different strategies have been proposed to reduce the amount of these toxins. Besides of preharvest, harvest and postharvest management which are generally suggested as the best strategy in prevention of mold growth and toxin production in raw materials and plants, utilizing chemical preservatives and decontamination/detoxification of contaminated products could be useful in the cases where mycotoxin contamination is inevitable in raw materials and foodstuff (Shetty and Jespersen, 2006; Blagojev et al., 2012).

On the other hand, consumer awareness and concerns about excess usage of chemical preservatives to control fungal growth have encouraged food industry and researchers to develop and substitute safer strategy in detoxification of mycotoxin in foods (Blagojev et al., 2012; Hassan et al., 2016). Among these, attentions have been drawn to utilize microorganisms in prevention/retarding fungal growth or removing mycotoxins (Magnusson et al., 2003; Cicoňová et al., 2010; Hassan et al., 2016). Lactic acid bacteria, probiotics, and yeasts are the most important microorganisms that potentially could be used for decontamination of mycotoxins (Blagojev et al., 2012; Zoghi et al., 2014; Ahlberg et al., 2015; Hassan et al., 2016). Due to the production of a wide spectrum of antimicrobial compounds (e.g., low-molecular weight metabolites, hydrogen peroxide, antifungal compounds, and bacteriocins), LAB can be considered as an efficient alternative to physical and chemical methods (Cicoňová et al., 2010; Khorshidian et al., 2016). Apart from the production of antifungal compounds produced by LAB, toxin compounds can bind to LAB and probiotics cell wall through physical interactions (Shetty and Jespersen, 2006; Niderkorn et al., 2009; Armando et al., 2012). Most of the studies that investigated cell wall ability to bind mycotoxins were related to different strains of LAB species, while *Saccharomyces cerevisiae* and *Candida krusei* are the yeast species that studies focus more on them (Shetty and Jespersen, 2006; Guo et al., 2012). Although the binding mechanisms of mycotoxin to microorganism are not clarified yet, the binding ability of nonviable cells (heat or acid-treated) indicates that this removal is a physical phenomenon established through noncovalent interactions such as dipole−dipole interactions or Van der Waals forces (El-Nezami et al., 1998; Haskard et al., 2000). In this sense, bacterial cell wall plays a substantial role, and the main components responsible are polysaccharides, peptidoglycan layer, and TAs (Hernandez-Mendoza et al., 2009; Oatley et al., 2000; Haskard et al., 2001; Lahtinen et al., 2004).

4.2.3.1 Aflatoxins

As mentioned before, mycotoxins have adverse acute or chronic effects on human health. Aflatoxins (AFs) are a type of mycotoxin that are produced mainly by *Aspergillus flavus* and *A. parasiticus*, but other strains such as *A. nomius*, *A. tamari,* and *A. pseudotamarii* might also produce AFs (Alberts et al., 2006; Quadri et al., 2013; Adebo et al., 2017). Among 18 different types of AFs, aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 are the most prevalent and important toxins in terms of the contamination of agricultural products and public health (Murphy et al., 2006; Dors et al., 2011). There are various strategies to reduce aflatoxin among which biological approaches recently have gained more attention. Therefore, many researches have been carried out to investigate the ability of different microorganisms especially LAB to decrease toxins in various media and food matrices.

Most studies utilized LAB, probiotics, and yeasts in various media and some foodstuffs to evaluate their ability to remove aflatoxin, and in all the cases, a reduction was occurred in aflatoxin content (Line and Brackett, 1995; El-Nezami et al. 1998, 2000; Sarimehmetoğlu and Küplülü, 2004; Pierides et al., 2000; Kabak and Var, 2008). In a study by Sarlak et al. (2017), the impact of *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. casei,* and *B. lactis* in reduction of 0.5 ppb of free aflatoxin M_1 (AFM_1) in Doogh (a traditional Iranian fermented milk drink) was studied, and it was shown that *Lb. acidophilus* had the highest ability of removing AFM_1. Mohammadi Sani et al. (2014) studied detoxifying ability of kefir starter (2%, 4%, 6%, 8%, and 10%) and *Lb. casei* (0.1%, 0.3%, 0.5%, 0.7% and 0.9%) in kefir prepared from milk spiked with 500 pg AFM_1. Although there was a reduction in all the treatments, the sample containing 6% kefir starter (88.17%) had the highest ability in toxin reduction (88.17%). Furthermore, 0.9% *Lb. casei* in combination with kefir starter (4%) showed the highest binding (82.12%). Binding ability of *Lb. acidophilus* (2%) and *B. lactis* (2%) individually or in combination to AFM_1 (50.2 ng/kg) in ultra-high temperature (UHT) skim milk was investigated during 3 days of incubation at 37°C. It was noted that the combination of bacteria resulted in the highest reduction (96.2%) after 2 days, and no AFM_1 was detected in the third day (El-kest et al., 2015). Elsanhoty et al. (2014) investigated yogurt starter alone or in combination with *Lb. plantarum* or *Lb. acidophilus* in preparation of yogurt samples from milk spiked with 50 μg/L AFM_1. They figured out that the highest reduction (87.7%) was related to the sample prepared by yogurt starter culture and *Lb. plantarum* at the end of storage (7 days). In a study by Sevim et al. (2019), binding ability of three LAB strains (*Lb. plantarum*, *B. animalis,* and *B. bifidum*) to AFM_1 along with inulin supplementation (4%) from contaminated yogurt either alone or in binary combinations for one and 10 days of storage was investigated. It was observed that yogurt starter + *B. bifidum-B. animalis* (60.8%) and yogurt starter + *Lb. plantarum-B. bifidum* mixtures (55.1%) after 1 day and 10 days cold storage periods, respectively, had the best AFM_1 binding. Addition of inulin increased the binding capacity of yogurt starter + *B. bifidum-B. animalis*, yogurt starter + *Lb. plantarum-B. bifidum*, yogurt starter + *Lb. plantarum-B. animalis* at the end of the 10-day storage period by inulin addition.

Unlike LAB and probiotics, less number of studies have been done on the use of yeast in reducing aflatoxin, and there was almost a decrease in most studies in which yeasts were used (Rahaie et al., 2012; Corassin et al., 2013; Karazhiyan et al., 2016). The binding capacity of viable, heat- and acid-treated *S. cerevisiae* to AFB_1 was determined in yeast-mold broth medium during incubation of 4, 12, and 24 h at 25°C. The result showed that the most decrease occurred after 4 h of incubation and further incubation time had no impact on binding efficiency, and the highest binding ability was related to acid-treated cells (Sahebghalam et al., 2013). Similarly, Karazhiyan et al. (2016) found that in the yogurt contaminated with various levels of AFM_1 (100, 500, and 750 pg/mL), highest adsorption level of AFM_1 to *S. cerevisiae* was obtained by acid-treated yeast (76.46%) followed by heat- (76.39%) and ultrasound-(75.99%) treatments, and viable yeast (74.2%). Although no specific mechanism has been identified in the reduction of aflatoxin, it seems that binding of toxin to cell wall is the most likely mechanism. Therefore, medium type, strain type, toxin concentration, chemical structure of toxin, pH, and ion strength of media can be effective in toxin removal (El-Nezami et al., 1998; Haskard et al., 2000). Furthermore, it has been reported that toxin reduction is highly strain-specific and dependent on microbial concentration (Kabak et al., 2009; Sarlak et al., 2017). Line and Brackett. (1995), El-Nezami et al. (1998), and Kabak and Var (2008) mentioned 1×10^9, 2×10^9, and 10^8 cfu/mL cell population for considerable removal of aflatoxin. Additionally, it was indicated that heat and especially acid-treated cells were more effective in reduction of toxin in comparison with the viable cells (Rahaie et al., 2012; Sahebghalam et al., 2013; Karazhiyan et al., 2016). It seems that releasing monomers from polysaccharides, decomposing the glycosidic linkages in monomers, protein denaturation, and Maillard reaction as a consequence of acid and heating condition change cell wall structure and provide new binding sites (Zlotnik et al., 1984; Bejaoul et al., 2004; Shetty et al., 2007; Rahaie et al., 2012).

4.2.3.2 Fumonisins

Fumonisins are a group of mycotoxin secreted by *Fusarium verticillioides* and *F. proliferatum,* which have been found in corn and corn-based products (Niderkorn et al., 2009; Al-Masri et al., 2011). Different health implications are ascribed to fumonisins in human and animals such as oesophageal and liver cancers, neural tube defects, and cardiovascular problems in humans (Marasas, 1995). Eighteen types of fumonisins have been recognized among which fumonisin B_1 (FB_1) which is produced by *F. moniliforme* has the highest toxicity (Seo et al., 2001; Niderkorn et al., 2009). There are only few studies that investigated the impact of microorganisms on fumonisin reduction. Mokoena et al. (2005) studied the impact of LAB fermentation on mycotoxin concentration in maize meal products. The results of this study showed that in the maize meal in which fumonisin B_1 was spiked, there was a decreasing manner in fumonisin B_1 during 4 days fermentation and finally it was not detectable in fermented products. Niderkorn et al. (2006) studied in vitro interactions between LAB and fumonisins, and they understood that most of the strains were able to reduce the toxin in acidified MRS broth (pH4.0) and LAB showed higher efficiency compared to *Propionibacterium* strains. Furthermore, they found that FB_2 was effectively decreased by

bacteria than FB_1 due to the presence of tricarballylic acid chain in FB_1 that was less available to interact with bacterial peptidoglycans. The toxin binding ability of the cells which were killed using different physical and chemical treatments was increased. Similarly, Niderkorn et al. (2009) found that physical treatment of the bacteria such as heating, freezing, and thawing resulted in an increase in reducing FB_1 and FB_2 concentration, while enzymatic treatment due to degrading peptidoglycan decreased toxin removal. The in vivo studies which were carried out by Al-Masri et al. (2011) and Khalil et al. (2015) indicated that probiotic bacteria and yeast administration had protective effect against fumonisin B_1 and could be considered as a confirmation for in vitro studies (Al-Masri et al., 2011; Khalil et al., 2015).

4.2.3.3 Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* ssp. (*A Aspergillus, A. ochraceus, A. melleus, A. sulphureus, A. nigri, A. carbonarius,* and *A. awamori*) and *Penicillium* spp. (*P. verrucosum, P. crysogenum*, and *P. nordicum*) has nephrotoxic, teratogenic, immunogenic, hepatotoxic, and carcinogenic activities (Bayman and Baker, 2006; Biancardi et al., 2013; Becker-Algeri et al., 2016). Similar to other toxin, LAB and yeast can remove and bind ochratoxin to cell wall. Böhm et al. (2000) observed that various strain of LAB and *S. cerevisiae* could significantly reduce OTA in broth medium. Similarly, Piotrowska and Zakowska (2005) found that among 29 strains of LAB that were able to remove OTA in liquid medium, *Lb. acidophilus* CH-5, *Lb. rhamnosus* GG, *Lb. plantarum* BS, *Lb. brevis,* and *Lb. sanfranciscensis* showed the highest decrease. Also, Luz et al. (2018) reported that *Lb. rhamnosus* CECT 287T (97%) and *Lb. plantarum* CECT 749 (95%) showed the highest binding capacity to OTA. In another study by Taroub et al. (2018), LAB from two grape varieties were isolated and their antifungal activity, probiotic properties and reduction of OTA in liquid medium had been evaluated. The results revealed that the strains with good antifungal activity against *Aspergillus* species were recognized as *Pediococcus pentosaceus* and *Lb. plantarum*. *P. pentosaceus* showed promising potential probiotic characteristics and had a high ability for OTA (10 ng/mL) removal after 48 h of incubation in both MRS and PBS media, and the OTA reduction was significantly higher in MRS (84%) than in PBS media (25%). Piotrowska (2014) stated that OTA removing was strain specific and thermally killed cells showed higher ability in removing OTA. Furthermore, it was reported that LAB could prevent fungal growth by production of antifungal component that inhibited OAT production (Fuchs et al., 2008; Belkacem-Hanfi et al., 2014; Essia Ngang et al., 2015; Fossi et al., 2016).

4.2.3.4 Patulin

Patulin is an unsaturated heterocyclic lactone which is produced by several species of *Penicillum*, *Aspergillus,* and *Byssochlamys* genera (Sewram et al., 2000; Hatab et al., 2012a). Like other mycotoxins, PAT has several human health implications such as carcinogenic, mutagenic, immunotoxic, and neurotoxic activities (Hopkins, 1993; Mahfoud et al., 2002; Sant'Ana et al., 2008). Occurrence of this mycotoxin in fruit juices

especially apple juice is high, and different approaches including washing, clarification, filtration, chemical addition, and ionizing radiation have been applied to eliminate or reduce PAT content (Acar et al., 1998; Yazici and Velioglu, 2002; Moake et al., 2005). Recently, using LAB is considered as an efficient, safe, and reliable method to detoxify PAT. In almost all studies, it has been concluded that removing PAT by LAB was considerably affected by strain type, concentration of toxins, cell density, incubation temperature, and physical treatment of cells especially heat treatment (Fuchs et al., 2008; Hatab et al. 2012a, 2012b; Guo et al., 2012; Wang et al., 2015). It is reported that by decreasing pH and increasing incubation temperature, efficiency of toxin removing increased (Hatab et al., 2012a). The Fourier Transform Infrared (FTIR) results reported by Hatab et al. (2012b) indicated that cell wall has important role in PAT adsorption. Moreover, Wang et al. (2015) stated that COOH and/or NH are the functional groups that participated in PAT adsorption indicating that polysaccharides and/or proteins were major functional component in binding PAT.

4.2.3.5 Other mycotoxins

Zearalenone, Deoxynivalenol (DON), and T-2 toxin are the other mycotoxins that some studies have carried out to determine their interaction with LAB and yeasts. Zearalenone is an estrogenic mycotoxin produced by different *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense*, *F. roseum,* and *F. semitectum* (Zinedine et al., 2007). Deoxynivalenol is a kind of trichothecene toxin produced by *Fusarium* species predominantly *F. graminearum* and *F. culmorum* (Bottalico and Perrone, 2002; Korosteleva et al., 2009). T-2 toxin is a non-macrocyclic trichothecenes mycotoxin produced primarily by *F. sporotrichioides* (Foroud and Eudes, 2009). The results of a few studies which focused on ZEA, DON, and T-2 toxin indicated that there were reductions in toxin content or fungal growth (Böhm et al., 2000; Meca et al., 2012; Armando et al., 2012; Oliveira et al., 2015). For example, Oliveira et al. (2015) studied the antifungal potential of LAB cell-free supernatant against *F. culmorum* growth and figured out that cell-free supernatants of *Lb. reuteri* R29 resulted in a 23% and 83% decrease in fungal growth and DON production, respectively. Zou et al. (2012) reported that among 5 strains of LAB, *Lb. plantarum* 102 showed the highest ability in reduction of DON and T-2 toxin in MRS broth during 72 h incubation at 37°C. Sangsila et al. (2016) studied the ability of eight strains of *Lb. pentosus* strains in removing ZEA from sodium acetate buffer solution, and the highest adsorption capability was obtained by strain JM0812 (83.17%) in the solution containing 74.70 μg/mL ZEA. Similarly, Armando et al. (2012) reported that various strains of *S.cerevisae* were able to remove not only ZEA but also OAT in PBS. Zhao et al. (2015) investigated in vitro detoxification of ZEA (100 μg/L) by 27 strains of *Lb. plantarum* isolated from traditional Chinese fermented foods. They found that the reduction of ZEA varied from 1.72% to 47.8%. Their results revealed that compared to the other strains, the strains Lp22, Lp39, and Lp4 had the highest ability and nearly 47.80%, 38.06%, and 39.50% of ZEA had been removed after 48 h of incubation, respectively. They found that apart from strain type, the removal of ZEA was significantly affected by bacterial cell concentration and incubation time.

4.2.4 Heavy metals

Heavy metals which may appear in different inorganic and organic forms are metallic elements with density of over 5 g/cm^3. Among these, Fe, Cu, and Zn considered as essential trace elements, while Cd and Pb are toxic even in very small quantities. Generally, Cd, Pb, Hg, and arsenic are the most toxic heavy metals (Zoghi et al., 2014). Heavy metals enter into water and food chain from fertilizers, sewage, and metal mines (Florea et al., 2005; Teemu et al., 2008). Besides anthropogenic sources, natural processes participate in contamination of water with heavy metals especially with arsenic (Singh and Sarma, 2010; Zoghi et al., 2014). Flocculation, precipitation, ion exchange, and membrane filtration are utilized to remove heavy metals from drinking water (Halttunen et al., 2007). However, in some cases, due to the inefficiency and expensive equipment and monitoring system requirements, new approaches such as using biomass have been developed to remove heavy metals (Zouboulis et al., 2004; Zoghi et al., 2014). Table 4.2 represents some studies related to the ability of microorganisms in binding heavy metals in different conditions.

Approximately all of the studies that used microorganisms in removing heavy metals indicated that reduction of heavy metals principally is carried out by binding to cell wall through ion exchange, adsorption, and chelation mechanisms (Halttunen et al. 2007, 2008; Teemu et al., 2008; Singh and Sarma, 2010). The result of transmission electron micrographs (TEM) of lyophilized *B. longum* 46 and *Lb. fermentum* ME3 which was reported by Teemu et al. (2008) confirmed reduction of heavy metals by binding at the surface of the bacteria. It seems that carboxyl and phosphoryl groups had key role in binding of heavy metals, and neutralizing the negative charge of carboxyl and phosphoryl groups by chemical modification resulted a decrease in *Lb. fermentum* ME3 and *B. longum* 46 ability to bind Cd and Pb (Halttunen et al., 2007; Zoghi et al., 2014). Similarly, Beveridge and Murray (1980) observed that cation uptake by isolated *Bacillus subtilis* cell walls decreased when phosphodiester and carboxyl groups reduced. Like other toxins, heating treatment augments the ability of bacteria to remove cadmium increased available binding site (Göksungur et al., 2005).

4.3 Conclusion

Detoxification of different toxicants present in food products by microorganisms have demonstrated that LAB, probiotics, and yeasts can decline mutagenicity and genotoxicity of these toxicants remarkably by physical binding or enzymatic degrading mechanisms. The efficacy of this protective activity depends on several factors such as strain type, medium type, incubation time, pH, growth phase, chemical structure of mutagen, mutagen concentration, and probably existence of different binding sites on the cell wall of bacteria or yeast. Thereby, considering the findings in various studies, this approach opens up a new prospect of reducing the bioavailability of food toxins. However, most of these studies have been carried out in vitro, and practical application of the approach needs further in vivo and clinical trials to support the obtained results and specify the real effects of microorganisms in human lumen and elucidate the underlying mechanisms.

Table 4.2 Binding of various mycotoxins and heavy metals to different LAB, probiotics, and yeast.

Microorganism type	Type of toxicant	Conditions	Removal (%)	Reference
Lactobacillus acidophilus NRRL B-4495	AFM_1	Incubation at 37°C for 3 h in PBS containing 10 ng/mL AFM_1.	22.75	Serrano-Niño et al. (2013)
Lb. reuteri NRRL B-14171	AFM_1		26.5	
Lb. rhamnosus NRRL B-442	AFM_1		24.54	
Lb. johnsonii NRRL B-2178	AFM_1		32.2	
Bifidobacterium bifidum NRRL B-41410	AFM_1		45.17	
Lb. delbrueckii ssp. *bulgaricus* CH-2	AFM_1	10 ng/mL AFM_1 and 4 h incubation in PBS and milk at 37 and 42°C, respectively.	18.7 in PBS 27.6 in milk	Sarimehmetoğlu and Küplülü (2004)
Streptococcus thermophilus ST-36	AFM_1		29.4 in PBS 39.2 in milk	
Lb. acidophilus	AFM_1	Incubation of contaminated UHT skim milk (50.2 ng/g) at 37°C during 3 days.	30.9 and 78.3 in first and second day, respectively.	El-kest et al. (2015)
B. lactis	AFM_1		54.8 (first day) 91.6 (second day)	
Lb. acidophilus + *B. lactis*	AFM_1		62.5 (first day) 96.2 (second day)	

Kefir starter (2%, 4%, 6%, 8% and 10%) and *Lb. casei* (0.1%, 0.3%, 0.5%, 0.7% and 0.9%)	AFM_1	Kefir prepared from milk spiked with 500 pg AFM_1 (48 h incubation).	81.83%−88.17% reduction for Kefir starter and 69.19−81.98 reduction for *Lb. casei.*	Mohammadi Sani et al. (2014)
Lb. acidophilus, *Lb. brevis,* *Lb. casei,* *Lb. delbruekii,* *Lb. plantarum*	AFB_1	Maize grain with 50, 100, 200, and 500 ng/g aflatoxin B_1 were incubated at 37°C.	The least reduction was related to the maize contaminated at 500 ng/g. The most efficient organism was *Lb. plantarum.*	Oluwafemi et al. (2010)
Mixed culture of *Streptococcus lactis* and *Lb. delbrueckii*	Fumonisin B_1 Zearalenone	Maize meal was spiked separately with FB_1 (0.5, 1.0, 2.0, and 3.0 μg/g). ZEA (0.25, 0.5, 1.0, and 2.0 μg/g) and fermented for 4 days.	A 56%−67% and a 68%−75% reduction was observed for FB1 and ZEA in the third and fourth days, respectively.	Mokoena et al. (2005)
Lb. paraplantarum CNRZ 1885 *Streptococcus thermophilus* RAR1	Fumonisin B_1 Fumonisin B_2	Physicochemical and enzymatic treated bacteria incubated at 25°C for 1 h in corn infusion containing FB_1 and FB_2 (5 μg/mL).	Freezing and thawing, heating, and acid treatment increased removal ability, while enzymatic treatments decreased toxin binding.	Niderkorn et al. (2009)
Saccharomyces cerevisiae LALVIN BM45 *S. cerevisiae* LALVIN Rhône 2226 *S. cerevisiae* UVAFERM 43 *S. cerevisiae* LALVIN Rho-ne 2323 *S. cerevisiae* LALVIN Rhône 2056 *S. bayanus* LALVIN QA23	OTA	Cells (6.7 g/L) were inoculated in yeast peptone glucose (YPG) and synthetic grape juice medium (SGM) containing 2 μg/mL OTA and incubated for 6 days at 30°C.	There was a decrease of OTA levels in YPG (11%−45%) and SGM (1%−35%) medium for many of the growing strains.	Bejaoul et al. (2004)

Continued

Table 4.2 Binding of various mycotoxins and heavy metals to different LAB, probiotics, and yeast.—cont'd

Microorganism type	Type of toxicant	Conditions	Removal (%)	Reference
S. cerevisiae LALVIN Rhône 2056 *S. bayanus* LALVIN QA23	OTA	Heat-treated cells (27 g/L) in SGM containing 2 μg/mL OTA over 72 h.	Approximately 90% reduction was observed within 5 min and up to 72 h of incubation.	Bejaoul et al. (2004)
S. cerevisiae LALVIN Rhône 2056	OTA	Heat-treated cells or yeast walls additive in the OTA-contaminated red grape juice (10 μg/L) for 2 h.	Approximately all of the OTA was removed by heat-treated cell after 5 min while for yeast wall, OTA adsorption was totally removed after 15 min.	Bejaoul et al. (2004)
S. cerevisiae RC008 *S. cerevisiae* RC009 *S. cerevisiae* RC012 *S. cerevisiae* RC016	OTA	Yeast pellets was suspended in PBS containing OTA (1, 5, 10, 40, and 100 μg/mL) and incubated for 1 h at 37°C. Furthermore, 10^7 cells were exposed to low pH (2), salt bile, and then were transferred into the PBS containing 100 μg/mL OTA or10 μg/mL ZEA for 1 h at 37°C.	46%−74%, 16%−39.2%, 14.5%−58%, 17.9%−39.2%, 56.7%−74.2% reductions at 1, 5, 10, 40, and 100 μg/mL OAT, respectively. The highest adsorption percentages were related to the RC012 and RC016 strains. Acid pH and bile salts significantly increased OTA binding only for RC008 and RC009 strains.	Armando et al. (2012)

Lb. plantarum *Lb. brevis* *Lb. sanfranciscensis*	OTA	Viable and heat-treated cells (1 and 5 mg dw (dry weight)/ml) in MRS medium and PBS buffer conaining1000 ng/mL OTA at 30 °C for 24 h.	More than 30% OTA was removed by *Lb. plantarum* and *Lb. sanfranciscensis*. The result of PBS was similar to MRS medium. Dead cells reduced OTA almost twice than viable cells.	Piotrowska (2014)
S. cerevisiae RC008 *S. cerevisiae* RC009 *S. cerevisiae* RC012 *S. cerevisiae* RC016	ZEA	Yeast pellets were suspended in PBS containing ZEA (1, 5, 10, 20, and 50 μg/mL) and incubated for 1 h at 37 Moreover, 10^7 cells were exposed to gastrointestinal conditions (GIT) and then were transferred into the PBS containing 10 μg/mL ZEA for 1 h at 37°C.	Binding percentage ranged from 48% to 87% and 41.1 to 6.7 at the lowest (1 and 5 μg/mL) and the highest (20 and 50 μg/mL) ZEA concentrations, respectively. Exposure to GIT conditions significantly increased RC009 and RC016 ZEA binding ability.	Armando et al. (2012)
Lb. delbrueckii lactis 22170 *Lb. rhamnosus* 6133 *Lb. rhamnosus* 20975 *Lb. rhamnosus* 23139 *Lb. rhamnosus* 6224 *Lb. helveticus* 6024 *B. animalis* 6174 *B. bifidum* 6071 *Enterococcus faecium* 20420 *E. faecium* 21605	Patulin	0.4 g of inactivated bacterial powder in 40 mL apple juice containing 100, 150, and 200 μg/mL PAT, incubation at 4, 30, and 37°C for 24 h.	There was a reduction (47%−80%) by all tested bacterial strains. Maximum PAT removal was occurred at 30°C. *Lb. rhamnosus* 6224 showed the highest reduction (80.4%).	Hatab et al. (2012b)

Continued

Table 4.2 Binding of various mycotoxins and heavy metals to different LAB, probiotics, and yeast.—cont'd

Microorganism type	Type of toxicant	Conditions	Removal (%)	Reference
S. cerevisiae YS3	Patulin	The treated and viable cells were suspended in 2 mL acetic acid solution (pH 4) containing 1 μg/mL patulin.	No significant difference between viable (53.28%) and heat-treated yeast cells (51.71%) in removing patulin.	Guo et al. (2012)
Lb. lactis 6020 *Lb. brevis* 1.12 *Lb. casei* 6103 *Lb. plantarum* 102 *Lb. plantarum* 8014	DON T-2 toxin	10^8 cfu/mL bacterial cell in MRS broth containing 1 μg/mL of DON or T-2 toxin and incubation at 37°C for 72 h.	The strongest toxin binding ability was related to *Lb. plantarum* 102	Zou et al. (2012)
L.b rhamnosus GG *Lb. casei* Shirota *Lb. fermentum* ME3 *B. longum* 2C *B. longum* 46 *B. lactis* BB12 *Lb. lactis ssp. cremoris* *Lb. lactis ssp. lactis* *Leuconostoc. mesenteroides* ssp. *cremoris* *Leuconostoc pseudomesenteroides* *S. thermophiles* *Lb. bulgaricus*	Pb and Cd	1 g/L lyophilized bacteria in solution containing 50 and 10 μg/ml Pb and Cd, respectively, incubation at 37°C for 5−240 min.	The highest binding was occurred at a pH close to neutral. The most effective metal removers were *B. longum* 46, *Lb. fermentum* ME3, and *B. lactis* Bb12.	Halttunen et al. (2007)

Lb. rhamnosus GG *L. rhamnosus* LC 705 *P. freudenreichii shermanii* JS *B. breve* Bbi99/E8	Cd and Pb	1 g/L bacterial biomass in ultra-pure water containing 50 and 0.1 μg/mL Cd or Pb, respectively.	*P. freudenreichii shermanii* JS had the most efficient removal of heavy metals (69.9%). Combination of strains resulted in lower toxin binding than single strains.	Halttunen et al. (2008)
Lb. fermentum ME3 *B. longum* 46	Cd and Pb	Native and chemically modified lyophilized bacteria (1 g/L) in solution containing 0.05 μg/mL Cd and Pb and incubation for 30 min at room temperature.	Methylation of carboxyl groups increased binding ability in comparison to phosphoryl group treatment.	Teemu et al. (2008)
Lb. acidophilus	Arsenic	Incubation of bacterial cell (1 or 2 mg dry wt/mL) in the water solution (pH = 4−10) containing 50−2000 μg/ml As for 4 h.	Maximum As removal was obtained at pH 7 within 3 h. By enhancing bacterial concentration (2 times), As removal was increased 1.16−1.66 times. By increasing initial metal concentration the amount of free As reduced.	Singh and Sarma (2010)

AFM_1, Aflatoxins M_1, *AFB_1*, Aflatoxins B_1;*OTA*, Ochratoxin A; *ZEA*, Zearalenone.

Acronyms

ACF	aberrant crypt foci
AFM_1	aflatoxin M_1
AIAs	aminoimidazoarenes
AαC	2-amino-9H-pyrido [2,3-b] indole
DiMeIQx	2-amino-3,4,8-trimethylimidazo [4,5-f] quinoxaline
DON	deoxynivalenol
DPN	diphenylnitrosamine
FB_1	fumonisin B_1
Glu-P-1	2-Amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole
HAA	heterocyclic aromatic amines
HCAs	heterocyclic amines
IARC	International Agency for Research on Cancer
IQ	2-amino-3-methylimidazo [4,5-f] quinolone
MeAaC	2-amino-3-methyl-9H-pyrido [2,3-b] indole
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MelQx	2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline
NDMA	N-nitroso-dimethylamine
NPIP	N-nitroso-piperidine
NPR	1-nitrosopyrrolidine
NPYR	N-nitroso-pyrrolidine
OTA	ochratoxin A
PAHs	polycyclic aromatic hydrocarbons
PAT	patulin
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine
Trp-P-1	3-Amino-1, 4-dimethyl-5H-pyrido(4,3-B) indole
Trp-P-2	3-Amino-1-methyl-5H-pyrido[4, 3-b]indole
ZEA	zearalenone

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Microbial bioprocessing of health promoting food supplements

5

Swati S. Mishra [1], *Sudhanshu S. Behera* [2,3], *Md Latiful Bari* [4], *Sandeep K. Panda* [5], *Steve C.Z. Desobgo* [6]

[1]Department of Biodiversity and Conservation of Natural Resources, Central University of Orissa, Koraput, Odisha, India; [2]Department of Fisheries and Animal Resource Development, Directorate of Fisheries, Government of Odisha, Odisha, India; [3]Centre for Food Biology and Environment Studies, Bhubaneswar, Odisha, India; [4]Center for Advanced Research in Sciences, University of Dhaka, Dhaka, Bangladesh; [5]School of Biotechnology, KIIT University, Bhubaneswar, Odisha, India; [6]Department of Food Process and Quality Control, University Institute of Technology of the University of Ngaoundere, Cameroon

5.1 Introduction

For centuries, human beings have utilized microorganism for their abilities to produce various bioactive compounds/metabolites of interest, such as food supplements or food ingredients. The food supplements include a vast array of products, i.e., vitamins, minerals, amino acids, polyunsaturated fatty acids (PUFAs), antioxidants (polyphenols, carotenoids), amino acids/peptides and their derivatives, etc., that are synthesized from microorganisms for providing health and nutrition to consumers. The data available from different sources like genomic, transcriptomic, proteomic, and metabolomic studies have provided ways to engineer microorganisms for development of food supplements of commercial importance. Synthetic biology/metabolic engineering envisages the use of genetic parts/tools [promoter, transcription factors, ribosomal binding sites (RBS), degradation tags, and transcriptional terminators] in microbial system for synthesis of biocommodities including food supplements. Microbial synthesis is an alternative to chemical methods for production of food supplements and uses organic feedstocks.

This chapter focuses on the classification and types of food supplements, various industrially important organisms involved, bioprocessing route, market trend, and regulatory issues.

5.2 Classification of food supplements

5.2.1 Food supplements, nutraceuticals, and food additives

A **food supplement** is a preparation that is intended to supply a nutrient that is missing from a diet. It can be vitamins, minerals, amino acids, fatty acids, and other substances. **Nutraceuticals** are supplements that contain a concentrated form of a substance that is

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not in food form but is derived from foods. For example, soy protein is a **food supplement**. However, ipriflavone is the synthetic derivative of isoflavone daidzein found in soy protein and is sold as a nutraceutical. On the other hand, **food additives** are substances that are added to food to maintain or improve the safety, freshness, taste, texture, or appearance of food. Some food additives have been in use for centuries for preservation—such as salt (in meats such as bacon or dried fish), sugar (in marmalade), or sulfur dioxide (in wine).

Food supplements can be categorized into two groups depending on their intended use as per the National Agency of Medicines: (a) Supplements as foodstuff; (b) Food for meticulous uses as a beverage, for different age group of people. Also, the supplements can be categorized as per the origin: supplements of natural or synthetic origin. Table 5.1 summarizes the classification of food supplements of microbial origin, and some examples of food supplements are described below.

Table 5.1 Classification and some examples of food supplements of microbial origin.

Food supplements	Microbial origin	References
Essential Fatty Acids		
[a]Omega-3 LC-PUFAs	*Aurantiochytrium* sp. strain TC 20	Chang et al. (2013)
Microbial lipids	*Rhodosporidium toruloides* DSM 4444	Tsakona et al. (2019)
Gamma Linolenic Acid (GLA)	*Cunninghamella echinulata* and *Mortierella isabellina*	Chatzifragkou et al. (2010)
Microbial lipids	*Mortierella alpina*	Diwan et al. (2018)
Vitamins		
Vitamin B1	*Bacillus subtilis*	Schyns et al. (2005)
Vitamin B1	*Aspergillus oryzae*	Tokui et al. (2011)
Vitamin B2	*Aphis gossypii*	Revuelta et al. (2018)
Vitamin B5	*Corynebacterium glutamicum*	Hüser et al. (2005)
Vitamin B6	*Escherichia coli*	Rosenberg et al. (2017)
Vitamin B12	*Pseudomonas denitrificans*	Li et al. (2008)
Amino acids		
Lysine	*Corynebacterium glutamicum*	Mitsuhashi (2014)
Glutamic acid	*Corynebacterium glutamicum*	Hirasawa and Shimizu (2016)
Essential minerals		
Zn, Ca, and P	*Microbial phytase*	Walk et al. (2013)
Probiotics		
Microflora	*Lactobacillus* genus	Argyri et al. (2013)

[a]Omega-3 LC-PUFAs: Omega-3-long chain polyunsaturated fatty acids.

5.2.1.1 Essential fatty acids

The essential fatty acids such as omega-3 fatty acid, alpha-linolenic acid (ALA), omega-6 fatty acid, and linoleic acid (LA) (Fig. 5.1.) are considered as nutritional supplements and are important for maitaining good health (Ji et al., 2015). The human body cannot synthesize these fatty acids on its own. Therefore, the essential fatty acids must be obtained from diet. For instance, fish oils are rich sources of fatty acids, e.g., docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which are widely used as nutritional supplements to the consumers (Ward and Singh, 2005; Finco et al., 2017). However, a limited number of plant oilseeds are good sources of other essential fatty acids (PUFAs). A promising alternative system for the production of omega-3 lipids is from microbial metabolism of yeast, fungi, or microalgae (protists and dinoflagellates). Marine protists and dinoflagellates such as species of *Crypthecodinium, Thraustochytrium,* and *Schizochytrium* are the rich sources of fatty acids (DHA), whereas microalgae like *Phaeodactylum* and *Monodus* are good sources of EPA (Ward and Singh, 2005). An isolated protists *Aurantiochytrium* sp. strain TC 20 was investigated using small-scale (2 L) bioreactors and found potential for the production of omega-3 long chain PUFAs (Chang et al., 2013). *Yarrowia lipolytica* is a model oleaginous yeast for the production of lipids-derived biofuels, biosynthesis of industrially important metabolites, and the essential fatty acids (Lazar et al., 2018). Chatzifragkou et al. (2010) reported the fungi *Cunninghamella echinulata* and *Mortierella isabellina*, capable of accumulating single cell oil containing γ-linolenic acid, and were cultivated on sugar-based media, at initial substrate concentration 60 g/L. Diwan et al. (2018) studied a nondetoxified rice straw hydrolysate to its application in lipid production from mold *Mortierella alpina*.

5.2.1.2 Vitamins

Vitamins are nutritional compound required in small quantities by the living organisms for growth, metabolism, and development (Ledesma-Amaro et al., 2013). Vitamins if

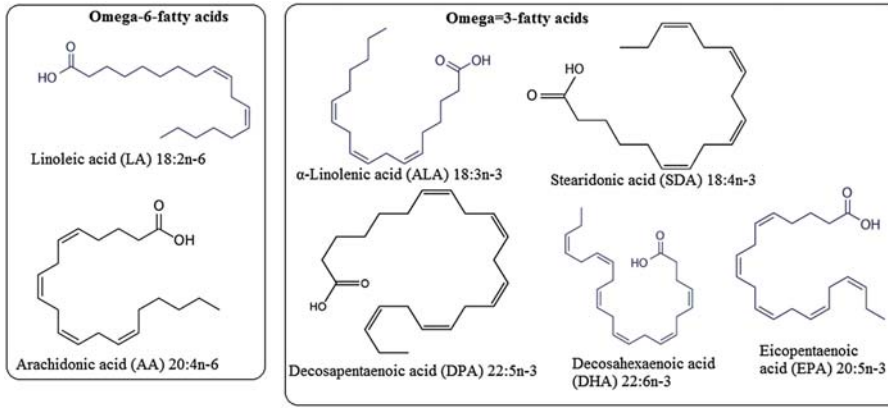

Figure 5.1 Chemical structures of some essential fatty acids.

not being synthesized in adequate amounts by the body should be acquired from food or food supplements. Human beings need 13 types of vitamins (classified as fat soluble (A, D, E, and K) and water soluble (C, B group) in the diet that are essential organic compounds (Acevedo-Rocha et al., 2019). The list of vitamins is as follows: K, C, E, D, A, Vitamin B12, Thiamine (B1), Niacin (B3), Pantothenic acid (B5), Riboflavin (B2), Biotin (B7), Vitamin B6, and Folate (B9). Vitamins such as vitamin E contain tocotrienols and tocopherols, and vitamin K includes both K1 and K2. Vitamin K1 is primarily found in leafy green vegetables, while K2 is most abundant in fermented foods and some animal products. Vitamin K2 may be absorbed better by the body, and some forms may stay in the blood longer than vitamin K1. These two things may cause K1 and K2 to have different effects on human health (Lyzak et al., 2017).

5.2.1.3 Amino acids

Proteins are chain of amino acids. Our body needs 20 different amino acids to grow and function properly. Though all 20 are important, only nine amino acids are classified as essentials. These are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valanine. Unlike nonessential amino acids, essential amino acids cannot be synthesized in our body and must be supplemented through protein-based diet. Certain nonessential amino acids are some time considered as conditionally essential such as arginine (when body is fighting certain disease like cancer). Protein-containing nutrients, either prepared-to-drink or as powder forms are sold as supports to patients suffering from sickness or trauma, and seeking to overcome the old-age sarcopenia (Colonetti et al., 2017), or for those who claim that heavy physical exercise raises amino acids requirements in the body (Stonehouse et al., 2016). Whey protein is a popular food supplement (Naclerio and Larumbe-Zabala, 2016; Colonetti et al., 2017).

5.2.1.4 Minerals

The body needs many minerals; these are called essential minerals. Just like vitamins, essential minerals help your body grow, develop, and stay healthy. The body uses minerals to perform many different functions—from building strong bones to transmitting nerve impulses. Some minerals are even used to make hormones or maintain a normal heartbeat. Essential minerals are sometimes divided up into major minerals (macrominerals) and trace minerals (microminerals). These two groups of minerals are equally important, but trace minerals are needed in smaller amounts than major minerals. A balanced diet usually provides all of the essential minerals. Potassium, sodium, chlorine, phosphorus, calcium, iron, magnesium, and sulfur (all macrominerals) and manganese, zinc, iodine, copper, molybdenum, chromium, cobalt, selenium, fluoride (all microminerals) are the essential minerals for humans. Potentially essential minerals are sold separately and in conjunction with vitamins and some other minerals, as food supplements.

5.2.1.5 Natural products

Nutritional supplements can be developed utilizing either complete materials or extracts from animals, vegetables, fungi, lichens, or algae among other examples as curcumin, *Ginkgo biloba*, cranberry, resveratrol, ginseng, collagen, and glucosamine (Prince, 2017).

5.2.1.6 Probiotics

Food and Agriculture Organization/World Health Organization defined probiotic as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Florou-Paneri et al., 2013). The dominated microflora considered as commercial probiotics are mainly of *Lactobacillus* genus with over 100 species recognized, including *Lactobacillus plantarum, Lb. acidophilus, Lb. rhamnosus, Lb. casei, Lb. bulgaricus, Lb. reuteri, Lb. helveticus,* and *Lb. delbrueckii*. However, *Lactobacillus* is generally recognized as safe (GRAS) organisms (Argyri et al., 2013). In the scientific literature, probiotic population of 10^6–10^7 CFU is established as therapeutic quantities in fermented foods (da Cruz et al., 2009; Behera et al., 2018a, b). While there are various alleged benefits to use probiotics, like preserving gastrointestinal wellbeing, reducing the risk and severity of constipation or diarrhea, and enhancing immune wellness, along with reduced risk and magnitude of severe respiratory tract diseases, not all of these claims are endorsed by adequate therapeutic evidence (Rijkers et al., 2011; Behera et al., 2018a).

5.3 System biology and industrial food microorganisms

Industrial food microbiology is the sciences to manufacture or produce food products in mass quantities. There are multiple ways to manipulate a microorganism (mutation, gene amplification using plasmids and vectors, etc) in order to increase maximum product yields. The plasmids and/or vectors are used to incorporate multiple copies of a specific gene that would allow more enzymes to be produced that eventually cause more product yield. Microorganisms play a big role in the food industry, with multiple ways to be used.

5.3.1 Filamentous fungi

Since primitive period, filamentous fungi and yeasts are utilized to produce beer, wine, bread, and cheese. The 20th century was a golden era for industrial food microbiology that exploited fermentation for production of number of enzymes, vitamins, amino acids, polymers, and many other useful compounds (Fig. 5.2). Filamentous fungi are used in industrial scale for manufacture of a huge range of beneficial products such as recombinant proteins and metabolites, all for the gain of human kind. These metabolic products consist of enzymes, amino acids, organic acids, exopolysaccharides (i.e., pullulan, xanthan), pigments, fatty acids, and food (mushrooms). The improvement of molecular biology strategies gives new methods to use yeasts and molds as microbial factories for manufacturing these high-value products. The choice is basically dependent on production yields and regulatory issues, importantly for fungi used in the food industry. Host lines are commonly selected from among these which have attained the so-called GRAS fame via the U.S. Food and Drug Administration (FDA).

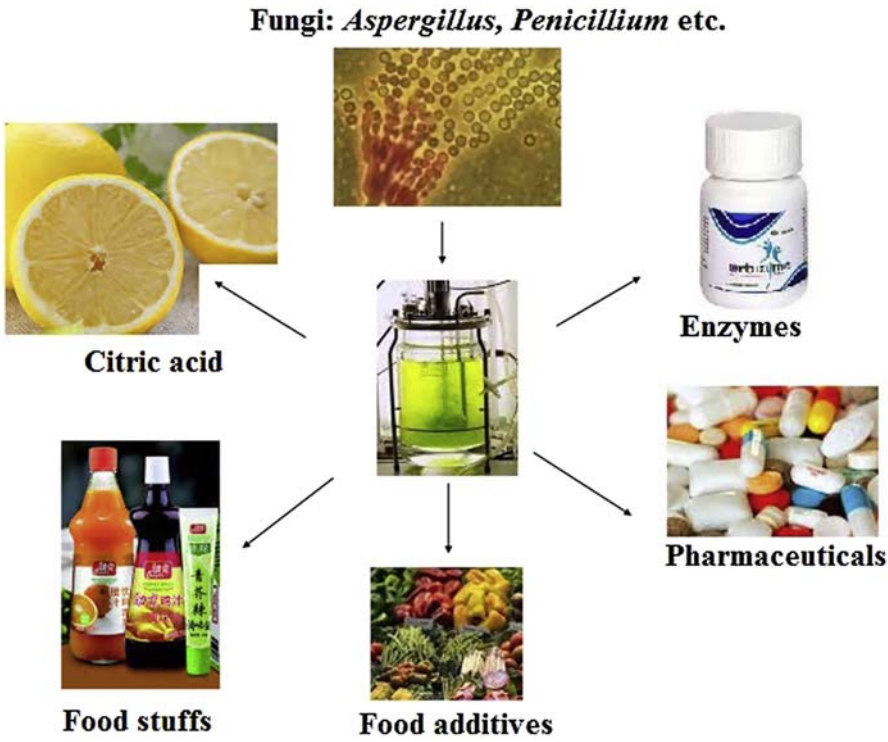

Figure 5.2 Industrial use filamentous fungi batch fermentation.

Filamentous fungi (i.e., *Aspergillus niger, A.oryzae,* and *Rhizopus oryzae*) provide a great opportunity for industrial fermentation. Two particularly important aspects are the high-yield coefficients and the ability to secrete products. The fermentation of soybeans into soy sauce by *A. oryzae* and *Aspergillus sojae* (Luh, 1995) and the layer fermentation of cheese and sausages (Trigueros et al., 1995) are excellent examples.

5.3.2 Industrial yeast

For millennia, yeasts were used to produce fermented foods (breads) and beverages (wine, beer, and sake). Nonetheless, selecting a particular strain of yeast for a particular industrial use is mostly based on historical criteria, instead of science (Steensels et al., 2014).

The development of superior industrial yeasts has resulted in the manipulation of established natural diversity through the use of technologies such as mutagenesis, protoplast fusion, cloning, genome shuffling, and guided evolution to produce artificial variety. In addition, recent technical advancements have allowed the production of high-throughput technologies, known as 'global transcription machinery engineering' (gTME), to stimulate genetic variability, generating a fresh source of genetic diversity for the yeast (Steensels et al., 2014).

Both conventional methods and modern gene manipulation approaches are implemented to produce yeast strains appropriate for work under particular commercial

conditions (Lane and Morrissey, 2010). The age-old yeast *Saccharomyces cerevisiae* is complemented in many applications with the use of less known non-*Saccharomyces* yeasts that are now used widely in food industry. While yeast is synonymous with *S. cerevisiae*, other biotechnologically important yeast strains were implemented to produce manufactured goods beyond conventional foods.

More recently, as industrial species for the heterologous development of enzymes and proteins, *Ogataea (Hansenula) polymorpha, S. cerevisiae, Komagataella (Pichia) pastoris,* and certain other yeast species were created (Branduardi et al., 2008; Johnson, 2013a, 2013b).

5.3.3 Industrial bacteria

Lactic acid bacteria (LAB) are used all over the world for industrial fermentations of milk, meat, fish, and vegetables. Their contribution in these fermentation methods consists of the formation of lactic acid from the available carbon source (matrices) ensuring acidification of the food. However, other than lactic acid forming capacity, LAB have the capability to make a contribution to product characteristics such as flavor, texture, and nutrition. LAB are additionally applied at an industrial scale in the fermentation of food supplements, i.e., mannitol, xylitol, sorbitol, and tagatose, as an end result of metabolic engineering (Monedero et al., 2010; Rice et al., 2019).

Anaerobic bacteria have played a significant role in the advancement of industrial biotechnology.The first mass fermentation was for the manufacturing of Acetone–Butanol–Ethanol (ABE) system by *Clostridium acetobutylicum* in the 1920s (Wolfe, 1999). Anaerobic microorganisms were also used for centuries in food fermentation (Goldstein, 1995). Food products (vinegar, cheese, and beer) created by yeast or bacteria through anaerobic fermentation acquire their properties through the production of substances like carbon dioxide, lactic acid, ethanol, propionic acid, and acetic acid. An approach to the study of chemical compounds and fuels derived from renewable sources is to use conventional food-fermenting bacteria for the chemical industry (Dishisha et al., 2012). One such example is the use of LAB (*Lb. bulgaricus*) in cosmetic, medicinal, and polymer formulations (Datta et al., 1995).

5.4 Bioprocessing (fermentation) technology in food supplement industry

The fermentation technologies are considered as cost competitive compared to chemical synthesis/methods to carry out microbial production of food supplements at an industrial scale (Lv et al., 2019). The various fermentation methods are described below.

5.4.1 Solid-state fermentation

Solid-state fermentation (SsF) is a fermentation process in which microorganism grow on solid materials without the presence of free liquid (Desobgo et al., 2017; Ray and

Behera, 2017). In SsF, the moisture necessary for microbial growth exists in an absorbed state or complexes within the solid matrix. At present, SsF techniques involve in the production of several groups of microbial products such as enzymes (amylase, glucosidase, cellulase, and pectinase), organic acids (citric acids, and lactic acids), microbial secondary metabolites (gibberellic acid, ergot alkaloids, penicillin, and cyclosporin), and other microbial metabolites, such as nucleotides, lipids, vitamins, and amino acids (Zhao et al., 2015; Panda et al., 2016). Some examples of food supplement production in SsF are described below.

A high production of β-glucosidase (27.4 U/mL) was obtained by cultivating the fungus *Lichtheimia ramose* by SsF using wheat bran with 65% of initial substrate moisture (incubated for 96 h at 35°C) (Garcia et al., 2015). SsF has potential to produce lignocellulolytic enzymes (cellulase and xylanase) (Behera and Ray, 2016).

Dhillon et al. (2013) evaluated the potential of different agroindustrial wastes (apple pomace, brewer's spent grain, citrus waste, and sphagnum peat moss) as substrate for solid state citric acid production using *Aspergillus niger* NRRL 2001. Among the four substrates, apple pomace resulted highest citric acid production (61.06 ± 1.9 g/kg dry substrate). Bartkiene et al. (2015) investigated the protein digestibility and formation of lactic acids (LA) during SsF of legumes (lupin and soya bean) using LAB. Protein digestibility of fermented lupin and soya bean was found higher on average by 18.3% and 15.9%, respectively, compared to untreated samples. The LAB produced mainly L-LA (D/L ratio 0.35−0.54), while spontaneous formation gave almost equal amounts of both LA isomers (D/L ratio 0.92−0.98). de Olmos et al. (2015) studied the ability of selected LAB strains (*Lb. paracasei* and *Bifidobacterium longum*) to grow on soy flour substrate with strain-dependent behavior on the SsF system. β-glucosidase activity was evident in both strains, and *Lb. paracasei* was able to increase the free amino acids at the end of fermentation under assayed conditions (50%−80% moisture, temperature incubation 31−43°C).

The production of single cell oils enriched with PUFAs, such as γ-linolenic acid (C18:3 n-6: GLA), is one of the main tasks for biotechnological production of nutritionally important lipids (Čertík et al. 2013). Significant activities in the biosynthesis of Gamma Linolenic Acid (GLA) have been described for species of the lower oleaginous fungi belonging to the genus *Cunninghamella, Mortierella, Mucor, Rhizopus*, and *Thamnidium* (Čertík et al. 2013). Jangbua et al. (2009) studied to maximize the yield of γ-linolenic acid grown on different substrates (polished rice, broken and spent malt grain) by a filamentous fungus, *Mucor rouxii*, using low cost production by SsF. The GLA content was highly accumulated in rice bran (6 g/kg fermented mass) in the fifth day culture grown at 30°C.

5.4.2 Submerged fermentation

In submerged fermentation (SmF), microorganism and substrate are homogeneously distributed in a liquid medium (Naz et al., 2017). For the microbial synthesis of food supplements, the SmF is favored mostly, due to more accessibility to nutrients, sufficient supply of oxygen, and demand of small time duration for the fermentation (Naz et al., 2017; Sharma et al., 2018). Hermansyah et al. (2018) reported the

production lipase by the cultures of *Pseudomonas aeruginosa* on agroindustrial waste product (palm oil mill effluent (POME)) using the SmF. The optimum value of the lipase activity unit (1.327 U/mL) was gained when 3% (v/v) of inoculum, 4 mM of Ca^{2+} ion, 0.4% (v/v) of olive oil, 0.9% (m/v) of peptone, and 0.9% of Tween 80 were added into the medium. Sharma et al. (2018) studied the isolation, purification, and characterization of potential lipase producing bacteria using SmF. The lipase producing bacteria were isolated and identified as *Bacillus methylotrophicus* PS3. The purification procedure (Sephadex G-100 gel column chromatography) resulted in 2.90 fold purification of lipase with 24.10% final yield. Oumer and Abate (2018) compared the production of pectinase by *Bacillus subtilis* in SSF and SmF using agricultural residues as substrate. The production of pectinase was enhanced more than a 6-fold in SmF and a fold in SSF. The highest productivity of pectinase using SmF from *Bacillus subtilis* was 10−66 U/mL whereas in SSF it improved from 800 U/g to 1272 U/g.

5.4.2.1 Batch and fed-batch fermentation

Batch fermentation, a cost-effective process, has been expensively used for the commercial production of various value-added products; during this process, nutrients were provided to the reactor while cells and products remained in the reactor until the end of fermentation (Laopaiboon et al., 2007). Fed-batch process, during which the medium was periodically withdrawn and substituted with fresh medium, was known to enhance the productivity of microbial fermentation as it saved the time for cleaning, sterilization, seed culture, and inoculation processes between batches (Qu et al., 2013). Abdel-Rahman et al. (2015) studied the fed-batch fermentation for the increased production of L-LA from glucose/xylose mixture using *Enterococcus mundtii* QU 25. A high L-LA concentration (129 g/L) with 99.5% optically pure was found in the fed-batch fermentation. A different fermentation processes, including batch, fed-batch, and repeated fed-batch processes by *Schizochytrium* sp., were studied (Qu et al., 2013) and compared for the effective DHA-rich microbial lipids production. The comparison between different processes showed that fed-batch process was a more efficient fermentation strategy for microbial lipids production (18.88 g/L) than the batch process (8.98 g/L).

Batch fermentation has several advantages over continuous fermentation, such as easy control of microbial contaminants and increased product quality per batch (Klasson et al., 1989). However, for large scale production, batch reactors require high capital investments and also require extensive labor (Wee and Ryu, 2009).

5.4.2.2 Continuous fermentation

A Continuous Stirred Tank Reactor (CSTR) can give a more consistent product and provides a steady rate of crude product to be processed in the recovery system. In employing a continuous fermentation process, the microorganism used must be rigid enough to withstand shear while still being easily pumped (Klasson et al., 1989). A continuous fermentation of lignocellulosic hydrolyzates yielded an LA productivity of 6.7 g/L/h using 30 g/L of corn steep liquor and 1.5 g/L of yeast extract as nutrients

(Wee and Ryu, 2009). Silbir et al. (2014) reported the levan (naturally occurring fructan) in continuous fermentation by *Zymomonas mobilis* B-14023. Continuous fermentation processes were performed in packed bed bioreactor using Ca-alginate immobilized *Z. mobilis* cells. The highest levan concentration (31.8 ± 0.21 g/L) was obtained at a dilution rate of 0.14/h while maximum volumetric productivity (6.556 g/L/h) was obtained at a dilution rate of 0.22/h.

5.4.3 Simultaneous saccharification and fermentation

The enzymatic hydrolysis and fermentation steps for microbial food supplements production can be performed as separate hydrolysis and fermentation (SHF) or as simultaneous saccharification and fermentation (SSF). The SSF process offers various advantages over SHF such as the use of a single-reaction vessel for both steps (allowing process integration with reduction of capital cost), rapid processing time, reduced end-product inhibition of hydrolysis, and increased productivity, which is obtained (Marques et al., 2008; Pleissner et al., 2017). Marques et al. (2008) reported the use of recycled paper sludge as an alternative substrate for LA production using *Lb. rhamnosus* ATCC 7469. The maximum production of LA was reported to 73 g/L, corresponding to a maximum productivity of 2.9 g/L/h. Pleissner et al. (2017) reported the food waste as carbon and nitrogen source in SSF using *Lactobacillus* sp. or *Streptococcus* sp. strains for L-LA production. The outcomes revealed a linear relationship between LA concentration and strain used in SSF. *Lactobacillus* sp., strains showed a productivity of 0.27−0.53 g/L/h and yield of 0.07−0.14 g/g, while *Streptococcus* sp., strains more efficiently degraded the food waste material and produced LA at maximum rate of 2.16 g/L/h and a yield of 0.81 g/g dry substrate.

5.5 Metabolic engineering of industrial organisms

Metabolic engineering is one of the most promising and emerging methods for the production of value-added bioproducts (Fig. 5.3). The metabolic pathway of several microorganisms has been successfully engineered for higher production of amino acids, vitamins, colorants, and organic acids compared to conventional methods. Table 5.2 summarizes the metabolic engineering of microorganisms for the production of food additives. Some of the relevant studies have been depicted below.

5.5.1 Production of polyunsaturated fatty acids

Polyunsaturated fatty acids are essential fatty acids required for human development and health and are typically categorized into two major classes: omega-3 (n-3) and omega-6 (n-6) fatty acids with the ω-3 fatty acids being the major focus of most industrial microbial engineering. They have many positive effects on human beings, such as antiinflammatory and antiblood clotting actions, lowering triglyceride level, reducing blood pressure, and reducing the risks of diabetes, some cancers, etc (Ren et al., 2010;

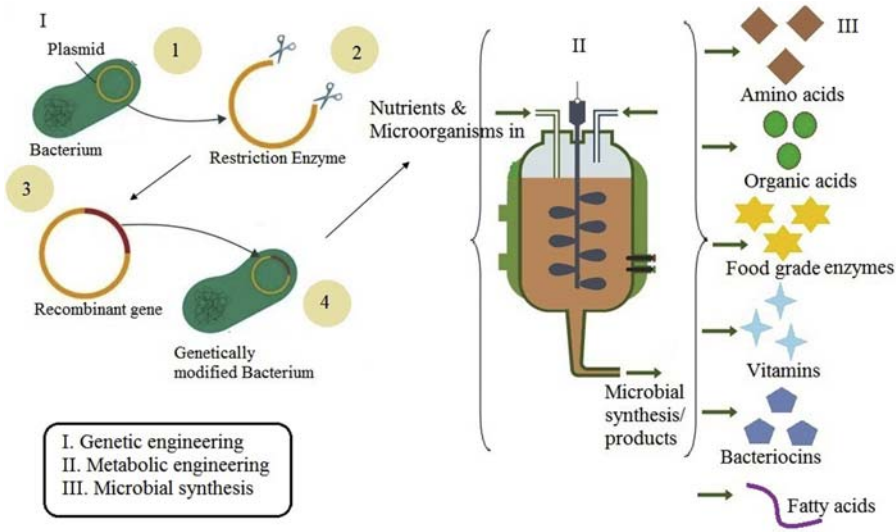

Figure 5.3 Microbial synthesis of Food supplements.

Xie et al., 2015). The human body cannot synthesize these fatty acids on its own. Therefore, the omega-3 fatty acids must be obtained from the diet (Ji et al., 2015). Commercially important ω-3 fatty acids include α-linolenic acid (ALA; C18: 3n − 3), eicosapentaenoic acid (EPA; C20:5n − 3) and docosahexaenoic acid (DHA; C22:6n − 3). Given the increased recognition of health benefits from these molecules, demand for ω-3 PUFAs is growing and expected to reach a global demand of 241 thousand metric tons with a value of $4.96 billion by the year 2020 (Yuan and Alper, 2019).

5.5.1.1 Eicosapentaenoic acid and docosahexaenoic acid

Eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) are two typical omega-3 fatty acids. Their traditional source is derived from cold-water fish oils. Alternatively, novel sources of omega-3 fatty acids can be green manufactured from marine algal or algae-like microbial oils, which could eliminate many of the taste and odor problems associated with fish and discard the shortcomings of fish oil—based process (Gupta et al., 2012). Currently, the most common algae or algae-like microorganism used for the production of DHA belong to the marine members of the families *Thraustochytriaceae* and *Crypthecodiniaceae* (discussed elaborately in Section 7.0).

EPA and DHA biosynthesis is typically pursued through the aerobic desaturase/elongase pathway although production is feasible through an anaerobic polyketide synthase (PKS) pathway (Xue et al., 2013). DuPont researchers used this aerobic pathway in *Yarrowia lipolytica* to generate a strain capable of producing EPA at 56.6% of the total fatty acids and about 15% of the dry cell weight, a value that is

Table 5.2 Metabolic engineering of microorganisms for the production of food additives.

Food additives/ supplements[a]	Engineering organism	Gene/factor involved	References
Essential Fatty Acids			
TGA	*Rhodococcus opacus* PD630	β-glucosidase	Hetzler and Steinbüchel (2013)
ALA	*Yarrowia lipolytica*	Δ12/Δ15-desaturase	Cordova and Alper (2018)
n-3 LC-PUFA	*Methanococcus* sp.	-	Sprague et al. (2017)
Vitamins			
Vitamin C	*Kluyveromyces lactis*	*GME*, *VTC 2,* and *VTC 4*	Rosa et al. (2013)
Vitamin B2	*Eremothecium gossypii*	*RIB*	Revuelta et al. (2016)
Amino acids			
L-arginine	*Corynebacterium glutamicum*	*pgi*/Increasing the NADPH level	Park et al. (2014)
Organic acids			
Citric acid	*Aspergillus niger*	SSADH	Yin et al. (2017)
L-Lactic acid	*Lactobacillus plantarum*	*ldhD*/D-LDH	Okano et al. (2018)
Succinic acid	*Actinobacillus succinogenes*	PCK, MDH, FUM	Guarnieri et al. (2017)
Acetic acid	*Acetobacter pasteurianus*	ADH	Wu et al. (2017)
Enzymes			
Amylase	*Saccharomyces cerevisiae*	Point mutation of *VTA1* gene	Liu et al. (2014)
Lipase	*Lactococcus lactis*	DNA insert	Raftari et al. (2013)
L-asparaginase	*Escherichia coli*	pET vectors, histidine tag	Einsfeldt et al. (2016)

Table 5.2 Metabolic engineering of microorganisms for the production of food additives.—cont'd

Food additives/ supplements[a]	Engineering organism	Gene/factor involved	References
Vitamins			
L-ascorbic acid	*Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*	ALO, LGDH	Sauer et al. (2004)
L-ascorbic acid	*Kluyveromyces lactis*	GME, VTC_2, VTC_4	Rosa et al. (2013)
Thiamine (vitamin B1)	*Bacillus subtilis*	*thiN*[b], *thiT*[c], and *thiW*[d]	Schyns et al. (2005)
Adenosylcobalamin (vitamin B_{12})	*E. coli*	AdoCbi-P	Fang et al. (2018)

[a]TGA: Triacylglycerols; ALA: α-Linolenic acid; Omega-3 LC-PUFAs: Omega-3-long chain polyunsaturated fatty acids; NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; SSADH: Succinate-semialdehyde dehydrogenase; D-LDH: D-lactate dehydrogenase; PCK: Phosphoenolpyruvate carboxykinase; MDH: Malate dehydrogenase; FUM: Fumarase; ADH: Alcohol dehydrogenase; ALO: D-arabinono-1,4-lactone oxidase; LGDH: L-galactose dehydrogenase; GME: DP-mannose 3,5-epimerase; VTC_2: GDP-L-galactose phosphorylase; VTC_4: L-galactose-1-phosphate phosphatase; AdoCbi-P: Adenosylcobinamide phosphate.
[b]thiamine pyrophosphokinase.
[c]thiamine permease.
[d]thiamine ABC transporter component.

the highest percentage among known EPA sources. The same group later developed a new commercial strain (*Y. lipolytica* Z5567) that optimized carbon flux toward EPA biosynthesis pathway, eliminated β-oxidation, and fine-tune regulated EPA transportation (Zhu and Jackson, 2015). When cultivated using a two-stage fed-batch fermentation process (using nitrogen-rich medium for growth phase and nitrogen-limiting conditions for oil production), this strain was capable of producing an oil comprising EPA at 50% and 25% dry cell weight (Xie et al., 2015).

In a recent study, the red yeast *Rhodosporidium toruloides* DSM 4444 was used in both in batch and fed-batch mode for production of microbial lipids rich in PUFAs, with oleic acid being the major fatty acid (61.7%, w/w) (Tsakona et al., 2019). Diversified mixed confectionery waste streams were used as the substrate for production of microbial lipids.

Consolidated bioprocessing (CBP) technology was used for coproduction of DHAs and bioethanol from rice straw biomass as substrate. Key points of the process were: (a) CBP of pretreated rice straw biomass to bioethanol (anaerobic fermentation) resulting into 1.8 g/L bioethanol and 29.40% solubilization of rice straw biomass; (b) utilization of spent lignocellulose derived sugars in microalgal fermentation (aerobic fermentation) with subsequent promising cell growth (2.77 g/L), substantial lipids (17.05%), and DHAs production (44.0% of total fatty acids). Other major fatty acids

(as total fatty acid %) were palmitic acid (13.95%), stearic acid (5.07%), EPA (7.24%), and docosapentaenoic acid (16.12%) (Singh et al., 2020).

5.5.1.2 α-Linolenic acid

An additional ω-3 fatty acid, α-linolenic acid (ALA), has been explored also in the oleaginous yeast *Y. lipolytica*. Biosynthesis of ALA requires a Δ15-desaturase to convert native unsaturated fatty acids of oleic acid (C18:1n − 9) and LA (C18: 2n − 6) into the ALA (Cui et al., 2016). Using a previously engineered strain of *Y. lipolytica* that can produce nearly 80% of lipids as an unsaturated C18 s, it was possible to create a platform for ALA biosynthesis. Specifically, heterologous expression of a codon-optimized, bifunctional Δ12/Δ15-desaturase from *Rhodosporidium kratochvilovae* coupled with a low-temperature fermentation (20°C) produced significantly increased ALA content. The resulting strain was capable of producing ALA to upwards of 30% of total fatty and achieving titers of 1.4 g/L ALA in fed-batch fermentation, the highest reported titer in a yeast host (Cordova and Alper, 2018). Collectively, these results highlight the use of microorganisms (especially oleaginous yeasts) for the production of nutritional fatty acids.

5.5.2 Production of amino acids

L-arginine, one of the valuable amino acids with wide range of applications as supplement, is produced through microbial synthesis. An interesting study was carried out for higher yield of L-arginine by engineering the metabolic pathway of *Corynebacterium glutamicum* (Park et al., 2014). The strain improvement of *C. glutamicum* ATCC 21831 was carried out through random mutagenesis. The mutagenic strain improved L-arginine production by increasing the NADPH level since biosynthesis of 1 mole of arginine requires 3 moles of NADPH. In order to generate higher concentration of NADPH, the *pgi* gene responsible for the production of glucose-6-phosphate isomerase was downregulated; as a result, the pentose phosphate pathway was activated in comparatively higher rate for improved production of NADPH. Additionally, it was observed that deletion of *Ncgl 1221 gene* enhanced the conversion of L-glutamate to L-arginine rather than releasing to the medium. Similarly, ornithine is one of the derivatives of arginine and has been reported for many health applications. The production can be improved by overexpression of *argCJBD* gene through plasmids (Shin and Lee, 2014). Lee et al. (2007) have demonstrated the production of higher rate of L-threonine through manipulating the metabolic pathway. Higher yield of L-threonine was observed (0.393/g glucose and 82.4 g/L in fed batch culture) by removal of the genes *thrA*, *lysC*, *thrL,* and *tdh* and mutation of genes *ilv A*, *thrA,* and *lysC* that are responsible for the inhibition of aspartokinase I and III; *thrL* represents transcriptional attenuation regulation, and *tdh* is known for degradation of threonine. Similarly, the gene of acetohydroxy acid synthase isoenzyme III, inhibited by valine, was removed. Also, the *ilvA, leuA,* and *panB* genes were deleted to make more precursors available for the overproduction of valine. Valine generation of 1.3 g/L was observed by overexpression of *ilvBN* genes (Park et al., 2011).

5.5.3 Production of organic acids

Organic acids are mostly used as food additives and preservatives. Among the weak acid groups, citric acid, lactic acid, acetic acid, and succinic acid are very prominent (Panda et al., 2016).

Yin et al. (2017) have reported the production of citric acid by different microorganisms (157 g/L by *Aspergillus niger* H915-1, 117 g/L by *A. niger*A1, and 76 g/L by *A. niger* L2). The higher rate of production of lactic acid by *A. niger* H915-1 was attributed to the mutation of 92 genes which included a succinate-semialdehyde dehydrogenase in the γ-aminobutyric acid shunt pathway. Also, the ATP-citrate lyases, which have important role in citrate synthesis, were upregulated.

A strain of *Lb. plantarum* was improved by deleting certain genes to obtain optically pure lactic acid, i.e., (L)-lactic acid, instead of the racemic mixture (l- and D-), produced by the wild strain. The gene encoding D-LDH (lactate dehydrogenase), *ldhD* was first deleted, and it was found to have no impact on inhibition of D-lactic acid production. However, the conversion of D-lactic acid to L-lactic acid was completely possible when the operon representing lactate racemase (*larA-E*) was disrupted. It may also be noted that the aforesaid mutant produced pure L-lactic acid (99.4% purity) of 87 g/L from 100 g/L glucose (Okano et al., 2018).

Succinic acid is often preferred as a flavoring agent and an acidity regulator in food and beverage industry. A study was conducted to improve the production of succinic acid in *Actinobacillus succinogenes*; the genes *pflB* (pyruvate formate lyase) and *ackA* (acetate kinase) were knocked out which blocks the competitive pathways for the production of formic acid and acetic acid, respectively. However, the mutated strains showed growth defects. Further, three different genes of three different strains were overexpressed phosphoenolpyruvate carboxykinase (PCK), malate dehydrogenase (MDH), fumarase (FUM), and the yield obtained were 31.3, 34.2, and 32.6 g/L, respectively, whereas the wildtype showed a production of 30.6 g/L (Guarnieri et al., 2017). In another study, enzyme subunits I (*adhA*) and II (*adhB*) of pyrroquinoline quinone-dependent alcohol dehydrogenase in *Acetobacter pasteurianus* were overexpressed resulting in higher production yield of acetic acid as compared to the wild strain (Wu et al., 2017).

5.5.4 Production of enzymes

Amylase is one of the important enzymes frequently used in food and beverage industries. Inverse metabolic engineering was applied to *S. cerevisiae* for the higher yield of amylase. Firstly, screening was conducted based on UV-random mutagenesis as well as selection for growth on starch. Later, the genetic mutations linked with overproduction of amylase were detected. S196I point mutation of *VTA1* gene encoding a protein engaged in vacuolar sorting resulted in higher amylase secretion by 35% (Liu et al., 2014). Similarly, genes linked with lipase transport in *Burkholderia cepacia* were amplified and subcloned in pNZ8148 vector; further transformation was done in *Lactococcus lactis* (Raftari et al., 2013). Recombinant lipase expression was observed to be higher ($\sim 152 \cdot 2$ μg/ml/h) than the wild strain. Asparaginase is an enzyme used in reduction of acrylamide during baking and also used as chemotherapeutic agent in

treatment of lymphoblastic leukemia. Einsfeldt et al. (2016) cloned the gene encoding L-asparaginase of *Zymomonas mobilis* in pET vectors along with a histadine tag. The enzyme was found to be expressed in *E. coli*. The recombinant protein was produced (0.13 IU/mL extracellular L-asparaginase and 3.6 IU/mL intracellular L-asparaginase) in a bioreactor after 4h when induced with induction of Isopropyl β-D-1-thiogalactopyranoside.

5.5.5 Production of vitamins

The demands of vitamins are increasing day by day. Hence, research is being conducted globally to enhance the production of microbe-derived vitamins through metabolic engineering. Successful results have been obtained for the production of provitamin A, vitamin C, and B vitamins. A study by Sauer et al. (2004) revealed the production of vitamin C by *S. cerevisiae* and *Zygosaccharomyces bailii* when incubated along with L-galactose, L-galactono-1,4-lactone, or L-gulono-1,4-lactone intermediates (of pathway for vitamin C production); however, in ordinary conditions, yeast do not produce vitamin C (Sauer et al., 2004). Yeast cells overexpressed with D-arabinono-1,4-lactone oxidase and L-galactose dehydrogenase were observed to convert 40% (w/v) of the raw material L-galactose. Vitamin C synthesis was carried out by engineering *Kluyveromyces lactis* with L-galactose biosynthesis pathway genes (L-galactose is the prime intermediate for the synthesis of L-ascorbic acid) of *Arabidopsis thaliana*. The genes isolated were *GME*, encoding GDP-mannose 3, 5 epimerase, *VTC 2* encoding GDP-L-galactose phosphorylase, and *VTC 4* representing L-galactose-1-phosphate phosphatase. The metabolically modified *K. lactis* strains could convert lactose and D-galactose to L-galactose for further synthesis of L-ascorbic acid (Rosa et al., 2013).

Synthesis of vitamin B1 was improved (titer 1.3 mg/L) in *Bacillus subtilis* mutation of salvage thiamine pyrophosphokinase (*thiN*), thiamine permease (*thiT*), and thiamine ABC transporter component (*thiW*) (Schyns et al., 2005). Similarly, threonine aldolase was overexpressed (*GLY1*), serine hydroxymethyltransferase encoding gene (*SHM2*) was disrupted in *Ashbya gossypii* for an overproduction of vitamin B2, i.e., more than 20 g/L (Abbas and Sibirny, 2011). It was also observed that *C. glutamicum* could produce 1000 mg/L of vitamin B5 with β-alanine as fed precursor when *ilvA* gene was inactivated and native ILvBNCD and PanBC enzymes were overexpressed (Hüser et al., 2005). Fang et al. (2018) have engineered an *E. coli* strain metabolically and application of genetic engineering. Genes encoding adenosylcobinamide phosphate (intermediate of vitamin B12 synthesis) were incorporated in *E. coli* for enhanced vitamin B12 yield (more than 250 fold).

Microbial vitamin K production was documented in *Bacillus subtilis*, *Propionibacterium freudenreichii*, and *Flavobacterium* sp., but the vitamin K biosynthesis model microorganism was not developed for mass production. Several researchers have thoroughly studied the process of synthesis of vitamin K_2 (menaquinone, MK) in *B. subtilis* (Mahdinia et al., 2018).

Microorganisms contribute to approximately 15% of the total industrial production of biocommodities. In past few years, many strategic approaches to metabolic engineering were established to generate carotenoids in various species (Bhatia and Victor,

2012) *Saccharomyces cerevisiae* and *Candida subtilis* were engineered to express genes of bacterial carotenoid secretion, supplying between 0.1 and 0.4 mg g/L dry cells with beta-carotene titers. *Escherichia coli* was also biologically engineered to generate the carotenoid using various techniques where gene expression from *Streptococcus pneumoniae* and *Enterococcus faecalis* were most active, reaching 460 mg/L titers (Ajikumar et al., 2010; Zhao et al., 2013). *S. cerevisiae* was updated to express carotenogenic genes through *Xanthophyllomyces dendrorhous*, resulting in levels of beta-carotene exceeding 6.3 mg/g dry cells (Verwaal et al., 2010; Yan et al., 2012). Other hosts that were currently being developed to develop carotenoids such as *Pichia pastoris* (out of which small amounts of β-carotene were developed to date: 0.34 mg/g dry cells) (Araya-Garay et al., 2012) and *Yarrowia lipolytica* (Sabirova et al., 2011).

Vitamin B2 is identified as riboflavin. At the moment, *B. subtilis* and *Eremothecium gossypii* genetically engineered expressing *RIB* genes are industrially used in manufacturing of riboflavin. Perkins et al. (1999) have produced a riboflavin producing strain comprising several versions of transformed *B. subtilis* riboflavin biosynthetic operons (rib operon) incorporated at two various sites in *B. subtilis* chromosome. Vitamin B2 is extracted from the fermented mixture via centrifugation through heat inactivation of the microorganisms.

5.5.6 Release of biominerals

Iron and zinc deficiencies are the major health problems worldwide. Phytic acid is the major storage form of phosphorous in cereals, legumes, oilseeds, and nuts. It is known as a food inhibitor which chelates micronutrients (i.e., iron, zinc, calcium, manganese, etc.) preventing them to be bioavailabe for monogastric animals, including humans because they lack enzyme phytase in their digestive tract. Some filamentous fungi (i.e., *Aspergillus ficuum* NRRL 3135, *Aspergillus oryzae*), yeast (*S. cerevisiae*), and bacteria (*B. subtilis* Natto and LAB) facilitate release of essential minerals from food matrices by virtue of possessing phytase (Singh et al., 2017). The phytase active LAB isolates belong to the species *Lactobacillus panis, Lactobacillus reuteri, Lactobacillus fermentum*, *Lactobacillus plantarum,* and *Pediococcus pentosaceus*. In a recent study, the highest extracellular phytase production was found in *Lb. panis* with a volumetric phytase activity of 140 U/mL. Phytate degradation in whole-wheat dough fermented with *Lb. panis* or *Lb. fermentum* was 90% and 70%, respectively (Nuobariene et al., 2015).

5.5.7 Production of bacteriocins

Bacteriocins are ribosomally blended antimicrobial peptides created by microscopic organisms. Numerous bacteriocins delivered by food grade lactic acids microscopic organisms display the possibility to control deterioration and pathogenic microbes in nourishment. Uncommonly few bacteriocins alongside their local antibacterial property likewise display extra enemy of viral and hostile to parasitic properties. Bacteriocins are by and large created by Gram + ve, Gram - ve and archaea microorganisms. Bacteriocins from Gram + ve bacteria particularly from lactic acid microbes (LAB)

have been completely explored considering about their incredible biosafety and expansive mechanical applications. LAB communicating bacteriocins were segregated from matured milk and milk items, rumen of creatures, and soil utilizing conceded opposition examine. Nisin is the main bacteriocin that has got FDA endorsement for application as a sustenance additive, which is created by *Lactococcus lactis* subsp. *Lactis*. At present, bacteriocins are exclusively connected in food ventures; however, they have an extraordinary potential to be utilized in different fields, for example, nourishes natural manures, ecological insurance, and individual consideration items. The eventual fate of bacteriocins is to a great extent reliant on getting FDA endorsement for utilization of different bacteriocins notwithstanding nisin to advance the examination and applications (Silva et al., 2018).

5.6 Other bioactive compounds used as food supplements

5.6.1 Food flavors

Flavor is typically the results of the presence of many volatile and nonvolatile elements possessing numerous chemical properties. The nonvolatile compounds contribute principally to the taste, while volatile ones influence aroma and flavor. These volatile compounds include alcohols, aldehydes, esters, dicarbonyls, short- to medium-chain free fatty acids, methyl group ketones, lactones, phenoplast compounds, and sulfur compounds (Longo and Sanromán, 2006).

5.6.1.1 Lactones

Lactone flavors with fruity, milky, coconut, and other aromas are widely used in the food and fragrance industries. *Trichoderma viridae* creates a trademark coconut flavor because of the generation of 6-pentyl-2-pyrone. The primary part of peach flavor is 4-decalactone which can be contributed by *Sporobolomyces odorus*. Both δ- and γ-lactones are utilized generally as flavor and scent compounds. A manufactured case of lactone creation is the microbial change of ricinoleic acid through incomplete β-oxidation toward γ-decalactone, which include a peach-like smell, by yeasts, such as *Yarrowia lipolytica* and *Sporidiobolus salmonicolor* (Vandamme and Soetaert, 2002; Lee et al., 2018).

The fatty acid biosynthetic (FAS-B) pathway of *Brevibacterium ammoniagenes* was used to produce triacetic acid lactone (TAL) from glucose rather than a petroleum-based raw material. The ketoreductase (KR) domain of the FAS-B was inactivated by mutating its key catalytic residue to enable it to produce TAL. It was assumed that the KR domain would include the sequence from amino acid residue 2051 to 2319. This sequence turned out to be a member of the short-chain dehydrogenase/reductase (SDR) super family. A replacement in the sequence was made to inactivate it. *Saccharomyces cerevisiae* yeast was transformed to express the modified

FAS-B and also phosphopantethiene tranferase (PPT1) from *B. ammoniagenes*. The yeast was able to produce TAL in vivo (Zhao, 2004).

A novel method to produce flavor lactones from abundant nonhydroxylated fatty acids using yeast cell factories was described. Oleaginous yeast *Yarrowia lipolytica* was engineered to perform hydroxylation of fatty acids and chain-shortening via β-oxidation to preferentially 12- or 10- carbons. The strains could produce γ-dodecalactone from oleic acid and δ-decalactone from LA. Through metabolic engineering, the titer was improved 4-fold, and the final strain produced 282 mg/L γ-dodecalactone in a fed-batch bioreactor (Braga and Belo, 2016).

5.6.1.2 Aromatic esters

Many sectors of industry, mainly food, cosmetics, and pharmaceutics, have increased their interest in esters due to their flavor property. Flavor esters that possess an aromatic ring in their molecular structure are also known as aromatic esters. These esters are widely found in nature (fruits and plants), and the synthetic (i.e., via chemical) and microbial routes (i.e., via fermentation/bioprocessing) are suitable for their biocatalysis. Almeida et al. recorded 94 distinct esters as being distinguished in brew. The majority of the esters found in brew are shaped through essential fermentation (Almeida et al., 2017). Several yeasts such as *S. cerevisiae, Pichia anomala*, *Candida utilis*, *Kluyveromyces marxianus,* and *Candida utilis* were found to age glucose to ethyl acetic acid derivation when developed on a medium constrained in iron. Ethanol could be changed over into either ethyl acetic acid derivation or acetaldehyde. Two pathways might be utilized to develop esters: (1) the alcoholysis of acyl-CoA components and (2) the immediate esterification of an organic acid with an alcohol (Loser et al., 2014).

5.6.1.3 Carbonyls

Diacetyl is one of the most significant carbonyls that imparts a nut-like flavor and can be extensively used as a food ingredient. The most significant diacetyl producing organisms are *Leuconostoc citrovorum*, *Leu. creamoris*, *Leu. dextranicum*, *Streptococcus lactis* subspecies *diacetylactis*, *S. thermophilus,* and certain strains of *Proprionibacterium shermani* (Chen et al., 2017).

In a recent study, diacetyl could be produced from the nonenzymatic oxidative decarboxylation of α-acetolactate during 2, 3-butanediol fermentation. In this study, the 2, 3-butanediol biosynthetic pathway in *Enterobacter cloacae* subsp. *dissolvens* strain SDM, a good candidate for microbial 2, 3-butanediol production, was reconstructed for diacetyl production. To enhance the accumulation of the precursor of diacetyl, the α-acetolactate decarboxylase encoding gene (*budA*) was knocked out in strain SDM. Subsequently, the two diacetyl reductases DR-I (*gdh*) and DR-II (*budC*) encoding genes were inactivated in strain SDM individually or in combination to decrease the reduction of diacetyl. Although the engineered strain *E. cloacae* SDM (Δ*budA*Δ*budC*) was found to have a good ability for diacetyl production, more α-acetolactate than diacetyl was produced simultaneously. In the end, by using the

metabolically engineered strain *E. cloacae* SDM (Δ*budA*Δ*budC*), diacetyl at a concentration of 1.45 g/L was obtained with a high productivity (0.13 g/L/h) (Zhang et al., 2015).

5.7 Microalgae in food supplements

Among the new passages in the food supplements segment, bioproducts containing microalgae as is representing a quickly growing business sector. The showcased items are essentially founded on three creation strains, i.e., *Spirulina* and *Chlorella*, followed by *Klamath*. It is a composite circumstance, since two of them are cyanobacteria and the subsequent one is eukaryotic (Klein-Marcuschamer et al., 2013; Koyande et al., 2019). Algal oil is still significantly more expensive than fish oil applications, although many groups are improving both the cost and quality of omega-3 oil from algal sources (Koyande et al., 2019).

Production of DHA mostly belong to the marine members of the families *Thraustochytriaceae* and *Crypthecodiniaceae*. The *Thraustochytrids* include the genera, *Schizochytrium* and *Ulkenia*, whereas dinoflagellate *Crypthecodinium* is a genus of the family Crypthecodiniaceae (Klok et al., 2014). Members of these genera are widely dispersed in the oceans of the world. By heterotrophically culturing these microorganisms, the omega-3 biotechnological processes for DHA production have gone into industrial scale (Ren et al., 2010). However, the production of EPA is still being restricted to laboratory scale. The traditionally used EPA producers are the algae *Phaeodactylum tricornutum*, *Nannochloropsis*, and *Nitzchia* (Wen and Chen, 2003). The relatively low accumulated biomass and slow growth rate of these algae hindered the industrial EPA production.

5.8 Food supplements global trend, environmental and regulatory issues

The estimated global food supplements market size is between 74.14 and 123.28 billion USD in FY 2019−20 and is projected to expand at a CAGR of 6.34%−8.2% during the forecasted period. The market is backed by rising health awareness globally among consumers of all age groups and growing awareness toward calorie reduction and weight loss in major markets including the US, China, and Italy is expected to promote food supplement, in addition, changing lifestyles and food habits are driving the product demand. Positive outlook toward sports nutrition market is also among major driving factors. Individual food supplements market analysis showed that global essential fatty acids market is expected to reach around USD 9.15 billion by 2026, growing at a CAGR of 9.8% between 2019 and 2026 (https://www.zionmarketresearch.com/report/essential-fatty-acids-market).Essential amino acids are generally produced through processes such as fermentation, which is the most widely used process and accounts for

major share in global production (9.3 Million Tons in 2019) of amino acids(https://www.grandviewresearch.com/industry-analysis/amino-acids-market).

Many scientific and regulatory challenges exist in research on the safety, quality, and efficacy of food supplements are common to all countries as the marketplace for them becomes increasingly global. Some of the key issues that commonly arise are (1) evaluating evidence for product claims as the market for food supplements has increased, (2) distinguishing between a food supplement and other categories such as conventional foods and biologics. To address these issues, US Congress passed the Dietary Supplements Health and Education Act (DSHEA) 1994, which defined dietary supplement as a product taken by mouth containing a dietary ingredient intended to supplement the diet. DSHEA then granted the U.S. FDA authority to establish regulations regarding dietary supplement manufacturing, regulating health claims, and labeling of dietary supplements, and creating governmental bodies to encourage research on supplements. On the other hand, the European Union (EU) in 2002 has created a legal and regulatory framework named as the "Food Supplements Directive 2002/46/EC" containing a list of nutrients and their chemical forms able to be used in food supplements. However, the maximum levels and conditions of use for other substances, such as botanicals, botanical preparations, and bioactive substances, such as lutein and glucosamine, are not harmonized and, therefore, fall under national legislation. While these products must comply with a series of European laws, the compositions of these products are still largely subject to national legislation, resulting in numerous trade barriers even between EU member states.

5.9 Challenges, future prospectives and concluding remarks

Challenges are basically the acceptance of these products worldwide with various diverse ethics, beliefs, habits, diet, and cultures. In agreement with the legislation, the labeling of these health supplements should embrace. Affirmation that it is a health/food supplement after the name of the product, the recommended dose of the product for daily consumption, the statement that the supplements do not substitute a balanced diet, statement about the storage of the product away from children are some of the critical issues. Until now, the spotlight has been on maximal biomass production while the dose effects of probiotics have not been extensively studied. The physiological state of the cells has also to be well thought-out to take advantage of health benefits. In this regard, technologies and operating conditions may be of significant importance and should be assessed.The exploitation of these microorganisms at a massive scale should be conducted to accomplish a better yield of food supplements for easy and cheap availability. Furthermore, screening of proficient isolates and subsequent efforts regarding strain improvement should be facilitated. Nutrition and health will continue to be the driving forces for exploiting the potential of microorganisms, to arrive at even more efficient processes for health supplements production.

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The lipases and their applications with emphasis on food industry

6

Akshita Mehta, Suman Guleria, Roji Sharma, Reena Gupta
Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

6.1 Introduction

Enzymes are considered as natural catalysts produced by the cell and responsible for high rate and specificity of one or more intracellular and extracellular biochemical reactions. Enzymes are used industrially because of their high catalytic power, specific mode of action, ecofriendly use, and reduced energy requirements (Marques et al., 2014). Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use (Sharma and Kanwar, 2014). Because of improved understanding of production chemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded their demand (Godfrey and West, 1996; Wilke, 1999). There is always a demand for new enzymes that may offer better properties for specific applications in ever-changing industrial activities (Konarzycka-Bessler and Jaeger, 2006). The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. Atleast 75% of all industrial enzymes (including lipases) is hydrolytic in action (Sharma et al., 2001). The basic enzymatic reaction is represented in Fig. 6.1.

Enzymes are widely used in pharmaceuticals, surfactants, cosmetics, laundry, food dressing, fuel industries, and dairy industries. Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds (Mohan et al., 2005; Akanbi et al., 2010). There are 75% of industrial enzymes that are hydrolytic and of microbial origin (including lipases) (Behera et al., 2019). Lipolytic enzymes are involved in the breakdown and thus, in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another. Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes the hydrolysis of the carboxyl ester bonds in triacylglycerols

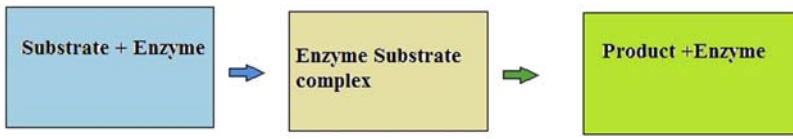

Figure 6.1 Diagrammatic representation of reaction catalyzed by the enzyme.

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(TAGs) to produce diacylglycerols, monoacylglycerols, fatty acids, and glycerol (Fotouh et al., 2016; Priyanka et al., 2019).). In addition, lipases catalyze the hydrolysis, acidolysis, aminolysis, and transesterification of other esters as well as the synthesis of esters (Gupta, 2016). Many lipases exhibit enantio-selective properties (Jaeger and Eggert, 2002; Gupta et al., 2004; Hasan et al., 2006). In contrast to esterases, lipases are activated only when adsorbed to an oil−water interface (Martinelle et al., 1995) and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues (Balashev et al., 2001). Lipase possesses the unique features of interface between an aqueous and nonaqueous phase which provide a new understanding of a rapidly moving field (Hasan et al., 2006). First lipase was discovered in a pancreatic juice in the year 1856 by Claude Bernard. Animal pancreatic extracts were traditionally used as the source of lipase for commercial applications. The number of available lipases has increased mainly as a result of achievements made in the cloning and expression of enzyme from microorganisms, as well as of an increasing demand for these biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature (Matos et al., 1987; Bornscheuer, 2000, 2002).

6.2 Sources of lipases

Lipases are widely distributed in animals, plants, and microorganisms (Bornscheuer, 2002; Bharathi et al., 2018); however, microbial lipases are commercially most important mainly because of ease of their cultivation and genetic manipulation to obtain higher yield (Hasan et al., 2006). Lipases are produced by both eukaryotes including animals, plants and fungi and prokaryotes including bacteria and archaea (Wang et al., 2009). Microbial lipases have received great attention due to the variety of their biochemical activities and ease of their isolation and production (Hasan et al., 2006). Microbial lipases are also more stable than plant and animal derivatives, and their production is safer for industrial and research applications (Ruiz et al., 2005). Because of their higher activities at neutral or alkaline pH optima, bacterial enzymes are more preferred than fungal enzymes. Moreover, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutritional needs, and easy screening procedures for desired properties (Hasan et al., 2006). Because of these properties, bacterial lipases have immense applications in food, detergent, pulp and paper, leather industries, and environmental management (Jeager and Eggert, 2002; Hasan et al., 2006; Narwal et al., 2015; Junior et al., 2017). Lipases that are capable of catalyzing reactions at low or moderate temperatures hold a great potential in certain areas of industry such as detergent, synthesis of heat labile compounds, and biodiesel production. One of the most obvious

advantages of using such enzymes is cutting down the cost of energy expenditures for processes that naturally require high temperatures (Tyndall et al., 2002). Different lipase-producing microorganisms are shown in Table 6.1.

6.2.1 Bacterial lipases

Many bacterial lipases are well studied compared to plants and animals. Bacterial lipase is a glycoprotein but some extracellular bacterial lipases are lipoprotein. The organisms are normally grown on nutrient medium containing carbon (oil, sugar, and mixed carbon sources), nitrogen, phosphorus sources, and mineral salts whereas the production of lipases mostly depends on inducer such as triglycerides, bile salts, and glycerol. Lipases from *Pseudomonas* and *Bacillus* sp. were probably the first studied and have preponderant role in industries, later on *Achromobacter* sp., *Alcaligones sp*., *Pseudomonas* sp., *Staphylococcus* sp., and *Chromobacterium* sp., have been exploited for production of lipases (Lang et al., 1991). Three dimensional structure of lipase from *Bacillus stearothermophilus* (PDB ID:1JI3) is shown in Fig. 6.2.

6.2.2 Fungal lipases

Fungal lipases have benefits over bacterial lipases due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. Lipase producers are widespread in the fungal kingdom. The chief producers of lipases *Aspergillus* sp., *Candida* sp., *Mucor* sp., *Rhizopus* sp. have been studied (Aguieiras et al., 2019). The thermophilic *Mucor pusillus* is well known producer of thermostable extracellular lipase.

6.2.3 Plant lipases

In plants, mostly lipases are present in food reserve tissues of growing seedlings or especially in those which contain large amount of TAGs. Lipase activity in plant seeds increases during germination because the TAGs are converted to soluble sugars by the action of lipase which is then transported to the growing tissues to supply structural carbon and energy to provide support for the growth of young plants.

6.2.4 Animal lipases

Animals are also rich sources of lipases but due to the availability of microbial lipases they are rarely studied, but still they have been isolated from many insects, fishes, and mammals. Animal lipase plays an important role in digestion of lipids in biological system (Walton et al., 1984). Fats require special digestive action before absorption because the end products must be carried in water medium (blood and lymph) in which fats are not soluble. Although little actual fat digestion occurs in the stomach, gastric lipase does digest already emulsified fats such as in egg yolk and cream.

Table 6.1 Lipase producing microorganisms.

Lipase producing microorganisms	Reference
Bacillus sp.	Imamura and Kitaura (2000); Kumar et al. (2012)
Bacillus subtilis	Ruiz et al. (2005)
Bacillus thermoleovorans	Rua et al. (1997)
Bacillus thermocatenulatus	Lee et al. (1999)
Pseudomonas sp.	Sarkar et al. (1998); Shukla and Desai (2016)
Pseudomonas aeruginosa	Chartrain et al. (1993)
Pseudomonas fluorescens	Kojima et al. (1994)
Pseudomonas fragi	Nishio et al. (1987)
Enterococcus faecalis	Kar et al. (1996)
Lactobacillus plantarum	Lopes et al. (1999)
Staphylococcus haemolyticus	Oh et al. (1999)
Staphylococcus aureus	Gotz et al. (1998); Nehal et al. (2016)
Staphylococcus warneri	Simon et al. (1996)
Staphylococcus xylosus	Moshbah et al. (2005)
Penicillium cyclopium	Chahinian et al. (2000)
Penicillium simplicissimum	Sztajer et al. (1992)
Aspergillus niger	Namboodiri and Chattopadhaya (2000); Sharma et al. (2016)
Aspergillus oryzae	Toida et al. (1998)
Botrytis cinerea	Commenil et al. (1995)
Chromobacterium viscosum	Taipa et al. (1995)
Streptomyces flavogriseus	Mostafa and Ali (1979)
Trichosporon asteroides	Dharmsthiti and Ammaranond (1997)
Trichosporon laibachii	Liu et al. (2004)
Rhizopus sp.	Macedo et al. (2003)
Rhizomucor miehei	Herrgard et al. (2000)
Geotrichum candidum	Jacobsen and Poulsen (1995)
Pichia burtonii	Sugihara et al. (1995)
Candida cylindracea	Muralidhar et al. (2001)
Acinetobacter sp.	Snellman et al. (2002)
Fusarium solani	Knight et al. (2000)

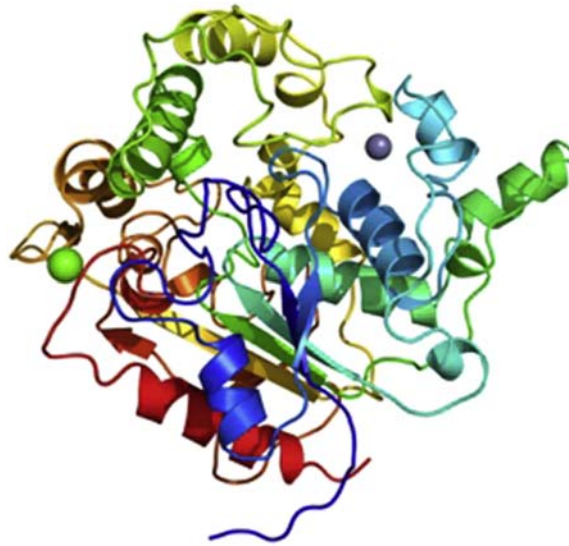

Figure 6.2 Structure of thermostable lipase from *Bacillus stearothermophilus* (PDB ID:1JI3) REF. Protein is shown as a cartoon colored from blue (N-terminus) to red (C-terminus). Zn^{2+} and Ca^{2+} ions are shown as gray and green spheres, respectively.
Tyndall, J., Sinchaikul, S., Gilmore, L., Taylor, P., Walkinshaw, M. 2002. Crystal structure of a thermostable lipase from *Bacillus stearothermophilus* P1. Journal of Biotechnology 323, 859−869. With Permission.

6.3 Process optimization of lipase

For last few years, to meet the demand of lipases, various qualitative and quantitative methods have been undertaken for uses in different fields. Lipases have much significance due to their substrate specificity and stability. They have low cost of production which promotes various applications. Microbial lipases are inducible, and their synthesis depends upon the presence of TAG, fatty acids, surfactants, vegetable oils, bile salts, etc. (Bradoo et al., 1999). The medium optimization and strain improvement is required for overproduction of lipase from the wild strains (Haq et al., 2009; Iftikhar et al., 2010a, 2010b).

Lipase production is affected by growth parameters such as type of nutrients and temperature; therefore, lipase activity can be increased by providing optimum culture conditions to the growing fungus (Shreya et al., 2018). Lipases can be produced by two processes such as submerged fermentation (SmF) or solid-state fermentation (SSF) although fungi grow better on SSF than yeast and bacteria (Gutarra et al., 2009). SmF has advantages in process control and production yields by which they can be widely used in the enzyme industry whereas SSF can produce industrial enzymes at minimum costs (Gutarra et al., 2009; Edwinoliver et al., 2010). SSF can be preferred over SmF because of lower operating costs, less demands for asepsis control, cheaper fermentation media, higher oxygen distribution, fewer operational troubles, simpler equipments and control systems, and lower energy consumption for microbial enzyme production (Damaso et al., 2008).

To achieve the diversified roles of enzymes, new technologies such as metabolic engineering, protein engineering, and directed evolution are required to be adopted.

6.3.1 Role of metabolic engineering

Metabolic engineering of microbes is widely used as an alternative to produce various products and chemicals (Lennen and Pfleger, 2013; Zhou et al., 2014; Pfleger et al., 2015). The extracellular production of lipase by metabolic engineering of *Pseudomonas fluorescens* allows the secretion of a thermostable lipase enzyme (Son et al., 2012).

Structural features of lipases are important characteristics for protein engineering to provide efficient biocatalysts for commercial exploitation. Protein engineering has been used extensively to tailor-design lipase enzymes for improved performance and durability (Kourist et al., 2010; Singh et al., 2013). Both rational design and directed evolution approaches have been successfully applied to redesign lipases for enhanced thermostability, tolerance to organic solvents, and substrate specificity (Kourist et al., 2010; Singh et al., 2013). Additionally, the production of lipase has been improved by optimal selection of host strains (Treichel et al., 2010) and utilization of metabolic engineering principles (Song and Ramkrishna, 2011; Ramkrishna and Song, 2012). The use of free enzymes has technical limitations due to difficulty of their recovery for reuse, which increases the process cost. Immobilization methods have been utilized to allow recycling of enzyme biocatalysts, which decreases cost and further improves their activity (Jegannathan et al., 2008; Tan et al., 2010). Finally, the biodiesel production process must be optimized to maximize yield of biodiesel while minimizing the process cost (Fjerbaek et al., 2009).

6.4 Crisper gene editing

A clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system is a prokaryotic adaptive immune system against invading mobile elements like phages and plasmids (Barrangou et al., 2007; Selle and Barrangou, 2015). Pancreatic lipase CRISPR/Cas9 KO Plasmid (m) is designed to disrupt gene expression by causing a double-strand break (DSB) in a 5′ constitutive exon within the Pnlip (mouse) gene.

6.5 In silico simulation and bioinformatic analysis

Bioinformatic analysis was used to identify subfamily-specific positions (SSPs)—conserved only within lipase and peptidase subfamilies, but different between them—that is supposed to be responsible for functional discrimination between enzymes with different catalytic activities.

Lipases belonging to serine hydrolase family are one of the largest group of enzymes in diverse catalytic functions, and they have α/β-hydrolase fold, catalytic triad, and an active site and the oxyanion hole (Ollis et al., 1992). To study structure—function relationship of multiple catalytic capabilities, α/β-hydrolases provide an excellent model within active sites. In this view, many efforts have been attempted to analyze and characterize significant structural data.

Comparative bioinformatic analysis of α/β-hydrolases with lipase activities has been performed, and high structural similarity of active sites has been observed despite different types of catalyzed chemical transformations, low sequence, and full-structure identity. While completely conserved positions in α/β-hydrolases define properties that are common for the entire family (for example, have a direct role in enzyme catalytic machinery), they do not explain functional diversity. Thus, bioinformatic analysis method was used to identify SSPs—conserved only within protein subfamilies, but different between subfamilies—that was supposed to be responsible for functional discrimination between lipases and peptidases. These hotspots were used to construct *in silico* library of *Candida antarctica* lipase B (CALB) mutants (Suplatov et al., 2012).

6.6 Applications of lipases

Lipases have attracted much interest in enzyme technology in recent years. This is partly because of modifications of the natural substrates of lipases; the TAG is of great technical interest. Lipases are essential components in the modern industrial process due to their ability to catalyze, depending on the thermodynamic conditions, hydrolysis reaction, as well as synthesis reactions such as esterification and transesterification (Casas-Godoyl et al., 2012). Lipases are commonly used in the processing of fats and oils, food processing, leather, textile, detergents and degreasing formulations, paper manufacture, synthesis of fine chemicals, and production of pharmaceuticals and cosmetics (Houde et al., 2004; Loli et al., 2015; Mouad et al., 2016; Garcia-Silvera et al., 2017; Tomke et al., 2017). Because of the vast applications, newer microbes are to be screened for production of lipases having desirable properties (Verma et al., 2012). Major applications of lipases are summarized in Table 6.2.

6.6.1 In food industry

Fats and oils are important constituents of foods. The nutritional and sensory value and the physical properties of a triglyceride are greatly influenced by factors such as the positions of fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturations (Ray, 2015). Lipases are able to modify the properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more fatty acid with new ones (Zorn et al., 2016). Cocoa butter has high fat value because it contains palmitic and stearic acids and has a melting point approximately 37°C (Ray, 2015). Melting of cocoa butter in the mouth produces a desirable cooling sensation in a product such as chocolate.

Lipases are used *ex situ* to produce flavor and to modify the structure by inter- or transesterification in order to obtain products of increased nutritional value, or suitable for parental feeding (Reetz, 2002). Lipases are also been used for in food to modify flavor by synthesis of esters of short-chain fatty acids and alcohol, which are known flavor and fragrance compounds (Macedo et al., 2003). Lipases facilitate the removal

Table 6.2 Industrial applications of lipases (Sharma et al., 2001).

Industry	Action	Application
Dairy foods	Hydrolysis of milk, fat, cheese ripening, and modification of butter fats	Development of flavoring agent in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Detergents	Hydrolysis of fats	Removal of oils stains from fabrics
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressing, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish product fat removal
Pharmaceuticals	Hydrolysis of expolyester alcohols	Production of various intermediates used in manufacture of medicine
Cosmetics	Synthesis	Act as emulsifiers and moisturizers
Pollution control	Hydrolysis and transesterification of oil and grease	To remove hard stains and hydrolyze oil and grease
Surfactants	Replace phospholipases in the production of lysophospholipids	Polyglycerol and carbohydrates fatty acid esters used as industrial
Fuel industries	Transesterification	Biodiesel production
Agrochemicals	Esterification	Herbicides such as phenoxypropionate

of fat from meat and fish products (Sharma et al., 2001). Cao et al. (1996) reported a lipase-catalyzed solid-phase production of sugar fatty acid esters. Lipases are used for the production of maltose and lactose like sugar fatty acid esters (Sharma and Kanwar, 2014). *Candida rugosa* lipases have many applications in food and flavor industry and in the production of ice cream (Ray, 2015).

6.6.1.1 *In dairy products*

Lipases have immense applications in food industry. They are used in the processing of various dairy products like milk, butter, ice cream, and cheese by increasing the fat stability (Libaek et al., 2006). They give characteristic flavors to cheese, through the free fatty acids produced by the breakdown of milk fats (Karaca and Guven, 2018). In contrast to lipases that hydrolyze a broad range of TAGs, phospholipases used in

dairy processing are more specific, such that flavor defects caused by the release of short-chain fatty acids can be avoided (Lilbaek et al., 2006). They can improve the characteristic flavor of cheese by acting on the milk fats to produce free fatty acids after hydrolysis (Jooyandeh et al., 2009). Different types of cheese can be made by using lipases from various sources, e.g., Romano cheese using kid/lamb pregastric lipase, Camembert cheese using lipase from *Penicillium camemberti,* and cheddar cheese using *Aspergillus niger* or *Aspergillus oryzae* (Aravindan et al., 2007). Many commercial lipases such as Palatase 20000 L, MER, AY30G, Snow plum blossom, and Calf PGE have been widely used in the manufacture of lipolysed milk fats (LMFs) to improve the consistency and aroma of the milk products (Saxena et al., 1999; Jooyandeh et al., 2009; Peng et al., 2014; Tambe et al., 2015). Lipase catalysis could improve the texture and softness of cheese. In addition to flavor improvement, most of the commercial lipases produced are utilized for processing of other foods, such as meat, vegetables, fruit, smoked carp, milk products, baked foods, and beer (Farahat et al., 1990; Nagodawithana and Reed, 1993; Ivic et al., 2016).

6.6.1.2 Application in baking

Baking is used for the production of many goods such as bread, biscuits, cookies, cake, and pastries. Since 1990, lipase is used for the production of baked products. Lipases are also used as flavor development agents in butter and margarine (Aravindan et al., 2007). Phospholipases produce the emulsifying lipids by degrading the wheat lipids, and they are used in improving the flavor content of bakery products by esterification reaction by which these enzymes are preferred over the traditional emulsifiers. These enzymes help to improve the texture and esthetic appeal of bread and other baked goods. Lipases from *B. subtilis* have been found to play a major role in bread making (Sangeetha et al., 2011; Ray, 2012). Other applications of lipases in baking industry are prolonging shelf-life, softness improvement, and volume improvement (Moayedallaie et al., 2010; Ray, 2012).

6.6.1.3 Flavor enhancement

The production of low molecular weight flavor esters is of great importance in food industry (Talon et al., 1996). Lipase is used as an additive for a wide variety of flavor and perfumes. Lipase from *Candida rugosa* was immobilized on silica to catalyze esterification reaction to produce ethyl butyrate, a flavor ester. *Pseudomonas fluorescens* lipase (PFL) and *Rhizopus japonicas* lipase (RJL) were employed for synthesis of (*R*)-(+)-citronellol and (*S*)-(−)-citronellol with low water content (Wang and Linko, 1995; Majumder et al., 2007). In addition, geranyl acetate and citronellyl acetate synthesis (75%−77% conversion) using *Mucor miehei* lipase (MML) was studied by Chatterjee and Bhattacharyya (1998). Lipases from *Bacillus aerius* and *Geobacillus* sp. have been used for the synthesis of isoamyl acetate and methyl salicylate, respectively, which have flavor enhancing properties in confectionary items such as chewing gums (Narwal et al., 2016).

6.6.1.4 Egg processing

Egg yolk is made of 16% protein, 32% lipids, and 50% water. About one-third of the lipids are phospholipids, mainly phoshatidylcholine (about 80%). Conversion of egg yolk phospholipids into lysophospholipids through lipases increases the emulsion stability. Phospholipases are used in enhancing the emulsifying power of egg yolk, thus, not only improving the performance of egg lipids but also reducing the requirement of egg yolk rate in food processing such as dressings and mayonnaise-like products. Emulsifying properties of egg come from its lipids. Phospholipase hydrolyzes egg lecithin and isolecithin. Processing of egg yolk increases the emulsifying capacity as well as temperature stability of the egg yolk. So, processed egg yolk is used for producing mayonnaises, custards, baby foods, sauces, etc (Aravindan et al., 2007). Eastern European countries and Russia are the main markets with approximately one-third of emulsified dressings being consumed there. Big global brands such as Nestlé, Kraft, and Unilever are a major player in this industry.

6.6.2 In detergent industry

The most commercially important field of application for hydrolytic lipases is as additives in detergents in industrial, laundry, and household detergents (Wiseman, 1995; Lodha et al., 2016; Garcia-Silvera et al., 2017). Lipase can reduce the environment load of detergent products as it saves energy by enabling a lower wash temperature to be used (Vakhlu and Kour, 2006). The detergent industry is the largest industry for this enzyme (Tambekar et al., 2013). The latest in detergent industry is lower wash temperatures which not only saves energy, but also helps to maintain the texture and quality of fabrics (Weerasooriya and Kumarasinghe, 2012). Detergent industries are primary consumers of enzymes, in terms of both volume and value (Verma et al., 2012). The use of enzymes in detergent formulations enhances the detergents ability, removes the tough stains, and makes the detergent environmentally safe. Nowadays, many laundry-detergent products contain cocktails of enzymes including proteases, amylases, cellulases, and lipases (Jeon et al., 2009). Lipases should have criteria to serve detergent additives, stability at alkaline pH, solubility in water, surfactants, and low substrate specificity (Rahman et al., 2006).

Genencor International introduced commercial bacterial lipases namely Lipomax from *Pseudomonas alcaligenes* and Lumafast from *Pseudomonas mendocina*, which were used as detergent enzymes in the year 1995 (Rahman et al., 2006).

6.6.3 In pulp and paper industry

The paper and pulp industry processes huge quantities of lignocellulosic biomass every year. The technology for pulp manufacture is highly diverse and numerous opportunities exist for the application of microbial enzyme (Verma et al., 2012; Choudhary and Bhunia, 2015). Lipase can be used in removing the pitch from pulp produced in the paper industry (Sharma and Kanwar, 2014). Pitch is the term used to describe collectively hydrophilic components of wood, namely, triglycerides and waxes, which

cause severe problems in pulp and paper manufacture (Gutierrez et al., 2009). The enzymatic pitch control method using lipase was put into practice in a large-scale paper making process as routine operation in the early 1990's and was the first case in the world in which an enzyme was successfully applied in the actual paper-making process (Bajpai, 1999). Lipases are also used to enhance deinking and bleaching in paper and pulp industry and waste treatment by increasing biological oxygen demand (BOD) and chemical oxygen demand (COD) (Srivastava and Singh, 2015; Singh et al., 2016).

6.6.4 In fat and oleochemical industry

The current trend in the oleochemical industry involves the use of immobilized lipases to initiate various reactions (hydrolysis, alcoholysis, and glycerolysis) using mixed substrates (Verma et al., 2012; Kiran et al., 2016). The use of lipases in oleochemical industry is enormous as it saves energy and minimizes thermal degradation during esterification, acidolysis, interesterification, and aminolysis (Vulfson, 1994; Bornscheuer, 2000). Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze alcoholysis (an acyl moiety displaced between an acyl glycerol and an alcohol), acidolysis (an acyl moiety displaced between an acyl glycerol and a carboxylic acid), and transesterificaton (two acyl moieties exchanged between two acylglycerols) (Sharma et al., 2001; Kataki and Salehi, 2016). *Mucor miehei* and *Candida antarctica* lipases were used for esterification of free fatty acids in the absence of organic solvent and transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols (Akoh, 1993). Immobilized MML in organic solvent has been used to catalyze the interesterification reactions for the production of vegetables oils such as corn oil, olive oil, sunflower oil, peanut oil, and soyabean oil containing omega-3 polyunsaturated fatty acid (Li and Ward, 1993). Interesterification and hydrogenation techniques are useful in the preparation of glyceride products for use in the production of margarine and butter.

6.6.5 In textile industry

The use of lipase in textile industry is becoming widely important (Ismail et al., 2015). Lipases are used for the removal of size lubricants in order to provide the fabric better absorbency for enhanced levelness in dying. It also reduces the frequency of cracks and streaks in the denim abrasion systems. Lipases with alpha amylases are used for desizing of the denim and other cotton fabrics at the commercial scale (Rowe, 2001). In the textile industry, polyester has certain important advantages including softness, high strength washabilty, stain resistance, stretch, machine resistance, and wrinkle resistance. It relates to modification of the characteristics of polyester fiber so that such polyesters are additionally susceptible to postmodification treatments (Hasan et al., 2006). The use of polyesterase that is closely related to lipase can improve the ability of polyester fabric to uptake chemical compounds, dyes, antistatic compounds, antistaining compounds, antimicrobial compounds, and deodorant compounds (Rowe, 2001).

6.6.6 In pharmaceutical industry

In the pharmaceutical industry, enzymes offer several advantages over chemical synthesis, thereby justifying the growing demands for lipase. Microbial lipases are used to enrich polyunsaturated fatty acid (PUFA) from animal and plants lipids, and their mono- and diacylglycerides are used to produce variety of pharmaceuticals (Dong et al., 1999). PUFAs are widely used as food additives, pharmaceuticals, and nutraceuticals because of their metabolic benefits. Lipase is a component of a hair-waving preparation in which it promotes penetration of the preparation (Saphir, 1967).

6.6.7 In cosmetics and personal care products

Lipases have potential application in cosmetics of its activities in surfactants and in aroma production (Metzeger and Bornscheuer, 2006). Monoacylglycerols and diacylglycerols are produced by esterification of glycerols and are used as surfactants in cosmetics and perfumes industries (Sharma and Kanwar, 2014). Lipases are produced by *Pseudomonas cepacia* and have been used to resolve racemic rose oxides produced by the bromomethoxylation of citronellol (Taneja et al., 2005). Methyl butyrate or methyl ester of butyric acid is an ester with a fruity odor pineapple, apple, and strawberry (Garlapati and Banerjee, 2013). Esters of aliphatic or aromatic acids and alcohols including terpene alcohols, aldehydes, and phenols are commonly present in flavor materials used in the perfumes and other personal care products (Franssen et al., 2005). Retinoids (vitamin A) are of great commercial importance in cosmetics and pharmaceuticals such as skin care products. Esters of cinnamic acid, ellagic acid, ferulic acid, and so forth are organic compounds of biotechnological relevance that could be suitably modified flavor/fragrance compounds, precursors of pharmaceuticals, and used as additives in foods, cosmetics, and sunscreens (Chandel et al., 2011; Saun et al., 2017). It has maximum UV absorption at 322 nm which falls between the UVB and UVA region, and, hence, can be used as a potential UV-absorbing substance for skin protection against sunlight (Kumar and Kanwar, 2011).

6.6.8 In medical applications

Lipases are used in medical applications. The level of lipases in blood serum can be used as diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury (Nagar et al., 2013). Lipases play an important role in modification of monoglycerides for use in emulsifiers in medical applications (Sharma et al., 2001). Lipases from *Candida rugosa* have been used to synthesize lovastatin, a drug that lowers the cholesterol level (Sharma and Kanwar, 2014). They act as a diagnostic tool, and their increasing levels can indicate certain infection or diseases. Obesity causes metabolic diseases and is a serious health problem around the world (Gupta et al., 2015; Loli et al., 2015). Lipases have medical applications such as in modification of castanospermine (a promising drug for the treatment of AIDS), antihypertensive agents such as angiotensin-converting enzyme inhibitors and in synthesis of calcium channel blocking drug such as diltiazem (Kumar and Ray, 2014).

6.6.9 In biodiesel production

Biodiesel is an alternative fuel for petroleum-based diesel and is biodegradable, renewable, noninflammable, and nontoxic. In biodiesel production, lipases are used as biocatalysts. The lipase-catalyzed transesterification reaction takes place between a lipid and short-chain alcohol to produce an ester and glycerol (Chen et al., 2009: Raita et al., 2010; Narwal et al., 2015). Biodiesel production from *Aspergillus* sp. using oil obtained from *Colophyllum inophyllum* seeds has been reported (Siva et al., 2015). The most commonly employed bacterial lipase for biodiesel synthesis is from *Pseudononas cepacia* (Li and Yan, 2010). The lipase from *Proteus mirabilis* is a particularly promising catalyst for biodiesel synthesis as it produces high yields of methyl esters even in the presence of large amounts of water and expresses very well in *Escherichia coli* (Korman et al., 2013). Soybean biodiesel production using the commercial product Novozym 435 within the temperature range of 45−70°C was the highest (92%) when 65°C was used (Brusamarelo et al., 2010). Lipases from *Candida rugosa, Pseudomonas cepacia*, and *Pseudomonas fluorescens* have been used for biodiesel production (Pandey, 2009). Specific lipases need gradual addition of methanol to achieve high yield between 80% and 90%; this is probably due to acyl migration of sn-2 to sn-1, which occurs spontaneously in glycerides. Huang et al. (2012) studied the methanolysis of soybean oil utilizing *Rhizopus miehei* displacing *Pichia pastoris* whole-cell biocatalyst.

6.6.10 In ester synthesis

The application of lipases in organic media is one of the most exciting facts of biotechnology industry in recent times. A variety of fatty acid esters are now being produced commercially using immobilized lipase in nonaqueous solvents (Kumar and Kanwar, 2011; Ruela et al., 2013; Saun et al., 2017). The esters produced from long-chain fatty acids (12−20 carbon atoms) and short-chain alcohols (3−8 carbon atoms) have been used in food, detergent, cosmetics, and pharmaceutical industries (Bauer et al., 1990). Esters prepared by reaction of long-chain acids with long-chain alcohols have important applications such as plasticizers and lubricants (Gandhi et al., 1995). Similarly, alcoholic esters of short-chain fatty acids are important flavor and aroma compounds, whereas esters of long-chain fatty acids are being explored for their use as fuel (biodiesel) and waxes in the oleochemical industries (Pandey et al., 1999; Saxena et al., 1999; Hasan et al., 2006; Narwal and Gupta, 2013; Gashaw and Lakachew, 2014; Eryilmaz et al., 2016).

6.7 Conclusion

Microorganisms are capable of producing several enzymes for their survival within a wide range of substrates. Among these enzymes, lipases are predominantly used in several applications. The growing demand for lipases has shifted the trend toward prospecting for novel lipases, improving the properties of existing lipases for established

technical applications and producing new enzymes for new areas of applications. They are one of the most versatile enzymes available in nature. These fat-splitting enzymes are attractive because of their applications in fields relevant to food, detergent, paper, oleochemical, pharmaceutical, cosmetics, medicine, and textile industry. The tremendous potential of lipases in various industries shows the need to develop novel cost-effective technologies for increased production, scaling up, and purification of this versatile enzyme.

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Conflict of interest

The authors confirm that this article content has no conflict of interest**.**

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Biogenic amines in fermented vegetables: food safety issues

7

Sudhanshu S. Behera[1,2], *Sandeep K. Panda*[3], *Ramesh C. Ray*[2]
[1]Department of Fisheries and Animal Resource Development, Directorate of Fisheries, Government of Odisha, Odisha, India; [2]Centre for Food Biology and Environment Studies, Bhubaneswar, Odisha, India; [3]School of Biotechnology, KIIT University, Bhubaneswar, Odisha, India

7.1 Introduction

Biogenic amines (BAs) are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones present in living organisms, and initiate numerous pharmacological and metabolic reactions (Naila et al., 2010). BAs are organic bases with low molecular weight and synthesized by microbial, vegetable, and animal metabolisms (Pessione and Cirrincione, 2016). In food and beverages, they are formed by the enzymes of raw material or are generated by microbial decarboxylation of amino acids. Exogenous/extracellular BAs are more frequently found in fermented foods such as wine, beer, dairy products, fermented meat and vegetables, sauerkraut, and soybean products (Behera et al., 2019b). Many factors may alter the BA contents in fermented foods including temperature, pH, salt content, the microbial load, and the storage conditions. BAs may lead to symptoms such as nausea, discomfort, hot flashes, cold sweat, palpitations, headaches, red rash, and abnormally high or low blood pressure in human beings.

Vegetables provide a significant part of human nutrition and source of natural antioxidants such as carotenoids, flavonoids and other phenolic compounds, vitamins as well as minerals and dietary fibers (Ray and Panda, 2007; Panda and Ray, 2016; Behera et al., 2019a). Fermented vegetables are considered to be healthy since apart from high amounts of vitamins, minerals and dietary fibers, they also contain high levels of glucosinolate hydrolysis products having anticarcinogenic activity (Ray and Panda, 2007; Ray and Shivkumar 2009). Traditionally, vegetables such as cabbage, cucumber, radish, carrot, green tomato, green pepper, and root crops such as tapioca, sweet potato, yams, and elephant foot yams are lactic acid fermented in large volumes for human consumption (Swain and Ray, 2016; Behera et al., 2020). Moreover, the consumers' demand toward fresh-like, high nutritional value, health promoting and rich flavor, ready-to-eat or ready-to-drink nonfermented/fermented foods, and beverages is increasing over the years (Di Cagno et al., 2015; Behera et al., 2018a). The fermentation is considered as the simplest and valuable bioprocess technology to keep and/or increase the nutritional, safety, sensory, and shelf life of vegetables (Ray et al., 2014). Although there is considerable literature pertaining to BAs in

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fermented food products, there is a paucity of information on BAs in fermented vegetables and associated metabolic disorders. This article focuses on the available literature on the occurrence and adverse effects of BAs in fermented vegetables, detection methods, strategy for removal, and safety issues.

7.2 Mechanism of BAs production

According to the chemical structure, BAs are classified into heterocyclic (histamine and tryptamine), aliphatic (putrescine and cadaverine), or aromatic (tyramine and phenylethylamine) compounds (Table 7.1) (Sahu et al., 2015; Biji et al., 2016). Generally, amines are basic nitrogenous compounds in which one, two, or three atoms of hydrogen in ammonia are replaced by alkyl or aryl groups. Amino acid decarboxylation (happens by removal of the α-carboxyl group) is the most common mode of synthesis of amines in foods (Fig. 7.1), and the aromatic amines may render the food toxic; for example, decarboxylase of histidine to histamine produces many varied effects within the body, including the contraction of smooth muscle tissues of the lungs, uterus, and stomach, the dilation of blood vessels, which increases permeability and lowers blood pressure, etc. When these amines and/or amino acids are formed by the action of living organisms through the decarboxylation and/or transamination (aldehyde and ketone moiety) in fermented commodities, they are designated as biogenic (Shalaby, 1996).

7.3 Traditional fermented vegetables and biogenic amines

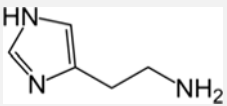
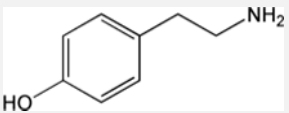
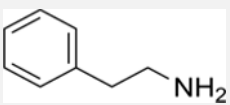
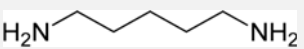
The fermented vegetables are popular in several African, Asian, European, and Latin American countries (Sivakumar et al., 2010). Many vegetables, including cabbage, cucumbers, turnips, cauliflower, green tomatoes, French beans, carrots, onions, celery, and radishes are fermented into nutritious products (Sivakumar et al., 2010; Behera and Panda, 2020). However, BAs have been found in some traditional fermented vegetables as a consequence of the activities of fermenting or contaminating microorganisms having amino acid decarboxylase activity (Peñas et al., 2010). Some examples of the occurrence of BAs in fermented vegetables are discussed in the following subsections.

7.3.1 Fermented leafy vegetables, radish, and cucumbers

7.3.1.1 Sauerkraut

Sauerkraut (sour cabbage) is one of the most popular traditional fermented vegetables produced by fermentation of white cabbage (*Brassica oleracea* L. var. *capitata* cv. Bronco) (Peñas et al., 2010; Ray et al., 2014; Behera et al., 2018b). It is the most used vegetable food during winter season in the Czech Republic (Liu et al., 2017). In *sauerkraut* fermentation, fresh cabbage is shredded and mixed with salt

Table 7.1 Biogenic amines, precursors, structure, and their decarboxylase enzyme found in fermented vegetables.

Biogenic amine	Amino acid precursor	Classification	Structure	Decarboxylase enzyme or pathway in LAB
Histamine	Histidine	Heterocyclic		Histidine decarboxylase (HDC)
Tyramine	Tyrosine	Aromatic		Tyrosine decarboxylase (TDC)
2-phenylethylamine	Phenylalanine	Aromatic		Tyrosine decarboxylase (TDC)
Cadaverine	Lysine	Aliphatic		Lysine decarboxylase (LDC)
Putrescine	Arginine	Aliphatic		Ornithine decarboxylase (ODC)
	Agmatine	Aliphatic		Agmatine deiminase (AgDI)

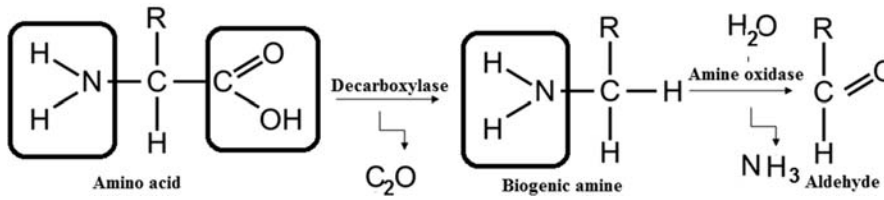


Figure 7.1 Decarboxyation of amino acid and lead to formation of biogenic amine.

(2.3%−3.0%) and allowed for natural fermentation (Wu et al., 2014). The fermentation process occurs in sequential steps involving *Leuconostoc* sp. in the initial phase, and *Lactobacillus* and *Pediococcus* spp. in the subsequent phases (Xiong et al., 2012). The BA level in *sauerkrauts* (21−122 mg/kg) (Peñas et al., 2010) is limited by fermentation conditions such as temperature and starter culture (Cvetkovic et al., 2015). The starter culture, *Leuconostoc mesenteroides,* and 5% salt concentration are the optimal requirements for production of *sauerkrauts* of good quality (Peñas et al., 2010). Cvetkovic et al. (2015) reported that most appropriate process parameters for low BAs and polyamines content (20−35 mg/kg) were found to be 1%−2% (salt concentration) and 18−20°C (temperature) during spontaneous fermentation of white cabbage (*Brassica oleracea*). Specific LAB (lactic acid bacteria) strains of the *Lactobacillus* genus can effectively prevent BA synthesis. However, limited content of BAs (33−52 mg/kg) in *sauerkraut* could serve as a healthy beverage for vegetarians and lactose-allergic consumers (Ren et al., 2016).

7.3.1.2 Kimchi

Kimchi, a traditional Korean fermented food, is made from fermenting vegetables such as salted radish, cucumber, and Chinese cabbage (beachu) along with spices, including garlic, ginger, and red pepper powder (Ray et al., 2014; Lee et al., 2015; You et al., 2017). Kimchi is fermented by LAB at low temperatures ensuring proper ripening, preservation, and has strong acidic taste (Jung et al., 2014; Lee et al., 2015). The classical identification of bacterial isolates from *kimchi* revealed that *Lactobacillus plantarum* and *Leu. mesentroides* are the predominant LAB species (Kim and Chun, 2005). Jeong and Lee (2015) reported that BA production is strain-specific and is more common among *Weissella* spp. than *Leuconostoc* spp in *Kimchi.* BAs, which are harmful to human health, have not been reported in *kimchi,* and generally *kimchi* is considered to have low levels of BAs because its major ingredients (i.e., cabbage, radish, garlic, ginger, and red pepper powder) do not contain high levels of BA precursors (Jeong and Lee, 2015). However, high levels of histamine (>5 mg/100 g) have been reported in *kimchi* sold in Taiwan (Tsai et al., 2005; Jeong and Lee, 2015).

7.3.1.3 Pao cai

Pao cai, one of the Chinese traditional lactic acid fermented vegetable, is usually treated in brines with high salt concentrations for long-term preservation. Four kinds

of *pao cai*: acidified cabbage (*Brassica oleracea* L.), radish (*Raphanus sativus* L.), pak choi (*Brassica chinensis* L.), and cowpea (*Vigna unguiculata* (Linn.) Walp.) are homemade popular side dishes used in China (Luo et al., 2015). The dominated LAB such as *Lb. plantarum*, *Lb. pentosus*, *Lb. fermentum*, *Lb. brevis*, *Lb. lactis*, and *Leu. mesenteroides* are found to deplete nitrite and inhibit the growth of nitrite-reducing bacteria during *pao cai* fermentation (Yan et al., 2008). A LAB coculture (*Lb. plantarum, Lb.buchneri,* and *Pediococcus ethanoliduran*) fermentation in *pao cai* increased the content of lactic acid (sourness), sucrose, and glycine (sweetness), along with a significant quantity of γ-aminobutyric acid (Zhao et al., 2016).

7.3.1.4 Gundruk

Gundruk is a fermented leafy vegetable consumed as pickle or soup in Nepal and Himalayas regions of India. In processing of *gundruk*, fresh leaves of vegetables locally named as "rayo-saag" [*Brassica rapa* L. ssp c*ampestris* (L.)], leaves of radish (*Raphanus sativus* L.), mustard [*Brassica juncea (*L.) Czern], cauliflower (*Brassica oleracea* L. var. *botrytis* L.), cabbages (*Brassica oleracea* L. var. *capitata* L.) are wilted for 1−2 days (Tamang and Tamang, 2010; Ray et al., 2014). However, there is a limited production of toxins and BAs (>100 mg/kg) associated with health risk reported from *gundruk* (Satish Kumar et al., 2013; Gautam and Sharma, 2015).

7.3.1.5 Sunki

Sunki, is a traditional, homemade fermented vegetables produced in the Kiso District of Japan, produced from the leaves of otaki-turnip. Otaki-turnip is boiled, mixed with zumi (a wild small apple), and dried *sunki* from previous year, and allowed to ferment for one or 2 months (Montet et al., 2014). It is produced in every house in Kiso during late autumn and winter. The bacterial community is found stable, and *Lb. delbrueckii, Lb. fermentum, and Lb. plantarum* are dominant LAB throughout the fermentation process (Endo et al., 2008). The dominant microorganisms in fermented *sunki* are reported to produce significant amounts of lactic acid (Vijayendra and Halami, 2015). Tomita et al. (2018) applied metabolomics to the investigation of the biochemical profiles of *sunki* samples. Different BAs such as α-aminobutyric acid, γ-aminobutyric acid, putrescine, cadaverine, tyramine, tryptamine, histamine, and phenethylamine were found during *sunki* fermentation, and the bacterial communities are most likely to have a direct impact on the metabolite profile. However, the levels of BAs such as tyramine and histamine detected in several *sunki* samples were estimated to be lower than 17.0 and 11.3 mg/L, respectively (Tomita et al., 2018).

7.3.1.6 Pickled vegetables

Nozawana-zuke is a low-salt pickle vegetable prepared from mustard, locally called *Nozawana* (*Brassica campestris* var. *rapa*) and consumed by the majority of Japanese people (Kawahara and Otani, 2006; Swain et al., 2014). Generally, the LABs isolated from the spontaneous *Nozawana-zuke* pickle fermentation are: *Lb. plantarum*, *Lb. brevis*, *Leu. mesenteroides*, *Pediococcus pentosaceus,* and *Enterococcus faecalis*

(Kawahara and Otani, 2006; Panda et al., 2007; El Sheikha and Ray, 2017). *Tursu*, a traditional fermented Turkish pickle, is made from vegetables such as cabbage, cucumber, carrot, pepper, turnip, eggplant, and beans (Çetin, 2011). LAB involved in the fermentation of *tursu* are *Leu. mesenteroides*, *Lb. plantarum*, *Lb. brevis,* and *Pediococcus pentosaceus*. BA (histamine) content of mustard pickle product is found to be in the range of 7.4−8.9 mg/kg of the retail and supermarket samples, although the average contents of each of the nine BAs were found less than 2 mg/kg (Kung et al., 2006; Saaid et al., 2009).

7.3.1.7 Sinki

Sinki is a nonsalted fermented radish taproot product traditionally consumed as pickle in some north-eastern states of India, Nepal, and Bhutan (Tamang et al., 2012; Ray et al., 2014).The optimum fermentation time is usually 12 days at 30 C when the pH of the fermenting mass drops from 6.7 to 3.3 (Tamang et al. 2012, 2016). The fermentation is initiated by heterofermentative *Lb. fermentum*, followed by heterofermentative *Lb. brevis,* and finally succeeded by homofermentative *Lb. plantarum* (Swain et al., 2014). However, the enzymatic activities of LAB such as peptidase, lipase, and esterase help in the development of flavor in fermented *sinki* (Tamang et al., 2012; Vijayendra and Halami, 2015). Tamang et al. (2009) conducted the screening of LAB strains for production of BAs in ethnic fermented vegetables of the Himalayas, and ornithine was detected in five strains of *Lb. brevis* from *sinki* samples.

7.3.1.8 Khalpi

Khalpi is a fermented cucumber (*Cucumis sativus* L.) eaten as pickle by adding mustard oil, salt, and powdered chillies. During the preparation, ripened cucumbers are cut into suitable pieces, sun dried for 2 days, and then put into a bamboo vessel, locally called "dhungroo" and made air tight before naturally fermenting for 4−7 days (Tamang and Tamang, 2010). Similar to *khalpi*, *Jiang-guais* is a popular traditional fermented cucumber in Taiwan. There has been no report of any food poisoning or infestation by consuming *khalpi* or *Jiang-guais* to BAs (Tamang and Tamang, 2010).

7.3.2 Fermented roots and tuber crop products

7.3.2.1 Gari

Tuber crops such as sweet potato, cassava, yams, and elephant foot yams are considered as vegetables (Ray and Shivkumar 2009; Behera and Ray, 2017; El Sheikha and Ray, 2017). *Gari* is the most popular fermented food consumed by the people of Nigeria and is derived from solid state fermentation of tapioca (syn.cassava) (El Sheikha and Montet, 2014). The initial stage of *gari* fermentation is dominated by *Corynebacterium* sp. with LAB succession (*Lb. acidophilus*, *Lb. casei*, *Lb. fermentum*, *Lb. pentosus*, *Lb. plantarum*, respectively) (Oguntoyinbo and Dodd, 2010; Behera

and Ray, 2017). During the preparation of *gari*, the cyanide content is reduced to below the safe level of 30 mg/kg of cassava (Ray and Shivkumar 2009). However, there is no report of BA toxicity in *gari*.

7.3.2.2 Lafun

Lafun is an African cassava fermented food product obtained by soaking peeled cassava tuber in water at ambient temperature (28−32°C) for 2−5 days (Padonou et al., 2010; El Sheikha and Montet, 2014; Behera and Ray, 2017). The dominated microflora such as *Lb. plantarum*, *Leu. mesenteroides* contribute cell wall−degrading enzymes (polygalacturonases and polygalacturonate lyases), and *Corynebacterium* sp. reported to contribute pectinolytic enzymes for softening cassava roots during *lafun* fermentation (Padonou et al., 2010). There is no report of BA toxicity in *lafun*s.

7.3.3.3 Lacto-pickle

The most common fermented foods are prepared using lacto-fermentation. Behera et al. (2018c) studied the production of lacto-pickle using elephant foot yam (EFY) in the presence of *Lb. plantarum* (MTCC-1325). The viability of the strain was determined during long-term storage at room temperatures, and viable cell counts are found to remain constant (Behera et al., 2019a). There is no report of BA toxicity in EFY lacto-pickle.

7.3.3 Fermented vegetable legume (soybean) products

Soybean, originated in Eastern Asia a 1000 years ago, is considered as one of the important protein-rich (around 40%) vegetable legume (Ordóñez et al., 2016). Additionally, fermented soybean foods contain various functional components, i.e., peptides and isoflavonoids (Ordóñez et al., 2016). As reported by "Health" magazine in 2006, soybean products are classified as one of the world's top five healthiest foods. Additionally, soybean is well known to be useful for preventing obesity, diabetes, heart disease, and breast cancer (Yang et al., 2011).

All foods that contain proteins are subjected to conditions enabling microbial or biochemical activity; BAs can be expected in them, including fermented and nonfermented soybean products (Toro-Funes et al., 2015). There are different types of soybean products (e.g., soy milk, soy curd, soy sauce, soybean paste, etc.) in which BAs can be detected. Fermentation is one of the major processes used in the production of food from soybeans (Yang et al., 2011). Primary sources of BAs in the soy foods include fermented foods such as *soy sauce* (China), *miso* (Japan), *nattō* (Japan), *stinky tofu* (China), and *tempeh* (Indonesia) (Ray et al., 2014; Jayachandran and Xu, 2019). Microorganisms form BAs during the fermentation process of soybean products (Kung et al., 2006; Kim et al., 2012). The microbial spoilage of food may be accompanied by the increased production of decarboxylases. Therefore, the presence of BAs might serve as a useful indicator of food spoilage in soybeans (Jayachandran and Xu, 2019).

Several species of molds, yeasts, and LAB are involved in the soy fermentation processes. Since soybeans are rich in protein, the synthesis of amines is predicted during the fermentation process (Shalaby, 1996). Several studies have shown that BAs in fermented soybean are most likely formed by the fermenting lactic microflora, and histamine and tyramine were found at various concentrations. The variability of BAs levels in the fermented soy products had been attributed to the variations in production processes (Shalaby, 1996; Jayachandran and Xu, 2019).

7.3.3.1 Doenjang

Soy *paste* (*Doenjang*) is a traditional Korean fermented soybean paste. Its name means "thick paste" in Korean language. *Doenjang* is produced through the fermentation of soybeans by naturally occurring bacteria, yeast, and mold, and has been consumed for centuries as a protein-rich source. This paste contains a relatively high concentration of amino acids degraded from soybeans and may be a source for BAs formation (Jayachandran and Xu, 2019). The mean values of BAs in *doenjang* samples found to be the range of 18.37 mg/100g. Decarboxylase activity has been described in different microorganisms, including *Bacillus*, *Citrobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pediococcus*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella,* and *Streptococcus* (Jung et al., 2016; Kim et al., 2016). Kim et al. (2016) isolated two BA-producing bacteria from traditional soybean pastes: one was histamine-producing *Clostridium* strain and the other was a tyramine-producing *Pseudomonas* strain. The *Meju*, a starter culture made by using different microbial strains (i.e., combination of bacteria and fungi), also used for the production of *doenjang* (Shukla et al., 2014). However, *doenjang* sample made from a mixed starter culture of fungal [i.e., *Aspergillus oryzae* J (A), *Mucor racemosus* 15 (M15), and *M. racemosus* 42 (M42)] and bacterial (i.e., *Bacillus subtilis* TKSP 24 (B)) strains reported as a potential source of natural antioxidants for the use in food and medicine industries (Shukla et al., 2016).

7.3.3.2 Chunjang

Chunjang is a Korean and Chinese traditional fermented sweet bean sauce produced by the fermenting steamed soybeans, flour, and salt by naturally occurring bacteria (most strains identified as *B. subtilis*). The BAs contents of *chunjang* sample contained histamine (1.85 mg/kg) and tyramine (19.57−31.35 mg/kg) (Bai et al., 2013). However, other remaining BAs were detected at safe levels below 30 mg/kg (Park et al., 2019).

7.3.3.3 Tofu-misozuke

Tofu-misozuke is a traditional fermented soybean product originating in Kumamoto, Japan, that usually contains BAs, but is toxic when consumed at high levels. Takebe et al. (2016) investigated the bacterial diversity and BAs content in *tofu-misozuke*. The tyramine synthesis was evaluated by PCR (polymerase chain reaction) detection of the tyrosine decarboxylase gene. It is claimed that the three tyramine-producing bacteria, i.e., *Enterococcus faecium*, *Weisselia viridescens,* and *Lb. curvatus,* were more prominent than others.

7.3.3.4 Soy sauce

Soy sauce is a condiment produced from paste of boiled soybeans, roasted grains, brine, and fermented by *Aspergillus oryzae* or *Aspergillus sojae* molds (Bai et al., 2013). Soy sauce is a traditional component in East and Southeast Asian cuisines, where it is used as a condiment and in cooking. The flavor in soy sauce may develop gradually during the fermentation and aging. Meanwhile, the free amino acids exist in soy sauce in high amount that could be potential sources of BA formation. There are variable factors that affect the production of BAs, i.e., the ratio of soy in the raw material, microbial flora, and duration of fermentation (Wei et al., 2013). Park et al. (2019) reported that the BA concentrations of retail soy sauce at safe concentrations lower than 20 mg/kg were the recommended limits for consumption. *Jijang* is a black soybean sauce made of fried *chujang* and commonly mixed the diced meat and vegetables before or after frying (Bai et al., 2013). Like other fermented soybean products, *jijang* contains abundant dietary amino acid precursors of BAs. However, relatively small amounts of BAs (>40 mg/kg) (compared to *chunjang* sample) have been detected in *jijang* samples (Bai et al., 2013).

7.3.3.5 Miso

Miso is a traditional Japanese paste. It is produced by fermenting rice, barley, and soybeans with salt and the fungus. This paste is used for sauces and spreads, pickling vegetables or meats and Japanese culinary staple (Ray et al., 2014). In *miso*, tyrosine decarboxylase bacteria have been identified as *Enterococcus faecium, Lb. bulgaricus,* and histamine decarboxylase have been associated with *Lactobacillus* species and *Lactobacillus sanfrancisco* (Nam et al., 2012). The BAs concentrations in miso had the levels of 1.49−17.81 mg/kg (e.g., putrescine, 2.73−17.81 mg/kg, histamine, and 1.49−4.62 mg/kg) and are within recommended limits for consumption (Toro-Funes et al., 2015). Lee et al. (2015) reported that the *miso* inoculated with *Lb. plantarum* as a starter culture had lowered histamine and total BAs content by 58% and 27%, respectively.

7.3.3.6 Nattō

Nattō is a traditional Japanese soy product made from soybeans fermented with *B. subtilis* (Ray et al., 2014). Tsai et al. (2005) identified some histamine-producing bacteria belonging to *Lactobacillus* species in *nattō* products manufactured in Taiwan. However, *Bacillus subtilis* is highly capable of producing β-phenylethylamine (∼30 mg/kg) and tyramine (∼100 mg/kg) in *nattō* products (Kim et al., 2012). Toro-Funes et al. (2015) reported that the BA content of *natto* was at safe concentration lower than 75.21 mg/kg, the recommended limits for consumption.

7.3.3.7 Sufu

Sufu (*furu*) is a kind of Chinese traditional fermented soybean curd made from soybean that is easily digested and a nutritious protein (Tang et al., 2011; Ma et al., 2013; Gu et al., 2018). The pure starter culture consists mainly of molds (*Actinomucor*, *Mucor*,

and *Rhizopus*) or bacteria (*Micrococcus* and *Bacillus* spp.) responsible for *sufu* fermentation. The process of *sufu* manufacture is itself carried out under nonsterile conditions, which caused microbial contamination and led to the formation of BAs. However, *Clostridium perfringens* grows in protein-rich media. Accordingly, this bacterium is often detected in the amino acid rich environment, including *sufu*. The type of dressing (salting and ripening) used determines the color, and subsequently the category of *sufu* (white, gray, brown, sesame oil, hot, and alcoholic *sufu*). In *sufu*, the commonly found BAs are putrescine, cadaverine, and tryptamine (Guan et al., 2013). However, the BAs (β-phenylethylamine, histamine, and tyramine) content of *sufu* exceeded recommended toxicity limits by a factor of approximately 0−0.17 mg/kg (Yang et al., 2020).

7.3.4 Fermented bamboo shoot products

Soidon (*soibum*) is a nonsalted fermented food prepared from the succulent bamboo shoot tip of *Schizostachycum capitatum* Munro by using traditional starter "soidon-mahi" in Manipur state of India (Jeyaram et al., 2010). *Ekung, eup,* and *hirring* are also some common indigenous fermented bamboo products of northeast India (Tamang and Tamang, 2009). The dominant strains of *B. subtilis*, *Lb. brevis, Lb. lactis, Lb. casei, Lb. xylosus, Lb. curvatus, Lb. plantarum,* and *Tetragenococcus halophilus* are generally responsible for developing starter as well as the production of quality ethnic fermented bamboo shoot (Jeyaram et al., 2010). These LAB are reported to govern several technological properties including acidifying and antimicrobial activities, degradation of phytic acids (19.33 U/mL) along with the production of BAs in *soidon* (<300 mg/kg) (Tamang and Tamang, 2009; Sonar and Halami, 2014).

7.3.5 Miscellaneous fermented vegetable products

7.3.5.1 Ziang-sang/Ziang-dui

Ziang-sang (Ziang-dui) is a fermented leafy vegetable product which is common to both Nagaland and Manipur states of India. It is made dominantly by Naga women and sold in local markets. The major representatives of the LAB involved in fermented *ziang-sang* are *Lb. brevis*, *Lb. plantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici,* and *Leuconostoc fallax* (Tamang et al., 2009). There is no report of BA poisoning in *ziang-sang*.

7.3.5.2 Jiang-guais

Jiang-guais is a fermented vegetable products prepared from cucumber, soy sauce, with ingredients of salt, sugar, and vinegar, which is originated in Republic of China, a 1000 years ago (Di Cagno et al., 2013). The LAB associated with the *Jiang-gua* are *Enterococccus casseliflavus*, *Leuconostoc lactis*, *Leuc. mesenteroides*, *Lb. pentosus*, *Lb. plantarum*, *Lb. paraplantarum*, *Lactococcus lactis* subsp. *lactis*, *Weissella hellenica*, and *Weissella cibaria* (Di Cagno et al., 2015). As mentioned above, there is no report of BA poisoning in *jiang-guais*.

7.3.5.3 Dhamuoi

Dhamuoi, another food prepared mainly from fermentation of cabbage and other vegetables, is prevalent in Vietnam. *Leuc. mesenteroides* and *Lb. plantarum* are the main LAB associated with fermented *dhamuoi* (Di Cagno et al., 2013). There has been no report of BA toxicity in *dhamoui*. Table 7.2 summarizes microorganisms and BAs reported in fermented vegetables.

7.4 Molecular techniques as modern detection tools for BAs

There are two reasons for the detection of amines in foods:

- Their potential toxicity and
- The possibility of using them as food quality markers (Shukla et al., 2011).

Early detection of BAs-synthesizing microorganisms is essential in the fermented food industry in order to avoid the risk of amines formation. Several traditional (i.e., paper chromatography, spectrofluorometry) as well as sophisticated techniques [i.e., PCR-DGGE (Denaturing Gradient Gel Electrophoresis)] are used for detection of BAs (Fiocco et al., 2007; Shukla et al., 2011). Enzymatic methods including radioimmuno assay and enzyme-linked immunosorbent assay (ELISA) were employed for the detection of BAs, i.e., histamine (Guesdon et al., 1986), with the advantages of rapidity and not requiring expensive instrumentation like High Performance Liquid Chromatography (HPLC) (Stratton et al., 1991). Lange and Wittman (2002) developed an enzyme sensor array method for the simultaneous detection of BAs (histamine, tyramine, and putrescine) in food samples within a span of 20 min.

PCR-based techniques are used to develop molecular detection tools targeting the genes encoding the enzyme amino acid decarboxylase and for that several primers are developed (Fiocco et al., 2007). Using target genes, all the microorganisms involved in BA synthesis in a given sample (i.e., fermented vegetables) can be detected. A multiplex PCR assay for the simultaneous detection of LAB strains, which potentially produce histamine, tyramine, and putrescine, in fermented foods has been developed (Coton and Coton, 2005). DNA is the preferred target for BAs identification, although targeting mRNA for a particular BA rather than DNA is probably a more suitable tool. Indeed, DNA may still be detectable when cells might have disappeared due to food process management, i.e., fermentation process or abiotic stresses encountered (e.g., high ethanol concentration, low pH), while detection of mRNA reflects the presence of effectively viable microorganisms. Detection of genes involved in amino acid decarboxylation is usually performed previously to bacteria isolation. Therefore, a molecular approach performed directly on food sample is preferred over traditional methods (Fiocco et al., 2007). It is evident from the aforementioned PCR-based approaches that it may be used for the selection and characterization of starter cultures as well as their use for the early detection of BA producers within a food production process including the food fermentation. In the fermentation case, several false positive results arise from PCR-based reactions because of the high sequence of diversity of genes encoding decarboxylases. For this reason, it is recommended to perform an additional

Table 7.2 Recent advances in molecular techniques on detection of dominant microflora inhabited fermented vegetable foods.

Fermented food products	Raw material/ substrate	Area of distribution/ country	Dominant microflora/starter culture involved	BA reported	Molecular techniques detected	Reference
Fermented vegetables						
Sauerkraut	Cabbage	Europe, USA, Australia, Canada	*Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lb. brevis*, *Lb. sakei*, *Pediococcus pentosaceus*, *Lb. pentosus*	33−52 mg/kg; 20−35 mg/kg	16S rDNA analysis	Ren et al. (2016), Cvetkovic et al. (2015)
Kimchi	Cabbage, green onion, ginger, hot pepper	Korea	*Lb. sakei*, *Lb. brevis*, *Lb. curvatus*, *Lb. plantarum*, *Leuc. mesenteroides*, *Leuc. citreum*, *Leuc. gasicomitatum*, *Leuc. holzapfelii*, *Leuc. Lactis*, *Leuc. gelidum*, *Weissella cibaria*	>5 mg/100 g	16S rRNA gene sequencing, MALDI-TOF MS	Tsai et al. (2005), Jung et al. (2014), An et al. (2014), Jeong et al. (2016), Hong et al. (2015)
Pao cai	Cabbage	China	*Lb. pentosus*, *Leuc. mesenteroides*, *Lb. plantarum*, *Lb. casei*, *Lb. rhamnosus*, *Alkali bacterium* sp.,	–	16S rDNA analysis, 16S rRNA PCR product analysis	Yan et al. (2008), Chang et al. (2013), Liang et al. (2018)
Gundruk	Leafy vegetable	Nepal, Bhutan, India	*Lb. plantarum*, *Lb. fermentum*, *Lb. casei*, *Ped. pentosaceus*, *Ped. acidilactici*, *Leuc. fallax*,	>100 mg/kg	16S rDNA analysis	Tamang and Tamang (2010), Satish Kumar et al. (2013), Gautam and Sharma (2015)
Sunki	Turnip	Japan	*Lb. delbrueckii*, *Lb. fermentum*, *Lb. plantarum*, *Lb. rapi*, *Lb. sunkii*, *Lb. otakiensis*, *Lb. kisonensis*	11.3−17.0 mg/L	PCR-DGGE	Endo et al. (2008), Tomita et al. (2018)

Nozawana-zuke	Mustard	Japan	*Lb. plantarum*, *Lb. brevis*, *Leu. mesenteroides*, *Pediococcus pentosaceus,* and *Enterococcus faecalis*	–	MALDI-TOF MS analysis	Kawahara and Otani (2006), Panda et al., (2007), El Sheikha and Ray (2017)
Tursu	Cabbage, cucumber, carrot, pepper, turnip, eggplant, and beans	Turkey	*Leu. mesenteroides*, *Lb. plantarum*, *Lb. brevis* and *Pediococcus pentosaceus*	2 mg/kg	16S rDNA analysis	Kung et al. (2006), Saaid et al. (2009)
Khalpi	Cucumber	Nepal, India	*Lb. plantarum, Lb. brevis, Ped. pentosaceus, Ped. acidilactici, Leuc. Fallax*	–	16S rDNA analysis	Tamang and Tamang (2010), Karki et al. (2016)
Sinki	Radish taproot	India, Nepal, Bhutan, Korea	*Lb. plantarum, Lb. casei, Lb. brevis, Lb. fermentum, Leuc. Fallax*	–	16S rDNA analysis	Karki et al. (2016), Tamang et al. (2016)
Fermented Roots and Tuber Crop Products						
Gari	Cassava	Nigeria	*Lb. acidophilus*, *Lb. casei*, *Lb. fermentum*, *Lb. pentosus*, *Lb. plantarum*	–	16S rDNA gene sequence, PCR–DGGE, PFGE	Oguntoyinbo and Dodd (2010), El Sheikha and Ray (2017)
Lafun	Cassava	–	*Lb. plantarum*, *Leu. mesenteroides,* Lb. fermentum and Weissella confusa	–	PCR-based methods and 16S rRNA gene sequencing	El Sheikha and Montet (2014), Behera and Ray (2017)

Continued

Table 7.2 Recent advances in molecular techniques on detection of dominant microflora inhabited fermented vegetable foods.—cont'd

Fermented food products	Raw material/ substrate	Area of distribution/ country	Dominant microflora/starter culture involved	BA reported	Molecular techniques detected	Reference
Fermented Vegetable Legume (Soybean) Products						
Doenjang	Soybean	Korea	Species of *Bacillus*, *Citrobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pediococcus*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella,* and *Streptococcus*	18.37 mg/100g	16S rRNA gene sequencing	Jung et al. (2016), Kim et al. (2016)
Chunjang	Soybean	Korea and China	*Bacillus subtilis*	19.57−31.35 mg/kg	–	Bai et al. (2013)
Tofu-misozuke	Soybean	Japan	*Weissella viridescens, Lb. sakei, Lb. curvatus*	136.1 mg/kg	16S rRNA gene sequencing	Takebe et al. (2016)
Soy sauce	Soybean with brine	Korea	*Aspergillus oryzae* or *Aspergillus sojae*	20 mg/kg	PCR−DGGE	Bai et al. (2013), Wei et al. (2013), Park et al. (2019)
Miso	Rice, barley, and soybeans	Japan	*Enterococcus faecium, Lb. bulgaricus, Lb. plantarum,* and *Lb.sanfrancisco*	1.49−17.81 mg/kg	16S rRNA gene sequencing	Nam et al. (2012), Toro-Funes et al. (2015)
Nattō	Soybean	Japan	*B. subtilis*	30−100 mg/kg	16S rRNA gene sequencing	Kim et al. (2012)
Sufu	Soybean	China	Molds (*Actinomucor*, *Mucor*, and *Rhizopus*) or bacteria (*Micrococcus* and *Bacillus* spp.)	0−0.17 mg/kg	16 rDNA gene sequencing	Gu et al. (2018), Yang et al. (2020)

MALDI-TOF *MS*: matrix-assisted laser desorption/ionization (**MALDI**), and the mass analyzer is time-of-flight (**TOF**) analyzer/mass spectrometry; *PCR-DGGE*: Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis; PFGE: Pulsed Field Gel Electrophoresis.

confirmation step which is the sequence analysis of PCR products potentially related to BAs coding genes (EFSA Panel on Biological Hazards 'BIOHAZ' 2011).

In general, the conventional culture techniques used for the detection of BAs-producing microorganisms are often tedious and unreliable. This highlights the importance of improved fast, reliable, and efficient culture-dependent and independent molecular approaches [e.g., Terminal Restriction Fragment Length Polymorphism (T-RFLP), Genome Probing Microarrays (GPM), PCR-DGGE, quantitative PCR (qPCR)] and proteomic methods [e.g., Matrix-Assisted Desorption Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF MS)] (Hong et al., 2015).

Like any other techniques, it should be stated that molecular approaches may also subject to numerous limitations. For example, the main drawback in dealing with in vivo PCR-based methods is the recovery of nucleic acids (DNA, RNA) which are suitable and pure enough for subsequent PCR experiments. The samples analyzed are usually full of PCR-enzyme inhibitors that may make difficult amplification process. The development of appropriate nucleic acids preparation will also allow the detection of those bacteria able to produce BAs but in the uncultivable state (Fiocco et al., 2007).

7.5 Technological factors affecting BA production

Several factors that influence growth and activity of microflora in the fermentation of vegetables also affect the production of BAs.

7.5.1 Temperature

The temperature regulates the metabolism and proliferation of microflora present in fermented vegetables, as well as the production of BAs (Marcobal et al., 2012). In general, the BA synthesis is lowered by reducing the fermentation temperature (Eom et al., 2007). When compared to the different type of strains, *Leu. mesenteroides* B-512F showed 2-fold faster growth rate and 20-fold higher enzyme activity during cultivation at 8°C than at higher temperatures (Eom et al., 2007). For example, tyrosine decarboxylase (TDC, EC 4.1.1.25) is an enzyme that catalyzes the decarboxylation of tyrosine to tyramine and CO_2. The increase of temperature (30−37°C) stimulated the activity of tyrosine decarboxylase (from *Enterococcus faecalis*) and showed highest decarboxylation efficiency and rapid accumulation of tyramine (Bargossi et al., 2015). In contrast, Zhang and Ni (2014) reported that the tyrosine decarboxylase from *Lb. brevis* dropped its relative activity and showed poor stability at 50°C after 1 h of incubation.

7.5.2 pH

The pH is a critical factor in the preservation and developing aroma and flavor of many fermented vegetables. Fermented vegetables below pH 4−5 are usually considered as safe (Padonou et al., 2010). As the pH decreases, most of the indigenous bacteria from raw materials will be eliminated by the acidic environment (An et al., 2014). In acidic stress condition, the transcription of genes of many decarboxylase clusters is induced by low pH and improves the fitness of cells (Kimura et al., 2009; Perez et al., 2015). Induction of the

gene cluster (*hdcA*, *hdcT*) of *Photobacterium damselae* encodes histidine decarboxylase, which takes histidine into the cytoplasmic space and excretes histamine from the cell (Kimura et al., 2009). These transcripts of gene cluster were increased under low pH and confer resistance to acid stress of bacterial species (Kimura et al., 2009). More recently, Perez et al. (2015) reported that tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of *Enterococcus faecalis* in acidic environments.

7.5.3 Salt concentration

Salting is an important step in vegetable fermentation. The preservation of vegetables by LA fermentation and packing in brine solution (equilibrated NaCl with additives) are generally allowed to undergo fermentation by natural flora (Suresh, 2008). Salt (2% w/v)-induced tyrosine decarboxylation showed higher production of tyrosine within the coculture of *Lactococcus lactis* subsp. *lactis* and *Lb. lactis* subsp. *cremoris* (Bunkova et al., 2011). However, the increased levels of salt concentration beyond 5% (w/v) found to decrease in BAs (PUT and CAD) production from *Serratia marcescens* CCM-303 under model conditions (Bubelova et al., 2015). Increased salt concentrations cause the reduction of BAs, due to lowering the metabolic activities of decarboxylating microorganisms in fermented vegetables (Bubelova et al., 2015).

7.5.5 Starter cultures

The use and selection of starter cultures is considered as an alternative for the industrial production of standard fermented vegetables, and in recent years, the demand of starter cultures is on the rise (Ray and Joshi, 2014; Lee et al., 2015). The quality of fermented vegetables is critically influenced by the microbial assortments in each step of fermentation (Jung et al., 2014). The starter (*Tetragenococcus halophilus* TS71, *Zygosaccharomyces rouxii* A22, and *Meyerozyma* (*Pichia*) *guilliermondii* EM1Y52) in reduced-salt (12% NaCl) concentration accumulated the lower amount of BAs in soy sauce (*moromi*) fermentation without undesirable effects (Singracha et al., 2017). However, the production of BAs is not a desirable property for LAB strains to be selected as starter cultures (Tamang and Tamang, 2009).

7.5.6 Effects of packaging

The major part of the vegetables is consumed as fresh, or minimally processed into various forms such as canned, dried, juice, paste, salad, and sauce and soup preparations. Minimally processed and fresh vegetables have short shelf life since they are subjected to rapid microbial spoilage and in some cases contagion by pathogens (Inatsu and Bari, 2014). Food packaging is designed to contain and protect foods. The primary function of packaging is to achieve the preservation and safety delivery of food products to the consumers' table. Food packaging envisages over all longer shelf life, convenience, safer and healthier food, and promotes authenticity and prevents food waste (Mastromatteo et al., 2010).

7.6 Physiological and toxicological effects of BAs

The occurrence of BAs such as histamine and tyramine, in the fermented vegetable matrices, are linked with financial losses along with complications and toxicological effect in human health (Marcobal et al., 2012). The physiological and toxicological impact of BAs on human health are very intricate and concentration dependant. In a limited concentration, the BAs support the metabolic functions of human beings; they have a role in cell membrane stabilization, regulation of the immune system, and modulate nucleic acid and protein synthesis. Specifically, serotonin produced through the decarboxylation of tryptophan acts as a vasoconstrictor and is also involved in neurotransmission. Serotonin is involved in many critical human physiological regulations such as sleep-regulation, mood swings, sex-related behavior, and the permeability of the blood−brain barrier (Erdag et al., 2018). Histamine found in many types of cells such as blood cells and neurons also supports in neurotransmission. Similarly, spermidine, cadaverine, and spermine functions as growth regulators; agmatine has pharmacological importance such as applications in the treatment of neuropathic pain and shields the brain from toxins and stroke (Ladero et al., 2010; Tofalo et al., 2016). Cadaverine is also known to partly regulate expression of genes and cell proliferation and differentiation.

However, above the threshold levels, they are risky, whereas moderate levels may lead to food intolerance. In higher levels, they cause severe food poisoning. Several types of complications are associated with the health in response to higher uptake concentrations of BAs. Researchers have tried to understand the level of tolerance of animals/human beings to different BAs. Endogenous histamine is produced whenever necessary by histadine decarboxylase in different types of cells and also gets deteriorated with immediate effect. Human beings have a maximum tolerance capacity of 180 mg of pure histamine when administered through oral routes. A few studies have claimed that there is no adverse effect or symptoms of histamine in human beings at a dose of 50 mg. Hence, this dose may be considered as a potential acute reference dose/person. Symptoms of histamine-associated allergies are associated with skin, digestive system, circulatory, nervous, and respiratory system (Ohnuma et al. 2001). Similarly, a concentration of 600−2000 mg of tyramine in food can elicit an increase in the systolic blood pressure. A high dose of tyramine is also associated with high brain hemorrhage, palpitations, nausea, and diarrhea (Ozogul and Ozogul, 2019). Another interesting study demonstrates the acute toxicity levels of BAs of the polyamine group in rats. The toxicity for putrescine was 2000 mg/kg of body weight for spermidine and 600 mg/kg body weight for spermine (Tofalo et al., 2016).

In the current scenario, the food regulatory bodies of different countries are only concerned with histamine (one among many BAs) in fish and related products (Ruiz-Capillas and Herrero, 2019). Hardly there is any regulation on the other BAs and other ranges of food products such as dairy products, alcoholic beverages, processed cereal products, etc. As per the European Commission Regulations (2073/2005, 144/2007, 365/2010), certain species of fish from the defined family should be sampled (Ruiz-Capillas and Herrero, 2019). Out of nine sampling units from the

same product, not a single replicate should exceed 200 mg/kg of histamine content; similarly, in the case of processed fish products, the limit of histamine content is double i.e., at maximum the content can be 400 mg histamine/kg with the same sampling process. Products with higher contents of histamine than the aforesaid limits shall be regarded as disqualified for human consumption (Leuschner et al., 2013). As per the regulation of Australia, New Zealand, and India, the histamine level should not exceed 200 mg/kg. However, FSSAI (Food Safety Standards Authority of India) allows the dried fish products, fermented fish, and fish pickles with histamine content up to 400 mg/kg (Restuccia et al., 2015).

7.7 Molecular basics of BAs in human metabolism

BAs are vasoactive components, and taking them in high amounts leads to change in blood pressure in humans and animals. It also functions as a neurotransmitter in human central nervous system. Moreover, BA takes effect by binding to the cardiovascular system (vasodilation and hypotension) and cell membrane receptors in several secretory glands (such as gastric acid secretion).

BAs toxicity varies from individual to individual depending on the ability of detoxication. During the fermented food intake process in the human gut, low amounts of BAs are metabolized to physiologically less active degradation products (Fig. 7.2). The detoxification system includes specific enzymes such as monoamine oxidase (MAO, EC 1.4.3.4) and diamine oxidase (DAO, EC 1.4.3.6).

However, when large amounts of BAs are ingested, the gastrointestinal system is unable to eliminate them favorably (Moret et al., 2005). However, polyamines, putrescine, spermidine, and spermine are known to regulate cellular metabolism. The most important is that the biochemical signaling processes stimulate the growth and development of cells, tissues, organs, and organ systems as a whole (Kalač, 2014). The association between the intake of fermented vegetables and the risk of several chronic diseases such as mental retardation, hyperactive syndrome (minimal brain dysfunction), schizophrenia, and migraine headaches shows that a limited daily intake of these foods promotes health (Boeing et al., 2012). The promotion of fermented food consumption for nutrition and health is a preferable strategy to decrease the burden of several chronic diseases in developing countries (Boeing et al., 2012).

7.8 Strategic management for removal of BAs

7.8.1 BAs—degrading microflora

In fermented foods, it is difficult to prevent the accumulation of BAs since the physicochemical and microbiological actions of fermentation cannot be easily modified (Xiong et al., 2012). The alternate approach is the manipulation of food microecosystem that would degrade BA (Alvarez and Moreno-Arribas, 2014). Many LAB species

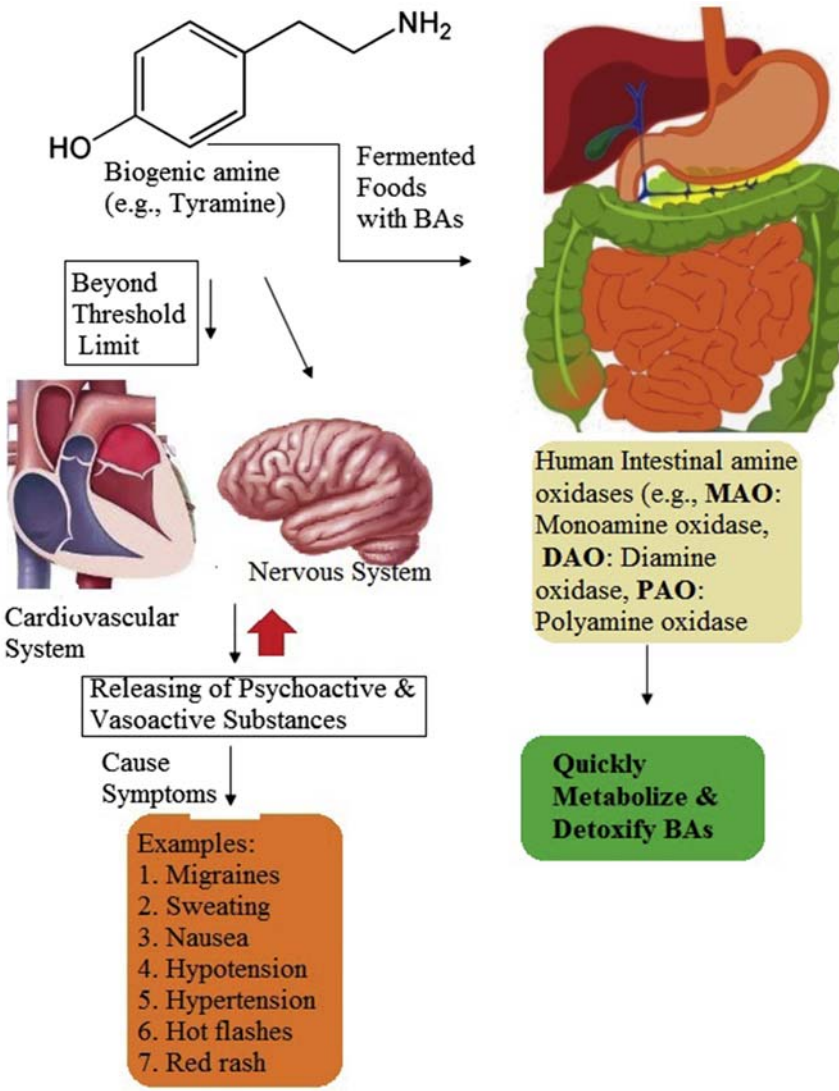


Figure 7.2 Proposed model: BAs in fermented vegetables and effects on human healths, and a general pathways of its detoxification by human intestinal system.

are used as starter cultures in several fermented vegetables, and their metabolic activities are responsible for the removal of BAs ensuring the quality and safety of these foods (Malinowska-Panczyk, 2012). In general, the choice of appropriate starter cultures is the priority to assure the quality of the final products. For this reason, the inability to form BA should be an important criterion in the selection of starter cultures for the production of fermented vegetables (Cvetkovic et al., 2015).

7.8.2 Probiotics

Probiotics are live, nonpathogenic bacteria that beneficially exert health effects on their host when ingested in adequate amounts (Chang et al., 2013). Health-promoting effects of probiotics have led to their increased use in fermented vegetables (Ryu and Chang, 2013). Fermented vegetables can be used as a potential source of probiotics as they harbor several LAB including *Lb. plantarum*, *Lb. pentosus*, *Lb. brevis*, *Lb. acidophilus*, *Lb. fermentum*, *Leuconostoc fallax,* and *Leu. mesenteroides* (Swain et al., 2014). These probiotics act as food supplements and also warrant toward health benefits (Ray et al., 2014; Swain et al., 2014). Isomaltooligosaccharides (IMO) are α-(1 → 6)-linked oligodextrans that show the prebiotic effect on *Bifidobacterium* spp. The *Leuconostoc* spp. expressing a highly active glycosyltransferase can be used for the synthesis of beneficial oligosaccharides in fermented vegetables (Cho et al., 2014). However, two probiotic strains, *Lb. casei* (TISTR 389) and *Lb. delbrueckii* subsp. *bulgaricus* (TISTR 895) were claimed to be the potential source of BA (histamine and tyramine) formers among the 15 tested probiotics strains (Priyadarshani and Rakshit, 2011). A similar consequence of tyramine production was found among LAB (*Lb. brevis*, *Lb. curvatus,* and *Leu. mesenteroides*) and other bacteria (*Saphylococcus hominis*) isolated from *kimchi* (Kim and Kim, 2014).

7.8.3 Microbial exopolysaccharides

Several microorganisms secrete exopolysaccharides (EPS) during growth that are released into the extracellular environment. EPS are recommended as an absorbent for BAs (Feng et al., 2012), which is a metabolic-independent process. It may be attributed to the interaction between heterocyclic or/and aliphatic or/and aromatic cautions of BA and negative charges of an acidic functional group of EPS (Wang et al., 2010; Feng et al., 2012).

7.8.4 Low-histamine technology

Food-fermenting LAB are generally considered to be nontoxic and nonpathogenic. Some species of LAB and some species of the genera *Bacillus*, *Enterobacter*, *Clostridium*, *Escherichia*, *Lactobacillus*, *Pediococcus*, *Pseudomonas*, *Proteus*, and *Salmonella* can produce BAs. Thus, in certain circumstances, fermented foods of contaminating microflora rather than the starter culture are responsible for the generation of increasing histamine levels (Jastrzebska et al., 2012).

7.8.5 Bacteriocins

Bacteriocins are recognized as "natural" compounds able to influence the safety and quality of fermented vegetables (Settanni and Corsetti, 2008). "Plantaricin A" group of bacteriocin produced by certain strains of *Lb. plantarum* isolated from cucumber fermentation was shown to be bactericidal toward four genera of LAB such as *Lactobacillus*, *Pediococcus*, *Leuconostoc,* and *Streptococcus* (Bali et al., 2016).

Enterococcus faecium Y13 is a class "IIa bacteriocin" producer found in traditional Chinese-fermented vegetables. The bacteriocin produced by Y13 strain has a broad inhibitory spectrum against BA-synthesized microorganisms (Liu et al., 2015).

7.8.6 Enzymes involved in degradation of BAs

The enzymes associated with LAB such as phytase, amylase, phosphatase, etc., isolated from fermented vegetables impact the compositional, processing, organoleptic properties along with the overall quality of fermented vegetables (Panda and Ray, 2016). *Leuconostoc* as a starter culture in the manufacture of the *kimchi, sauerkraut,* and pickled cucumbers and the enzyme, dextransucrase isolated from this bacterium, are used to synthesize dextran polymers or prebiotic oligosaccharides (Eom et al., 2007). In general, monoamine-oxidases (MAO, E.C.1.4.3.4) are flavoproteins present in LAB that regulate the oxidative deamination of BAs and form the corresponding degradable products (aldehydes, hydrogen peroxide, and ammonia) (Wang et al., 2013). The enzymes histamine oxidase and putrescine oxidase isolated from *Arthrobacter crystallopoietes* KAIT-B-007 and *Rhodococcus erythopolis* NCIMB 11,540, respectively, catalyze oxidative deamination of BAs (histamine and putrescine) into aldehydes, hydrogen peroxide, and ammonia (Sekiguchi et al., 2004).

7.8.7 Modulation of strain-specific determinant

The safety and technological properties of BAs are found to be strain-specific and deeply linked with the specific determinant (Jeong et al., 2016). Phenotypic antibiotic resistance, hemolysis, biofilm, and coagulase-negative properties are main determinant attributes in fermented vegetables (Jeong et al., 2016). Jeong et al. (2016) reported that none of the isolates (e.g., *Staphylococcus succinus*) exhibited antibiotic resistance or hemolysis activities in Korean-fermented soybean foods. The coagulase-negative *Staphylococcus* isolates cause to express strain-specific protease and lipase activities in soybean fermentation (Jeong et al., 2016). The different genetic determinants/heterogeneity within enterococci regulate the tyrosine decarboxylase activity. The genetic characteristics of different enterococci strains and correlate specific mutations guided the metabolic tyraminogenic activity of the strains producing BAs (tyramine) (Bargossi et al., 2015). Moreover, the synthetic medium containing tyramine accumulation suggested the possible regulation at transcript level on the tyrosine decarboxylase or the membrane transport systems of different BA accumulation trend (Bargossi et al., 2017).

7.8.8 Existing and emerging technique

Traditionally, BA formation in fermented vegetables has been prevented primarily by limiting microbial growth through chilling and freezing (Barrett and Lloyd, 2012). Existing and emerging secondary control measures of BAs in fermented vegetables include controlled atmosphere packaging and storage at temperature below 5°C, the application of hydrostatic pressure and irradiation, microwave preservation, use of

the food additives and preservatives, and most important is the "microbial modeling" of histamine-synthesized bacteria (Naila et al., 2010). These methods primarily delay the formation of BAs through the inhibition of bacteria growth or microbial decarboxylase enzyme activity accounted for BAs formation (Naila et al., 2010).

7.8.9 Food safety facets of fermented vegetables

BAs are considered as a food hazard, even though there is not a threshold for these molecules in the European legislation, except for histamine (Spano et al., 2010; Carocho et al., 2014). The control of BAs in fermented vegetables is gaining importance in order to monitor production or to know their quality to monitor food safety. Food safety involves several factors:

- Legislation (to establish the minimum hygiene requirements),
- Official controls (to check food business operators' compliance),
- Food business operators (to establish and manage food safety programmes), and
- Procedures based on the Hazard Analysis and Critical Control Point (HACCP) principles (Erzettia et al., 2009).The HACCP system is a tool to help food business operators attain a higher standard of food safety (Erzettia et al., 2009).

7.8.10 Future strategy to biocontrol BAs in fermented vegetables

Starter cultures, such as probiotic *B. subtilis*, genetically designed cultures needs to be considered to ensure the healthy aspects as well as the safety of fermented vegetables in the future. The application of the knockout technique on *odc* and *ldc* genes or loss-of-function of *B. subtilis* mutants in fermented soybean foods may lead to prevent or reduce BAs formation in the fermented foods (Mah, 2015). Hence, the genetically designed starter should be engineered carefully and precisely to avoid any unintended and undesired gene products. For the purpose of future, this technology requires more studies.

7.9 Legislative regulations regarding BAs in fermented vegetables

The reports on the occurrence of BAs in foods have been increasing that warrants for consumers' safety. The variations in clinical response depending on both technological and physiological factors result in a confused awareness to the problem of food safety. Consequently, the ingestion of food containing high levels of BA is generally underestimated and poorly understood (Russo et al., 2010).

Indigenous fermented vegetables including soy products (i.e., *miso* and soy sauce) can contain high concentrations of histamine and other BAs although they have not been incriminated in histamine toxicity. Whereas, in Japan many cases have been

reported for histamine toxicity citing fermented fish that had been seasoned with *soy sauce* or eaten with *miso*. Even though the incriminated fish was the source of histamine in these cases, the BAs present in the soy sauce or *miso* could not be ruled out (Stratton et al., 1991). Until today, there is no specific legislation regarding BA content in many fermented foods including vegetables. Thus, the need is to establish regulations limiting the amounts of BA in fermented vegetables as a first stage to achieve the safety control of the final product and also to increase the awareness of consumers.

7.10 Conclusion and future perspectives

The growing demand for secure fermented vegetables is stimulating innovation and new product development in the food industry worldwide. The synthesis of BAs is strain-dependent and influenced by the growth conditions of the fermenting microflora and the substrates (fermented vegetables). As known, safety is the basic requirement that must be satisfied in food processing. Although high levels of BAs would affect consumer's health, their concentration limits in fermented vegetables are not standardized by regulatory agencies. Understanding the microflora of fermented vegetables has to be increased exponentially by the applications of "omics" technologies that will open up new horizons for the industrial production of fermented vegetables with good taste, quality, and nutritional safety.

Conflict of interest
All authors have declared that they don't have conflict of interest for publishing the article.

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Nanobiotechnology applications in food sector and future innovations

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Yogita Lugani[1], *Balwinder Singh Sooch*[1], *Poonam Singh*[2], *Sachin Kumar*[3]
[1]Enzyme Biotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India; [2]Organic Building Materials, CSIR-Central Building Research Institute, Roorkee, Uttarakhand, India; [3]Biochemical Conversion Division, Sardar Swaran Singh National Institute of Bio-Energy, Kapurthala, Punjab, India

8.1 Introduction

The concept of nanotechnology was given by Nobel laureate Richard P. Feynman in 1959 during his famous lecture "There's Plenty of Room at the Bottom" (Feynman, 1960) where he demonstrated the concept of manipulation of matter at molecular and atomic level, i.e., nanoscale. Nanotechnology is one of the emerging and rapidly growing fields which has shown tremendous revolutionary developments in different fields of science including physics, chemistry, biology, and engineering, and its meaning varies with each field. The widely used definition of nanotechnology is synthesis of nanoscale materials (with size less than 100 nm) possessing new functions and properties (physical, chemical, electrical, optical, and magnetic) by understanding, controlling, and restricting of matter at nanometer level (NSTC, 2007). The nanomaterials used in different sectors for applications are metallic nanoparticles, carbon nanotubes, quantum dots, nanowires, nanoceramics, dendrimers, liposomes, and fullerenes, and these materials can be synthesized by top-down or bottom-up approaches (Lugani et al., 2018a).

Nanotechnology is gaining interest for research by many government and private organizations for research due to their myriad applications in different sectors such as food, cosmetics, energy, paints and coatings, textiles and clothing, medicines and drugs, and defense and security (Bryksa and Yada, 2012; Lugani et al., 2018a,b). Nanotechnology has been revolutionized in food sector for food processing, packaging, storage, and development of innovative products due to inimitable properties of such small particles like controlled release of nutraceuticals and food supplements, antimicrobial characteristics, enhancement of shelf life of food, and improvement of taste, flavor, texture, consistency and stability of food products, and mechanical and heat resistant properties due to unique properties of nanoparticles (Berekaa, 2015), as given in Box 8.1.

Development of nanosensors for the detection of contaminants and foodborne pathogens from foods is another advancement of nanotechnology in food sector, and many electrical companies are focusing toward developing electrically conducting materials

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Box 8.1 Unique properties of nanoparticles

- ❖ Improve nutritional value and texture of food products.
- ❖ Improve food consistency and prevent lump formation.
- ❖ Improve physical performance of food.
- ❖ Fortification of minerals and vitamins in foods.
- ❖ Enhance product shelf life.
- ❖ Reduce fat and sugar content.
- ❖ Provide controlled release at target site.
- ❖ Increase gas permeability, water resistance, and flame resistance.
- ❖ Nano-biosensors can detect foodborne pathogens.
- ❖ Able to bind and remove food contaminants.
- ❖ Help in innovative, lighter, stronger, and active packaging.

for manufacturing sensors (Wesley et al., 2014). The global market of nanotechnology is enhancing tremendously in food sector due to commercial applications of nanomaterials in food products. Further, advanced techniques like microfluidics, microelectromechanical systems, and DNA microarrays help in realization of use of nanotechnology for food applications for rapid sampling of biological and chemical contaminants, bioseparation of proteins, smart delivery of nutrients, and nanoencapsulation of nutraceuticals (Ravichandran, 2010). There are many industries in USA, UK, Germany, Switzerland, and Netherlands where nanoencapsulated food additives have been commercialized (Food Encapsulation Market, 2018–2024). However, nanoparticles have shown hazardous effects on environment and human health, which are the major concerns for their industrial use. Table 8.1 shows the benefits and risks of nanoparticles in food sector. The greater toxicity of nanomaterials compared to larger particles is due to their bioavailability and greater chemical reactivity, but it is unclear whether nanomaterials can accumulate in the food chain, and what levels of these materials can harm the environment and human health (Bumbudsanpharoke and Ko, 2015). Hence, it has been recommended to conduct *in-vivo and in-vitro* toxicity studies with nanomaterials before recommending for public use.

This book chapter provides a comprehensive overview on approaches used for synthesis of nanoparticles, innovations of nanotechnology in food sector, and global market of agribusiness. Further, a brief outlook has been given to safety aspects of nanoproducts with special emphasis to toxicity and health concerns with their use.

8.2 Types of nanoparticles

Nanoparticles are broadly classified into two categories i.e., organic and inorganic based on their composition (Moghtaderi and Abargouei, 2018). There are three types of organic nanoparticles i.e., carbohydrate-based, protein-based, and lipid-based nanoparticles (fat crystals, micelles, oil droplets, and vesicles) which are highly used in

Table 8.1 Benefits and risks of nanoparticles in food sector.

Benefits	Risks
• Keep foods fresh for long duration. • Remove foul smell and provide antimicrobial effects. • Help in dispersion and bioavailability of nutrients in foods, and retention of volatile ingredients. • Temperature, pH, and moisture triggered controlled release. • Nanosilver materials provide natural and powerful antibiotic, antioxidant, and antibacterial properties. • Nanocomposite materials improve mechanical and rheological properties of foodstuffs. • Synthesis, and applications in food sector are under standards of FDA (Food and Drug Administration), USA. • Able to bind and remove food contaminants. • Provide better absorption, better stability, and targeted delivery of nutraceuticals. • Use for agrochemicals delivery,	• Excess use results in environmental poisoning. • Promote allergic pulmonary inflammation. • Accumulation in different tissues and organs like skin, liver, lung, kidney, spleen, brain, vascular, and reproductive tissue. • Altered absorption profile and metabolism in body. • Abnormal cellular morphology, cell shrinkage, and chromosomal damage. • Induce oxidative stress, and alter cell signal transduction pathways, which may result in carcinogenesis. • Occurrence of autoimmune diseases such as rheumatoid arthritis, scleroderma, and systemic lupus erythematosus. • Enhanced antigen-specific immune reactions and hypersensitivity responses.

commercial food products (Shin et al., 2015). Inorganic nanoparticles mainly consist of metals, and metal oxides such as silver, titanium dioxide (TiO_2), zinc dioxide, silicon dioxide, and iron oxide (He and Hwang, 2016). Organic nanoparticles are claimed to be less toxic than inorganic ones due to their easy digestion within gastrointestinal tract (Clements and Xiao, 2017). Nanostructure materials are classified into zero dimensional (nanoparticles, nanoclusters, quantum dots, and fullerenes), one-dimensional (nanorods and nanotubes), two-dimensional (thin films), and three-dimensional (dendrimers and nanocomposites) based on structural elements (Pathakoti et al., 2017). Metallic nanoparticles are used in biosensors, drug delivery, and in treatment of cancer, and among these, silver and gold nanoparticles are of prime importance for medical use (Nikalje, 2015). The metallic nanoparticles which are used in food sector are silver, titanium, zinc, and silica (Martirosyan and Schneider, 2014). Quantum dots are inorganic semiconductor fluorescent nanoparticles which consist of CdSe core, and they are functionalized by coating with different polymers. They are used in food industry for detection of pathogenic bacteria and proteins (Bonilla et al., 2016).

Different types of nanomaterials used in food management are liposomes, micelles, dendrimers, nanospheres, nanocapsules, nanofilms, and nanoconjugates (Predhan et al., 2015). Liposomes are types of nanocapsules which are small in size (50−200 nm), biocompatible, versatile with good entrapment efficiency, and they

are used for specific and controlled delivery of additives, antimicrobials, enzymes, nutrients, and vitamins (Godwin et al., 2009; Nikalje, 2015). Dendrimers are hyper branched, tree like structures with size less than 10 nm, and they are used for controlled long circulatory delivery of bioactive materials (Nikalje, 2015). Carbon nanotubes are small macromolecules and possess unique physical properties, size, and shape. Nanocapsule is defined as nanovascular system with a core-shell structure consisting of polymer membrane or coating and the target drug or food formulation is added within the cavity (Sekhon, 2010). Nanocapsules provide several benefits in food sector like consecutive release of multiple active ingredients, change in flavor character, ease of handling, enhanced bioavailability, efficacy and stability, long-lasting organoleptic perception, moisture and pH triggered controlled release, protection against oxidation, retention of volatile ingredients, antibacterial properties, and improved taste making (Chaudhry et al., 2008). Nanofilms are nanoscale materials used in sunglasses, cameras, and computer displays due to water repellent, antimicrobial, antifog, ultraviolet (UV) and infrared-resistant, self-cleaning, antireflective, electrical conductive and scratch-resistant properties (Bhattacharyya et al., 2009). The antibacterial action mechanism of nanoparticles is shown in Fig. 8.1.

Carbon nanodots are nanomaterials of size below 10 nm, having strong fluorescence. They are obtained through electrophoresis. According to Nuclear Magnetic Resonance (NMR) study, C-nanodots are derived from candle soot which is sp^2 hybridized with no saturated sp^3 carbon atoms. C-nanodots are good electron donor and electron acceptors too. They can be used as nanoprobes for sensitive ion detection, bioimaging as they have high PL quantum yield and photostability (Li et al., 2012). Fullerenes are cage-like molecules constituents, the third form of pure carbon. C60, the archetype, is the roundest molecule that can possibly exist. Other than C60, C20, C70, and C82 also exist, which are mainly used in catalysis and fullerene containing polymers. High fullerene containing living polymers are synthesized using norbornene derivative of fullerene (Nimibofa et al., 2018).

Nanoclusters composed of small number of atoms can be of single or multiple elements. Due to different electric structure and unusual physical and chemical properties, nanoclusters developed as new branch of fluorophores. They can be used in sensors and bioimaging because of attractive characteristics like ultrasmall in size, good dispensability, and good biocompatibility (Zhang and Wang, 2014). One of the noticeable aspects of properties of nanoclusters is superatom structure, ascribed to which nanoclusters exhibit similar properties to those of atoms of periodic table (Liu and Astruc, 2018).

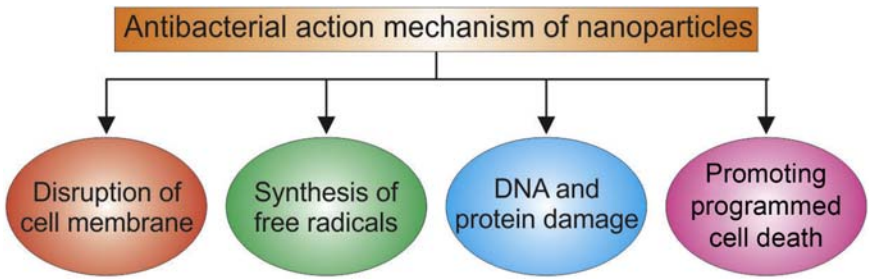

Figure 8.1 Mechanism of antibacterial action of nanoparticles.

8.3 Nanoparticle synthesis approaches

There are two common methods for preparation of nanomaterials i.e., top-down (size reduction from bulk material) and bottom-up (synthesis of materials from atomic level). In top-down approach, physico-mechanical methods such as crushing, electroplating, laser-ablation, lithography, milling, and grinding are used for production of nanomaterials from bulk materials (Abobatta, 2018). Basically, nanolithography is a method of fabrication, which is used to print the desired shape and structure on any light sensitive material of minimum one dimension i.e., size range of 1−100 nm. But, this process is comparatively expensive. To synthesize metal nanoparticles, laser ablation is proved to be a reliable method, which involves production by various solvents with the help of laser beam that condenses a plasma plume to fabricate nanoparticles. Thermal decomposition is another chemical method which is one of the top-down processes and produces particles endothermally by breaking chemical bonds. But, the synthesis of secondary products is the main challenge with this process. For milling and postannealing of particles, mechanical milling is believed to be the most popular method (Ealias and Saravanakumar 2017).

In bottom-up approach, there is production of uniformly distributed complex nanomaterials by self-assembly of small molecules. Different techniques like chemical vapor deposition, laser pyrolysis, liquid phase techniques, molecular self-assembly, and sol−gel processing are used for the synthesis of nanostructures using this approach. The ratio of chemical concentration and selected capping material are the main determinant for estimating the shape and size of nanostructure (Abobatta, 2018). Sol−gel is a wet chemical method, and it is commonly used because of its versatility as wide variety of nanoparticles can be synthesized. Chemical solution used in this method acts as precursor for the integrated system. The synthesis of nanoparticles can also be carried out by spinning disc reactor. Reactor is equipped with rotating disc inside with temperature controller and filled with nitrogen gas to avoid any chemical reaction due to the presence of oxygen.

Chemical vapor deposition is another method, which is basically the deposition of gaseous reactant on substrate in thin film form. Reaction is occurred by combination of substrate and combined gaseous molecules at ambient temperature followed by deposition. For industrial scale production of nanoparticles, pyrolysis is the best process. It proceeds by burning of a precursor in flame at high pressure (Ealias and Saravanakumar 2017).

8.4 Nanobiotechnology in food sector

Nanobiotechnology is an innovative technology gaining momentum in food sector which results in improvement of food quality and safety along with development of novel food products having thermal stability, better solubility, and oral availability (Semo et al., 2007). Various applications of nanomaterials and their impact in food sector are summarized in Table 8.2. This is one of the emerging technologies to meet global food demand and enhance incomes in developing countries (Roco, 2002). There are many positive effects of nanomaterials in agriculture in different forms like nanofertilizer (Abobatta, 2019), nanopesticide (Corradini et al., 2010), nanosensors (Das et al., 2009), postharvest

Table 8.2 Applications of nanomaterials in food sector.

Food application	Nanomaterial	Positive effect	Reference
Food preservation and packaging	Nanosilicates	Reduce food spoilage and rancidity by acting as gas, and moisture barrier in films.	Neethirajan and Jayas (2007)
	Zinc oxide nanosensor	Reduces change in color, and flavor of foods by blocking ultraviolet light.	Neethirajan and Jayas (2007)
	Nanosilver	Maintain healthy conditions on food surface with reducing microbial growth.	Travan et al. (2009)
	Nanocomposites	Lighter, stronger, and fire resistance packaging with better thermal properties and less permeability to gases.	Llorens et al. (2012)
	Nanolaminates	Enhance quality and shelf life of coated foods by incorporating active functional agents like antioxidants, antibrowning agents, antimicrobials, colors, enzymes, flavors into films.	Wesley et al. (2014)
	Chitosan nanoparticles	Possess broad spectrum antibacterial, antiviral, and antifungal activity.	Beyth et al. (2015)
Food contact material (crockery and cooking equipment)	Silver nanoparticles	Enhance antibacterial properties.	Miller and Senjen (2008)
Nutritional supplement	Silicamineral hydride complex	Act as antioxidant, and enhance potency and bioavailability.	Miller and Senjen (2008)
	Lipid nanoparticles	Improve bioavailability and retention of active biochemicals result in providing high-loading capacity and improved stability.	Gong et al. (2012)
	Selenium nanoparticles	Promote human health with additional antimicrobial and anticancer properties.	Skalickova et al. (2017)

Table 8.2 Applications of nanomaterials in food sector.—cont'd

Food application	Nanomaterial	Positive effect	Reference
Nutritional drink	Iron nanoparticles	Improve toddler health by increasing bioavailability and reactivity.	Miller and Senjen (2008)
Pathogen detection	Specified protein on silica chip	Detects specific foodborne pathogens by luminescence.	Homer et al. (2006)
	Luciferase nanosensor	Emission of light or fluorescence by attachment of dye with *Salmonella* and *Campylobacter*.	Fu et al. (2008)
	Carbon nanotubes and silicon nanowire transistor	Detection of cholera and staphylococcal enterotoxin B toxin.	Mousavi and Rezaei (2011)
	Immunogold nanoparticles	Detection of *Cronobacter sakazakii*.	Aly et al. (2018)
Testing of food quality	Nanobarcodes	Detection of quality of agricultural products.	Coles and Frewer (2013)
	Nano-smart dust	Detection of environmental pollution.	Coles and Frewer (2013)
	Gold and silver nanoparticles	Detection of food contaminants like melamine and malathion.	Paul et al. (2017)

technology (Meetoo, 2011), biosensors for aquaculture (Kumar et al., 2013), waste management (Bharathi et al., 2016), plant growth regulators (Choy et al., 2007), and agricultural engineering aspects (Melendi et al., 2008) due to improved targeted activity (Lu et al., 2002) and controlled release (Raliya et al., 2015) with safe and relaxed transport. It has been reported that nanotechnology is an emerging technology in animal husbandry in under developed countries (Eguchi et al., 2013).

8.4.1 Nanotechnology for food security

The UN millennium goal can be achieved by applications of nanotechnologies in agriculture and food sectors to ensure food security and safety (Sabourin and Ayande, 2015). Different crop management techniques have been improved significantly using nanotechnology-based agrochemicals. Nanotechnology helps in increasing the efficacy

of pesticides, herbicides, and fertilizers through controlled release, and under environment friendly manner. In a previous study, chitosan and sodium alginate was used for encapsulation of imidacloprid which resulted in enhancement of its efficacy in soil applications (Guan et al., 2010). Nanomaterials are used in processing of meat products, its storage, and marketing (Gallocchio et al., 2015). Nano-feed, produced by nanosized additives and nanoclay, is used to provide better feed to animals by removing pathogens and toxins from processed foods for improving resistance against diseases, encouraging activation of animal's own self-healing forces, improving bone growth and phosphate utilization, and reducing mortality rate (Sekhon, 2014). Nanotechnology promises to improve feeding effectiveness and nutrition of animals and plant pathogen detection, reduce animal losses due to diseases, targeted genetic engineering, enhances conservation and management of crops, fisheries, and animal production (Singh, 2016a; Pramanik and Pramanik, 2016). This technology can be used to improve crop yield by developing healthy seeds and improving the effectiveness of fertilizers and pesticides (Teng et al., 2011; Seabra et al., 2013). It is also used for soil and water cleaning, remediation, and genetic engineering and molecular-based crop breeding to enhance the crop production (Parisi et al., 2015; Wani and Kothari, 2018). Nanocarriers are used for controlled release of plant growth regulators, herbicides, and pesticides. In a recent study, treatment of mustard plant (*Brassica juncea*) with atrazine (using poly epsilon-caprolactone as carrier) nanocapsules showed improved herbicidal activity, decreased photosynthetic rates and stomatal conductance, increased oxidative stresses, weight loss, and growth reduction (Oliveira et al., 2015). Nanoparticle-mediated DNA or gene transfer in plants has been used for developing insect resistant plant varieties by some researchers (Khot et al., 2012; Sekhon, 2014).

8.4.2 Nanotechnology for food preservation and storage

Nanotechnology is observed to be efficient for extending shelf life of food products with minimum loss of nutrients. Efficient and improved techniques such as nanosensors, nanocomposites, and nanoparticle in packaging are available for food preservation and storage. These techniques help in providing better food stability, bioavailability, preservation of color, etc. This technology has the potential to enhance storage period of fruits and vegetables (Parisi et al., 2015; Wani and Kothari, 2018). Different forms of nanosystems such as nanobarcodes, nanosensors, nanocapsules, nanocomposites, nanofibers, nanoparticles, and nanotubes have been used in food processing, packaging, and preservation as shown in Fig. 8.2 (Duncan, 2011; Bajpai et al., 2018). Nanocomposites are made of nanoparticles and polymers, and they are used in food sector for enhancing the shelf life of food products, keeping the products fresh, devoid of microbial action, and providing gas barrier to minimize the leakage of carbon dioxide from carbonated beverages (Pandey et al., 2013; Hamad et al., 2018). Guard IN Fresh is a nanocomposite-based commercialized product used for ripening of fruits and vegetables by scavenging ethylene gas (Gupta and Moulik, 2008). Nano-Ceram PAC is an ecofriendly nanocomposite-based coating material which helps in rapid absorption of unpleasant components of food, results in avoiding foul odor, and repulsive taste (Bordes et al., 2009).

Figure 8.2 Different forms of nanosystems used in different food applications.

8.4.3 Nanotechnology in food packaging

Nanotechnology has provided better packaging options for food products by enhancing shelf life, better traceability of food products, and above all safety. One of the future advancement of this technology is use of polymer composites which provide active, flexible, and intelligent packaging (Aigbogun et al., 2017). Biologically synthesized silver and gold nanoparticles from *Fusarium* sp., *Pseudomonas struzeri, P. aeruginosa*, and *Penicillium* sp. are used for antimicrobial packaging (Sadowski et al., 2008; Khalilabad et al., 2013). Metallic nanoparticles like silicate nanoparticles, titanium oxide, and zinc oxide (ZnO) are used to remove chemicals and pathogens from foods, by reducing the flow of oxygen in the packaging containers, leakage of moisture, and keeping the food fresh for longer time (Nam et al., 2003; Horner et al. 2006). Nanoparticles such as ZnO, silver nanoparticles, inorganic nanoceramics (Arshak et al., 2007), silicon dioxide (Coma, 2008), TiO_2 (Acosta, 2009), and polymeric nanoparticles (Bouwmeester et al., 2009) are used in food packaging and preservation. It has also been reported that use of active coating by incorporating cinnamon

oil and solid wax using nanotechnology provide protection to different foods such as bakery, cheese, and sliced meat against spoilage (Rodriguez et al., 2008). Active packaging films also results in selective control of oxygen transmission and aroma affecting enzymes, and hence avoid unnecessary oxidation of fats and oils, rancidity, off-odor, and flavor problems in packed foods (Rivett and Speer, 2009). Nanosilver, montmorillonites, and silver zeolite nanoparticles were used for producing chitosan-based edible nanoparticle films (Rhim et al., 2006). The use of nanoclay in plastic bottles stiffen the packaging and keep the beer fresher by minimizing loss of carbon dioxide from products, enhancing product shelf life and reducing gas permeability (Zhao et al., 2008). The incorporation of ZnO nanoparticles into plastic packaging provide antibacterial protection, improve strength and stability of plastic films, and reduce the chances of food contamination (Neethirajan and Jayas, 2011). One of the widely used nanomaterial for food packaging is nanoclay due to its low cost, corrosion resistance, thermal, mechanical, and barrier properties (He et al., 2019). A significant improvement in interlaminar fracture toughness and enhanced glass transition temperature by 6°C was observed with 3% nanoclay loaded woven carbon fiber/compatibilized polypropylene nanocomposites (Gabr et al., 2015). The nanoclay materials which have been listed under GRAS (Generally Regarded As safe) status and in effective Food Contact Substances (FCS) by U.S. FDA are montmorillonite and bentonite (He et al., 2019). The organic chemicals based edible coatings used for food packaging of perishable products are lemon grass essential oil (Trujillo et al., 2015), pectin from apples (Gorrasi and Bugatti, 2016), and quinoa protein/chitosan (Robledo et al., 2018).

8.4.4 Nanomaterials as antimicrobials

The conventional techniques used for controlling the microbial growth in food are thermal processing and chemical preservation. Recently, there is increased demand of natural food products for better health by consumers. Nanotechnology provides better alternate materials for providing antimicrobial activity with minimum toxic and undesirable side effects compared to physical and chemical methods. Moreover, this technology is one of the powerful tools to improve the shelf life of agri-food chain through technological advancement. The antimicrobial properties of nanoparticles result by inhibition of microbial growth on nonsterilized foods, and prevention of post-contamination of pasteurized foods. Silver nanoparticles are quite stable with broader spectrum of antimicrobial activity, and these nanoparticles are reported to be safe for biological system when incorporated within standard limits approved by Food and Drug Association (Zhao et al., 2008). The other nanoparticles which have been reported for antimicrobial activity are copper and copper oxide, chitosan, cadmium, magnesium oxide, selenium, single-walled carbon nanotubes, and telluride (Arshak et al., 2007). The antimicrobial activity of silver-coated nanocomposites, ZnO, and pediocin incorporated nanoparticles in nanocomposite films, and PEG (polyethylene glycol) coated with garlic oil composite has already been published (Moraru et al., 2003). Nanoparticles of ZnO possess antibacterial and antifungal activity against *Aspergillus niger, A. flavus, A. fumigatus* (Rajiv et al., 2013), *Fusarium graminearum* (Dimkpa et al., 2013), *F. culmorum*, and *F. oxysporum* (Rajiv et al., 2013). In a recent

study, the antibacterial properties of *Lactobacillus plantarum* were enhanced through the induction of mild stress by pullulan nanoparticles (Hong et al., 2019). In another study, green synthesized gum stabilized nanoparticles loaded with flavonoids showed antimicrobial activity against infections caused by brain eating amoebae and multi-drug resistant bacteria (Anwar et al., 2019).

8.4.5 Nanotechnology in nutraceuticals production and their delivery

Nanomaterials are used in food processing methods for incorporation of nutraceuticals like vitamin and mineral fortification, nanoencapsulation of flavors, and gelation and viscosifying agent and their delivery to the target site (Huang et al., 2010; Bajpai et al., 2018). β-carotenes, lycopene, and phytosterols like nutraceuticals are incorporated into foods in nanoformulations for effective delivery (Mozafari et al., 2006). The controlled delivery of nutraceuticals, antimicrobials, enzymes, food additives, etc., has been achieved using lipid-based nanoemulsions like archaesomes, nanoliposomes, and nanocochleates (Mozafari et al., 2008). Nanoemulsions are also used in food processing in the form of proteins (milk, egg, and vegetable proteins), carbohydrates (alginate, pectin, carrageenan, dammar gum, guar gum, sucrose-acetate isobutyrate, and xanthan) for improving the texture and uniformity of ice creams, reducing creaming and sedimentation, dispersion and availability of food nutrients, and production of food products like sweeteners, salad dressing, beverages, and other processed foods (Oberdorster et al., 2007; Kang et al., 2007; Fernandez et al., 2008). Nanotechnology is used for delivery of nutraceuticals and bioactive compounds available in functional foods like β-glucan from oats, β -carotene from carrots, conjugated linoleic acid from cheese, isoflavones from soyabean, omega-3-fatty acid from salmon oil, lycopene from tomato (Chen et al., 2006). The nanosized micelles produced by milk protein, casein, is used as vehicle for delivery of sensitive health-promoting ingredients including vitamin D2 (Semo et al., 2007). Fig. 8.3 represents the processing of raw materials for their introduction in foodstuffs.

There are several benefits of nanoencapsulation system such as enhanced stability and integrity, change in flavor, enhanced bioavailability, easy handling of food products, pH- and moisture-triggered controlled release, long-lasting organoleptic perception, retention of volatile ingredients, taste making, protection against rancidity and oxidation, and consecutive delivery of multiple active ingredients (Shefer, 2008). Nanoencapsulation

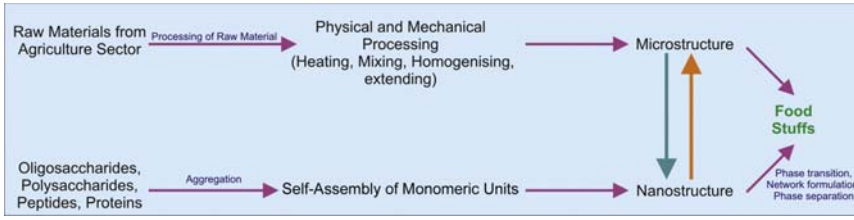

Figure 8.3 Preparation of nanoparticles for their applications in foodstuffs.

with calcium alginate is observed to improve the viability of probiotic microorganisms such as *Bifidobacterium* sp., *Lactobacillus casei, Lb. acidophilus*, and *Lb. rhamnosus* in freeze-dried yogurt (Duncan, 2011). George Weston Foods, Australia, has integrated nanocapsules into bread to avoid unpleasant taste, and odor from tuna fish oil, and hence provides controlled release of beneficial probiotic microorganisms to promote gut health (Neethirajan and Jayas, 2007). Encapsulation of curcumin into hydrophobically modified starch showed improvement in its anticancerous property (Yu and Huang, 2010). Polylysine nanoparticles are smaller than phytoglycogenoctenyl succinate nanoparticles and used as antioxidant in foods to prevent unnecessary oxidation of oils (Scheffler et al., 2010). The nanocoating alginate/lysozyme nanolaminate is used to preserve the quality of fresh foods during storage (Medeiros et al., 2014), chitosan/nanosilica coatings (Shi et al., 2013), chitosan film with nano-SiO_2 (Yu et al., 2012), and gelatin-based edible coatings containing cellulose nanocrystals (Fakhouri et al., 2014). Nanomaterials provide promising approach for improving the bioavailability of nutraceutical compounds due to their subcellular size (Singh et al., 2017). Carotenoid nanoparticles showed improved bioavailability of carotenoids and nano-based mineral supplements (nano-iron and nano-calcium). Nanometer-sized micellar systems are also available for delivery of minerals, phytochemicals, and vitamins (Singh, 2016b). The most frequently used nano-formulations for food supplements are micelles, nanoemulsions, nanocapsules, nanosponges, nanogels, nanofibers, nanoliposomes, core−shell nanoparticles, solid lipid nanoparticles, layered double hydroxides, mesoporous silica nanoparticles, and cyclodextrin complexes (Jampilek et al., 2019).

8.4.6 Nanosensors in food sector

Nanosensors are electronic devices which possess a sensing part and an electronic data processing part. The sensing part is used for the detection of gases (hydrogen, ammonia, sulfur dioxide, hydrogen sulfide, and nitrogen oxides), heat, light, foodborne pathogens, humidity, and chemicals, and electronic data processing part produces electrical signals (Rubio et al., 2006; Kang et al., 2007). Zhao et al. (2004) has developed 60 nm fluorescent biosensor for *in-situ* pathogen quantification by using Ab-conjugated silica in ground beef. Similarly, many fluorescent dye biosensors and magnetic nanoparticles−based biosensors have been developed for the detection of *Salmonella* (Fu et al., 2008), *Campylobacter* (Stutzenberger et al., 2007) and *Escherichia coli* (Cheng et al., 2009) in food samples. In a recent study, luminescence oxygen biosensor is reported to be relatively cheap and more compatible for smart food packaging (Kelly, 2017). A fluorescent nanobarcode detection system has been developed for detection of several foodborne pathogens such as *E.coli*, anthrax, tularemia bacteria, Ebola, and severe acute respiratory syndrome (SARS) virus by different color codes in a computer scanner (Li et al., 2005). A Dip-pen nanolithography technique has been developed by NanoInk, Skokie, for detection of food products and pharmaceutical pills. This detective tool is currently used by Barcode, a registered US company, for traceability to ensure wholeness (Zhang et al., 2009). Reflective interferometry was used previously for the detection of *E. coli* contamination in packaged foods (Wanekaya et al., 2006). In a previous study,

metal-based nanosensors (palladium, platinum, and gold) were used in food packaging for detection of change in food color and gases produced by spoilage (Kang et al., 2007).

Similarly, single-walled carbon nanotubes and DNA were used for detection of presence of pesticides on the surface of fruits and vegetables, and monitoring of soil conditions required for growth of crops (Sozer and Kokini, 2009). Several immunosensors have been developed for detection of toxins such as cerium oxide immunosensor for ochratoxin A, and chitosan-based nanocomposites, silicon nanowire transistors, and carbon nanotubes for Cholera toxin and Staphylococcal enterotoxin B (Rai et al., 2012). Nano-smart dust is observed to be useful in food packaging for detection of any kind of environmental pollution. Biomimetic sensors and smart biosensors were used for detection of presence of mycotoxins, bacteria, viruses, and other pathogens (Coles and Frewer, 2013). In a recent study, the use of nanomaterial-based sensors have been reported for sustainable management of agricultural soil, detection, and protection against pathogens, for food quality and safety, for improvement of crop practices, food quality, and packaging methods (Kim et al., 2018).

8.5 Other applications of nanotechnology

There are tremendous applications of nanotechnology in different sectors like food and agriculture, paper and pulp, paints and coatings, textiles and clothing, defense and security, medicines and drugs, bioengineering, optical engineering, medicine, cosmetics, electronics, energy, space exploration, and transportation (Mihindukulasuriya and Lim, 2014). Liposomes, TiO_2, ZnO, dendrimers, nanoemulsions, and nanocrystals are used in sunscreen, moisturizers, makeup, and hair care products. The use of ionic or metallic silver has been reported in different sectors such as sunscreen lotions, steel coatings, wastewater treatment, and textile fabrics (Duran et al., 2007). In modern cosmetic world, nanosized components are reserving their place in products like moisturizer, hair products, and makeup products. The main interest is liposome-based antiaging topical formulation. Nanoparticles such as TiO_2 and ZnO are used as UV fillers. The main reason behind the use of nanoparticles in cosmetic industry is to improve the penetration capacity of certain ingredients like vitamins, unsaturated fatty acids, and antioxidants along with the stability of these ingredients. Due to certain properties shown by nanoparticles, they increase the efficiency and tolerance of UV filters on skin (Mu and Sprando, 2010).

Hydrogen economy is gaining huge attention and so is the implementation of nanotechnology in storage of hydrogen and advances in the use of nanomaterials for solar hydrogen nanostructure of semiconducting metal oxides as TiO_2, ZnO, and Fe_2O_3 are stable than common solar cell materials and cheaper too that make the commercial viability (Krol et al., 2008). Along with disciplines of biology, chemistry, engineering, or medicine nowadays, nanotechnology is expanding its uses and wide interest in interdisciplinary research like cancer nanotechnology. Issues in dealing with cancer nanotechnology are cancer detection, diagnosis and treatment which are quite different

from conventional therapeutics like nanosystems can diagnose the problem as well can carry therapeutic payload. Alongside multiple cells can be targeted with high affinity. Nanosystems are designed to accommodate multiple drug molecules for cancer treatment like toxicity in normal cell can be monitored (Misra et al., 2010).

The field of drug delivery is improved by enhancing the treatment methods like artificial implant and organ transplants with the help of nanotechnology and the advances in the reproduction and repair of damaged tissues methods. Nanotechnology makes the process of construction quicker, safer, and cheaper. With the use of nanoparticles, mechanical properties of composites can be improved, and it provides better blocking of light and heat penetration. Addition of relevant nanoparticles enhances self-healing ability to provide insulation. These paints can be used for coating purpose to prevent from salt water attack because of its hydrophobic properties. For the treatment of surface water and to remove heavy metals, pathogens, and organic contaminants, nanotechnology is the leading remediation. Nanoparticles can also be used to clean oil spills as well as for the treatment of sludge and industrial wastewater produced (Ealias and Saravanakumar, 2017). As per studies, nanoparticles and nanoclusters show appreciable applications for catalysis purpose. In the case of gold, nanoclusters are proved to be more reactive than that of nanoparticles because of well-defined size and structural properties shown by them (Safari and Zamegar, 2014; Nasiruddin et al., 2018).

8.6 Global status of nanotechnology in food sector

The companies which are making advancements in the field of nanotechnology all around the world include Adnano Technologies, Dabur Pharma, Meda Biotech, Nano-Bio Chemicals, NanoShel, NanoXpert Technologies, Sisco Research Laboratories, Quantum Corporations, and Velbionanotech (http://www.foresight.org/policy/brief1.html). The major players of nanoencapsulated food additives in the world are Advances BioNutrition Corporation, ABCO Laboratories, Inc., Aveka Group, Aveka Inc., Balchem Corporation, Coating Place Inc., Encapsys LLC, LycoRed Ltd., Maxx Performance Inc., Sensient Technologies Corporation, Cargill Inc. (USA), Royal DSM N.V, FrieslandCampina Kievit (The Netherlands), Firmenich SA (Switzerland), Symrise AG (Germany), and Taste Tech Ltd. (UK) (Food Encapsulation Market, 2018−2024). Nanoencapsulation with canola active oil has been used by Shemen in Haifa, Israel, for several commercialized food products (Bikker and Kruif, 2006). Nanoencapsulation is also used by other companies for different food products such as Fortified Fruit Juice by High Vive, NanoResveratrol by Life Enhancement, Spray for Life Vitamin Supplements by Health Plus International, Daily Boost by Jamba Juice Hawaii, Color Emulsion by Wild Flavors, Nanoceuticals Slim Shake Chocolate by RBC Life Sciences Inc., and Nanotea by Qinhuangdao Taiji Ring Nano-Products Co. Ltd (Cobb and Macoubrie, 2004; Donaldson and Seaton, 2007). A sponsorship program has been launched in 2007 by Working Party on manufactured nanomaterials of the Organization for Economic Cooperation for

Development for the testing of manufactured nanomaterials (OECD, 2007). The share of nanotechnology in global food and beverage industry is multitrillion dollars (Cushen et al., 2012), and the worldwide economic impact of nanotechnology is targeted to three trillion dollars by 2020 (Roco et al., 2011). Second Regulatory Review on nanomaterials by EU (European Union) recommended the compulsory labeling of nanoingredients in food products and this policy was applicable from December 2014 (EU, 2012a). EU MEMO (2012) has made regulations for nanomaterials in environmental legislations such as water, waste, and air (EU, 2012b). Taiwan FDA guidelines (2017) consider nanomaterials as new food contact substances and enforce safety assessment of food packaging nanomaterials with premarket approval (https://www.fda.gov.tw/TC/newsContent). In USA, the R&D agencies which are actively indulged in nanobiotechnology research especially in food sector are Agriculture and Food Research Initiative (AFRI), Center of Nanoscale Science and Technology (CNST), National Nanotechnology Initiative (NNI), National Institute of Health (NIH), National Institute of Food and Agriculture (NIFA), and US Department of Agriculture (USDA). NNI is announced to provide nearly $1.4 billion in 2019 for basic research, early-stage applied research, and technology transfer efforts in the field of nanotechnology (http://www.nano.gov/about-nni/what/funding). The funding bodies of Europe in the field of nanotechnology are Austrian Nano Initiatives (ANI), Biotechnology and Biological Sciences Research Council (BBSRC), European Commission (EC), Engineering and Physical Sciences Research Council (EPSRC), Medical Research Council (MRC), and Regional Developmental Agencies (RDA) (http://atip.org/index.php/atip-publications-2/atip-reports/2008-1/6214-atip08-007-nanosciencenano-funding-in-europe-an-overview). The funding bodies in India which provide funds for innovations in the area of nanotechnology are Council of Scientific and Industrial Research (CSIR), Defense Research Development and Organization (DRDO), Department of Atomic Energy (DAE), Department of Biotechnology (DBT), Department of Science and Technology (DST), Indian Council of Medical Research (ICMR), and Ministry of New and Renewable Energy (MNRE) (McClements and Rao, 2011).

8.7 Safety issues of nanoproducts in food

Nanomaterials can cause harmful effects on environment and human health due to their participation in different chemical reactions resulting by their unique physical and chemical properties. The enhanced concern on safety issues of nanomaterials by private and government sectors are due to their extensive use in food products as color additives or flavoring agents. Nanomaterials have been disposed of by many manufacturing industries, and these nanomaterials can accumulate in soil and water which results in disruption and alteration of microbiota of that region. Nanoparticles are observed to accumulate on the surface of marine bodies, which lead to toxic effects on phytoplanktons that in turn affect the pelagic species. The benthic species also get affected when surface nanoparticles accumulate at the bottom of marine bodies. The inhibition of plant growth by aluminum nanoparticles has also been reported (Morones et al., 2005). It has

been pointed in an editorial note entitled "Nanofood for Thought" that benefits of nanotechnology can reap in food industry if issues related to their safety are addressed honestly and companies are more open about what they are doing (Nature Nanotechnology, 2010). Several previous studies have revealed the toxicity effects of nanomaterials used in food ingredients and packaging. For the evaluation of risk assessment of nanomaterials, it is essential to quantify various factors like biological molecules, commensal microbes, osmotic concentration, physical factors, chemical forces, osmotic concentration, absorption, distribution, metabolism, and excretion (He et al., 2015). The toxicity effects of nanomaterials on human organs directly depends on their physicochemical characteristics such as their concentration in food, amount of that food consumed, bioavailability, and biodistribution (He et al., 2015; He and Hwang, 2016; Wani and Kothari, 2018).

Absorption, distribution, metabolism, and excretion of nanoemulsions change with change in size, and it is one of the major complications for ingestion of nanofoods (Chawengkijwanich and Hayata, 2008). Nanoparticles behave similar to asbestos (Hett, 2004), and these particles may settle in brain or trigger immune response (Scrinis, 2008). The small sized nanoparticles possess large surface area, and hence, they can easily pass through biological barriers and accumulate in tissues like central nervous system (Borm and Kreyling, 2004). Chemical composition of nanoparticles is very crucial to estimate the toxicity level of semiconductor nanoparticles and carbon nanotubes. Different cytotoxic effects have been reported with different levels of titanium oxide crystallinity (Stolle et al., 2009), and iron ions may accelerate cellular oxidative stress (Murray et al., 2009). Toxicological effects of absorbed nanoparticles in human system are influenced by their surface chemistry, charge, roughness, and hydrophobicity (Kirchner et al., 2005). Positively charged nanoparticles are found to be more toxic than negatively charged and neutral nanoparticles, and hydrophilic polymer i.e., PEG decreases the toxic effect of nanomaterials (Sukhanova et al., 2018). Solubility is another important parameter affecting the toxicity of nanoparticles, and soluble titanium oxide nanoparticles are reported as more toxic than insoluble ones (Oberdorster, 2001).

Similarly, the carcinogenic effects of soluble nickel compounds have already been published (Salnikow and Kasprzak, 2005). There are different effects of digestible organic nanoparticles (carbohydrates, proteins, lipids, and surfactants), and nondigestible inorganic nanoparticles (metals and minerals) on the body (Chen and Evans, 2005). One of the major factors affecting the toxicity of nanoemulsion is bioavailability of components within biological system. The low bioavailability of component within nanoemulsion but its high absorption rate may result in bioaccumulation of component within living system. Consumption of large amount of nanoemulsions may show adverse toxic effects in human system due to chemical nature of solvents and surfactants (Cushen et al., 2012). Silver nanoparticles have been reported to show adverse effects on human lung fibroblast by increasing reactive oxygen species, damaging DNA and mitochondria, chromosomal aberrations, and decreasing ATP content. Hence, cytotoxic, genotoxic, and carcinogenic effects of silver nanoparticles have been detected on biological system (Kim et al., 2007).

Carbon nanotubes, which are commonly used in food packaging, showed toxic effects on human skin and lungs (Mills and Hazafy, 2009). The gradual accumulation of TiO_2

nanomaterials in the body has been reported by Jovanovic (2015) after consuming chewing gum containing TiO_2 nanomaterials. In a similar study, SiO_2 nanoparticles have been found to accumulate in the gut epithelium after consuming food containing E551 (Athinarayanan et al., 2014). Nanomaterials promote the allergic pulmonary inflammation by increasing the production of reactive oxygen species (Syed et al., 2013), and by inducing nanoparticle-specific immune response (Hirai et al., 2014). Increased gene expression of interleukins (IL-4, IL-10, IL-13) and allergy-associated Th2 cytokines were observed when exposed with small sized (22 nm) carbon black nanoparticles (Lefebvre et al., 2014). Different metallic nanoparticles such as ZnO (Fukui et al., 2012), Ag (McShan et al., 2014), and CuO (Karlsson et al., 2013) can show adverse effects into food stimulants by enhancing intracellular reactive oxygen species, which may result in lipid peroxidation and DNA damage (Fukui et al., 2012). The toxic effects of nanoparticle components have also been approved by Great Britain, which recommends the toxicity assessment of nanomaterials by scientific institutions before their approval for use in food products (Krishnan et al., 2018). However, traditional methods such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or casein AM (CAM) are not useful to evaluate the toxicity of various nanostructures like quantum dots, fullerene, and single-walled carbon nanotubes (SWCNT). Moreover, there are limited toxicological studies on nanomaterials used for edible coatings and food packaging, and hence, future *in-vivo, in-vitro*, and *in-silico* evaluations are needed to establish standard protocols for risk assessment and control the safety issues associated with use of nanomaterials in food sector.

8.7.1 Global concerns about safety issues of nanoproducts

The regulatory bodies that govern use and applications of nanosystems in food in different regions are Consumer Product Safety Commission (CPSC), National Institute for Occupational Safety and Health (NIOSH), Occupational Safety and Health Administration (OSHA), US Patent and Trademark Office (USPTO), USDA, Environmental Protection Agency (EPA), European Food and Safety Authority (EFSA), and Food and Drug Administration (FDA) (Qi et al., 2004). Food industries involved in food processing, packaging, and its preservation need to follow these regulations. Size of nanoparticle is another concern for consumer, and regulations on particle size have met by European Parliament and Council Legislation (Carvajal et al., 2010). The food industries have to strictly adopt precautionary principle to avoid incorporation of engineered nanomaterials in the food (Rhim and Ng, 2007). Other regulations approved by EC states that engineered nanomaterials should be free from mycotoxins, heavy metals (Scampicchio et al., 2008), prevent change in inherent, and organoleptic properties of food (Silva et al., 2012) should not promote deterioration of food and harmful health effects, and nanocomponent should be assessed as food additive before its use as packaging material (Sondi and Sondi, 2004).

The emerging applications of nanotechnology in agriculture, food processing, packaging, preservation, water treatment, and safety concerns with use of nanomaterials have been discussed in the conference entitled "Nanotechnologies in the Food and Agriculture Sectors: Potential Food Safety Implications" held by Food and Agriculture Organization of the United Nations (FAO) and World Health Organization

(WHO) in 2009. Experts from 13 countries (Australia/New Zealand, Brazil, Canada, China, European Union, Indonesia, Japan, Malaysia, Mexico, The Republic of Korea, South Africa, Switzerland, and United States) around the world have attended this meeting and shared the safety concerns of nanomaterials on human health, and recommended early consideration of their safety (FAO/WHO, 2010, 2013). In 2012, FAO and WHO have released an article entitled "State of the art on the initiatives relevant to risk assessment and risk management of nanotechnologies in the food and agriculture sectors", and this article includes the applications of nanomaterials in food packaging, recent activities in risk assessment, and management of nanomaterials at national and international levels, and current status of nanosafety management in the participatory countries (FAO/WHO, 2012). Requirement of one-time reporting and record keeping for control of have been proposed by nanoscale materials EPA, US, in 2015 under Toxic Substances Control Act, Section 8(a) (EPA, 2015). The use of selenium nanoparticles in multilayer films for food packaging was observed to be safe by European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes, Flavorings, and Processing Aids (Bergeson and Hutton, 2018).

The regulatory norms are still not strictly followed in many developing countries, where no specific regulatory framework is followed. However, it is utmost essential to set strict regulatory guidelines of nanoparticles at global level for safe use of nanomaterials in food products.

8.8 Conclusions and future recommendations

It has been concluded that nanobiotechnology is one of the emerging field of nanotechnology in food sector due to its potential applications. Food shortage is one of the major challenges which is expected to be faced by the world in the near future due to expanding population growth, pressure on environment, and efficiency on production system. The major challenge of food sector is development of better food manufacturing, preservation, and storage techniques to provide healthier and nutritious food for human welfare. Hence, nanobiotechnology is an alternate opportunity to overcome global problem and help in providing authenticated, nutritious, safe, secure, shelf-stable, high-quality, fortified, and therapeutic food products to future generations. It is widely accepted that nanofoods will be available largely to consumers worldwide in coming years. Nanotechnology can offer reduced chemical inputs, improved plant growth, use of nanofertilizers and nano-pesticides, and improved crop yield, and productivity for sustainable future. This technology is considered as one of the six "Key Enabling Technology" by EC to support greener farming and growth in food sector. Therefore, nanotechnology can be integrated with agriculture to reduce poverty, food security, agriculture growth, management of environment sources, and securing social outcomes. Moreover, many innovative hybrid technologies can be developed by integrating nanotechnology with pharmaceuticals, biotechnology, molecular biology, and computational technology.

Nanoparticles have great potential to enhance the bioavailability of nutraceuticals, and bioactive compounds, their controlled release with fine-tuning of bioavailability, pharmacokinetics, and bioefficacy. The major concerns for food scientists and regulatory bodies are consumers' benefit and safety of food products. Hence, it is essential to invest financial resources, sufficient time, and technological means to achieve commercialization of nanoproducts in the market. There are many regulatory agencies like European Food Safety and FDA, which stress upon the importance of nanoparticle characteristics like particle size, and surface properties, and association of nanomaterials with intestinal absorption along with hazardous analysis of tiny molecules. The industries should strictly follow the regulatory guidelines given by regulatory bodies like FDA and WHO to evaluate the safety of food, food packaging, storage, and use of food supplements. There is limited information about safety after oral administration of nanomaterials, their absorption, distribution, metabolism, and excretion. It is also required to develop novel and standardized tests for testing the toxic hazardous effects of nanomaterials on environment and human health, tissue distribution, and risks associated with their exposure. In addition, it is essential to set strict regulatory guidelines of nanoparticles at global level for safe use of nanomaterials in food products. The use of instrumentation and computational science can help researchers and scientists to gain unprecedented information about toxicological effect of nanomaterials on human cell lines. Hence, technological, scientific, and social considerations are required to enhance the applications of nanotechnology in different sectors in the coming years. Further, some focus is required to engineer nanomaterials with modern and advanced technologies to make this technology more efficient in food industry in coming years.

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Perspectives of microbial hyaluronic acid utilization in wound healing

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Nicole Caldas Pan, Cristiani Baldo, Hanny Cristina Braga Pereira, Josiane Alessandra Vignoli, Maria Antonia Pedrine Colabone Celligoi
Department of Biochemistry and Biotechnology, Centre of Exact Science, Londrina State University, Londrina, Parana, Brazil

9.1 Introduction

Hyaluronic acid (HA) is a natural and linear polymer composed of repeating disaccharides units of β (1 → 4)-glucuronic acid (GlcUA) and β (1 → 3)-N-acetylglucosamine (GlcNAc). In human, HA exists in all tissues and is abundant in umbilical cord, synovial fluid, eyes vitreous humor, and skin (Kogan et al., 2007; Liu et al., 2011). In human, its production occurs predominantly by mesenchymal cells. In normal biological conditions, HA functions in space filling, hydration, lubrification of joints, and provision of a matrix through which cells can migrate. During tissue injury, the polymer act in the regulation of tissue repair and disease process, such as inflammatory cells activation, regulation of epithelial cells behavior, and fibroblasts (Liang et al., 2016).

Commercially, the HA can be extracted from animal tissues like rooster comb or produced by Lancefield group A and C streptococci bacteria. The HA microbial production is preferred because of viral infection risk of the HA extracted from animal tissues (Chong et al., 2005). Additionally, the use of cheaper substrates such as a residue and agroindustrial byproducts in the fermentation medium allows the reduction of HA production cost (Amado et al., 2017; Pan et al., 2017). The values of HA products and derivatives range from US $2000 to $60,000/kg (Pires et al., 2010).

Due to its biological functions, research articles referring to HA have grown exponentially in recent years (Liang et al., 2016). The viscoelasticity, high moisture retention capacity, and biocompatibility of HA makes the polymer find a wide-range of applications in pharmaceutics and medical fields such as drug delivery (Zanin et al., 2017), osteoarthritis treatments (Yu et al., 2014; Altman et al., 2018), ophthalmic surgery (Kretz et al., 2014), periodontitis (Rajan et al., 2014; Chen et al., 2019), and cutaneous wound healing. In the wound treatment, exogenous applications of HA promote wound healing by facilitating cell migration and proliferation (Su et al., 2014; Ferrari et al., 2015; Shin et al., 2016).

This review is focused on the microbial HA production and its utilization on wound healing treatment. Due to the HA intrinsic role regarding tissue repair, it has been shown a great potential concerning wound healing treatment, and many formulations based on HA have been currently used in medical practice.

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9.2 Hyaluronic acid

9.2.1 Structure

Hyaluronic acid is a nonsulfated linear glycosaminoglycan that consists of repeating disaccharide units of β (1 → 4)-glucuronic acid (GlcUA) and β (1 → 3)-N-acetylglucosamine (Kogan et al., 2007). The molecular mass of HA can ranges from 10^4 to 10^7 Da, depending on the source and the extraction and purification procedures used (Lapcik et al., 1998).

In addition to primary structure, HA exhibits the secondary and tertiary structure in aqueous solution. The secondary structure is stabilized by inter- and intramolecular hydrogen bonds between carboxyl groups and GlcNAc that generates hydrophobic faces formed by axial hydrogen atoms (Scott et al., 1991). Such hydrophobic patches energetically favor the formation of meshwork-like β-sheet tertiary structure due to a molecular aggregation. The tertiary structure is stabilized by hydrophobic, electrostatic interactions, and hydrogen bonds (Brown and Jones, 2005; Hascall and Laurent, 1997). Furthermore, increase of the HA concentration and molecular weight induces viscoelasticity increase. The viscoelasticity is dependent on the pH and is affected by the ionic medium strength. The pKa value of D-glucuronic acid carboxyl groups is about 3.0, and a pH change will affect the HA chains ionization, altering the of polymer molecular interactions and its rheological properties (Brown and Jones, 2005).

9.2.2 Properties

The unique physicochemical and biological properties of HA such as hygroscopic, viscoelastic, and antioxidant make it a material of interest for applications in the medicine and cosmetics fields, including osteoarthritis treatment (Yu et al., 2014; Huang et al., 2019), ophthalmic surgery (Kretz et al., 2014; Taban and Shamie, 2018), plastic surgery and esthetic treatment (Coleman, 2006; Dai et al., 2019), drug delivery (Tripodo et al., 2015; Suner et al., 2019), periodontitis (Fujioka-Kobayashi et al., 2017; Chen et al., 2019), skin moisturizers, and wound healing (Shin et al., 2016; Beninatto et al., 2019).

9.2.2.1 Hygroscopic properties

HA is highly hygroscopic. In aqueous solution, hydrogen bonding occurs between adjacent carboxyl and N-acetyl groups allowing the HA to maintain conformational stiffness and to retain water (Dahiya and Kamal, 2013). According to Sutherland (1998), the water-binding capacity of polymer is correlated with its molecular mass. HA exerts an important modulation in the tissue hydration and osmotic balance (Necas et al., 2008). This osmotic property is particularly relevant for cell proliferation and migration (Chen and Abatangelo, 1999). The HA hygroscopic properties are explored in filling facial lines and wrinkles (Moheit and Coleman, 2006).

9.2.2.2 Viscoelasticity properties

The HA viscoelasticity markedly depends on the concentration of solution and molecular mass. Above the entanglement point, the viscosity of HA increases exponentially with concentration and the elasticity increases with higher molecular mass and concentrations (Laurent et al., 1996). The HA viscoelasticity nature can influence in cell functions and may contribute to retardation of viral and bacterial passage through the hyaluronan-rich pericellular zone. As a component of the vitreous humor of the eye and synovial fluid, the HA viscoelasticity presents an important role to cushioning and lubricating (Chen and Abatangelo, 1999). The viscoelasticity of HA is the main feature responsible for its application in ophthalmic surgery. The high viscosity of the HA solution allows to manipulate ophthalmologic tissues and to maintain the surgical space (Schiraldi et al., 2003).

9.2.2.3 Biocompatibility, biodegradability, and nonantigenicity

The well-known biocompatibility, biodegradability, and nonantigenicity of HA make it a suitable material for ophthalmic (Calles et al., 2016), fillers (Moheit and Coleman, 2006), periodontal tissue (Miranda et al., 2016), tissue engineering (Beninatto et al., 2019; Ebrahimi-Hosseinzadeh et al., 2016), and drug delivery (Sgorla et al., 2016; Yegappan et al., 2019) applications. These properties are present for both HA extracted from animal sources and HA produced by bacteria. The microbial HA is chemically identical to mammalian polysaccharide and thereby ensures its nonimmunogenicity (Chong and Nielsen, 2003). Additionally, the biocompatibility and biodegradability of natural polymers such as HA ensure a similarity of the polymer to the extracellular matrix, a property that is explored in the medicine field, for wounds and burns dressing (Mogoşanu and Grumezescu, 2014).

9.2.2.4 Antimicrobial activity

The HA antimicrobial activity is controversial, and few studies have been conducted to evaluate, if any, this polymer effect. Pirnazar et al. (1999) suggested that HA of high molecular mass may prove beneficial in minimizing bacterial contamination in wound healing. However, these authors observed that 1 mg/mL of high molecular mass HA (1.300 KDa) had no bactericidal effect, but the greatest bacteriostatic effect inhibiting the growth of *Actinobacillus actinomycetemcomitans*, *Prevotella oris*, *Staphylococcus aureus*, and *Propionibacterium acnes* strains. Kang et al. (2011) observed that HA retarded the growth but not kill *Candida* cell. Ardizoni et al. (2011) showed that different microbial species and, sometimes, different strains belonging to the same species are differently affected by HA. A dose-dependent microbial growth inhibition by HA was observed to *Staphylococci, Enterococci, Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida glabrata,* and *C. parapsilosis*, but had no effect on *E. coli* ATCC 13768 strain. *Candida albicans* and *Streptococcus sanguinis* were transiently favored by HA at 4 mg/mL (Ardizzoni et al., 2011). HA antiviral activity was also observed by this researcher group (Cermelli et al., 2011). On the other hand, Cerbo et al. (2013) observed no antimicrobial activity when tested with several

lactic acid bacteria strains, but, on contrary, they seemed to enhance the bacterial viability, suggesting that HA may be applied in a new probiotic oral formula. No HA bacteriostatic effect was also showed on *Proteus mirabilis*, *E. coli*, *C. albicans*, *P. aeruginosa,* and *S. aureus* (Tang et al., 2002). Costagliola et al. (1996) studied bacterial ability to use Na-hyaluronate as a nutrient in vitro and observed an increase about 50% in the *Staphylococcus* and *Streptococcus* growth at 72 h using 0.7% of HA. Zhang et al. (2007) also observed an increase of *S. pyogenes* biomass in HA-enriched media, as well as upregulates several virulence factors. According to these authors, the ability to degrade HA should be considered as a streptococcal virulence factor.

In some HA applications such as wound healing, ophthalmic surgeries, and periodentitis treatments, infections are not still completely avoided (Lequeux et al., 2014). According to Kemp et al. (2009), glycosaminoglycans are sugars and can support microbial growth when used in these applications. Due to its controversial antimicrobial activity, HA has been used in combination with antimicrobial agents such as silver nanoparticles (Kemp et al., 2009; Abdel-Mohsen et al., 2013; Chudobova et al., 2013; Mohandas et al., 2015), antibiotic (Ahire and Dicks, 2016), and chitosan (Hoyo-Gallegoa et al., 2016; Valverde et al., 2019). Kemp et al. (2009) observed a minimal inhibitory concentration of 0.025 and 0.1 μM to HA-silver nanoparticle solutions to *S. aureus* and *E.coli*, respectively, while the use of HA solution did not exhibit antimicrobial activity for concentrations up to 1 μM for both bacteria. No antimicrobial activity was found for HA solution between 1 and 10 mg/mL against *S. epidermidis* and *S. aureus* bacteria, while HA combined with nisin exhibited a great antimicrobial property on both bacterial species. In this study, an antibacterial activity of about 99.50% was observed against *S. epidermidis* for HA-$N_{0.01}$ (0.01 eq of nisin) used in the reaction relative to one carboxylic acid of HA solution at 1 mg/mL and approximately 99.95% against *S. aureus* for HA-$N_{0.01}$ solution at 2 mg/mL (Lequeux et al., 2014). Hernández-Montelongo et al. (2016) synthesized nanofilms of HA/chitosan associating the capacity of HA forms a soft, highly hydrated and nontoxic film with the antimicrobial chitosan properties, and this film exhibited an antibacterial surface for *Xylella fastidiosa*. Pérez-Álvarez et al. (2019) studied a multiactive antibacterial multilayers of HA and chitosan onto poly(ethylene terephthalate). These multilayers inhibited *E. coli* adhesion onto developed surfaces, and when triclosan and rifampicin were added in the coatings, better antibacterial activity was observed.

9.2.2.5 Antioxidant activity

HA exhibits antioxidant effect acting as free radical scavenger agent that contributes to increase the tissue repair capacity and the skin protection against UV radiation (Guillaumie et al., 2010). The mechanism by which HA reduces the free radicals damages is based on its structure, which has crosslinked carboxylic groups. Thus, these carboxyl groups can interact with metal ions such as Cu^{2+} and Fe^{2+}, allowing these molecules act as metal chelators (Campo et al., 2004).

Studies have revealed that HA has antioxidant activity in vivo and in vitro (Rosa et al., 2008; El-Safory and Lee, 2010; Kanchana et al., 2013). *In vitro,* antioxidant

activity could be determined using different methodologies: inhibition of lipid peroxidation, scavenging activities of hydroxyl radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation, and superoxide anion scavenging activity. Ke et al. (2011) showed strong inhibition (92.86%) of lipid peroxidation from 1.45×10^5 HA molecular weight at 1120 μg/mL compared to moderate DPPH radical (59.38%) at 1600 μg/mL. The DPPH radical scavenging activity of 1.35×10^6 Da HA molecular weight at 1000 μg/mL was 41% (Pan et al., 2017). The HA antioxidant effect on DPPH scavenging is due to their hydrogen-donating abilities since DPPH accepts an electron or a hydrogen radical to form a stable diamagnetic molecule (Anraku et al., 2015). Anraku et al. (2015) evaluated scavenging activity of HA on DPPH and ABTS radical and suggested that HA is a potential antioxidant. The authors showed an inhibitory concentration of 50% for HA about 1.69 mg/mL on DPPH, and the ABTS radical cation reduction reached 100% at 2.5 mg/mL HA within 5h (Anraku et al., 2015).

In vivo, the HA antioxidant activity with high molecular weight (1000 kDa) was studied in human corneal epithelial cells, using the ethylenediaminetetraacetic acid (EDTA) as the oxidative stress. The results showed that the incubation at 0.2% of HA with cells for 30 min led to a decrease in the synthesis of reactive oxygen species as well as a decrease in DNA damage induced by EDTA (Ye et al., 2012). Studies in patients with osteoarthritis showed that intracellular HA injections reduce levels of H_2O_2 and O_2^- in synovial fluid (Yu et al., 2014). In a cyclophosphamide (CY) induced immunosuppressed mice model, Ke et al. (2011) also showed that orally administration of low HA molecular weight was able to overcome CY-induced immunosuppression and significantly raised the activity of superoxide dismutase, catalase, glutathione peroxidase, and total antioxidant capacity in immunosuppressed mice. Trabucchi et al. (2002) reported that low HA molecular weight is able to permeate the skin, protecting the granulation tissue from reactive oxygen species and stimulating the wound healing.

9.2.3 Biological functions

In vertebrates, HA is an important extracellular matrix constituent (Gall, 2010). High HA concentrations are found in umbilical cord (4.1 mg/mL), synovial fluid (1.4−3.6 mg/mL), and eyes vitreous humor (0.14−0.34 mg/mL). However, the greatest HA concentration is observed in skin that retains 50% of the HA of human body (Kogan et al., 2007; Marcellin et al., 2009). Indeed, the average 70 kg person has approximately 15 g of HA in the body (Gall, 2010).

The HA function is associated with its rheological properties. While HA fragments stimulate gene expression through different cells that regulate inflammatory responses and tissue repair, the whole HA molecule provides the protection against tissue damage caused by the environment and promotes regeneration and repair (Liang et al., 2016). In synovial fluid, due to its viscoelasticity and moisturizing properties, HA provides the joint lubrication, acting as a shock absorber, reducing friction during bone movement and reducing the joint wear. In the cartilage, HA acts as a structural element of the extracellular matrix, forming a central aggregation for proteoglycans, which retains the matrix macromolecular structure. In the skin, the polymer retains water in the tissue, changing the

dermal volume compressibility. Changes in HA is observed with aging, wound healing, and degenerative diseases (Kogan et al., 2007). In wound healing process, HA and its fragments play essential role promoting the increase of wound contraction and epidermal proliferation, cytokines regulation and molecules adhesion, increasing of collagen deposition, and stimulation of neovascularization. In addition, due to its antioxidant effect, HA protects the wound against free radicals (Chen and Abatangelo, 1999; Brown, 2004).

9.3 Microbial production of hyaluronic acid

Originally, the native HA used to be extracted from animal tissues like rooster combs. However, mainly due to the limited tissues sources and viral infection risks, this technique is progressively being replaced by the microbial fermentation. Indeed, the microbial production presents several advantages over animal-derived products as the production parameters are reproducible, renewable resources, easy to be manipulated, biocompatible, and apparently nontoxic. Microbial products have found a very large applications field in clinical medicine (Chong et al., 2005; Liu et al., 2011; Moscovici, 2015).

Groups A and C *Streptococcus* of Lancefield and *Pasteurella multocida* are bacteria that produce HA naturally. *Streptococcus equisimilis, S. pyogenes,* and *S. uberis* are also capable of producing HA. However, among these, the species *Streptococcus equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* are the most commonly used (Oliveira et al., 2016; Marcellin et al., 2014).

Alternatively, metabolic engineering has been used to obtain HA producers from recombinant strains. Initially, recombinant *Bacillus subtilis* and *Escherichia coli* strains were created for the HA production (Yu and Stephanopoulos, 2008; Mao et al., 2009; Jia et al., 2013; Westbrook et al., 2018). HA has also already been produced by a wide variety of heterologous host as *Lactococcus lactis* (Chahuki et al., 2019), *Pichia pastoris* (Jeong et al., 2014; Westbrook et al., 2018), *Agrobacterium*, *Corynebacterium glutamicum* (Cheng et al., 2016), *and Pasteurella* (Chu et al., 2016).

9.3.1 *Biosynthesis of hyaluronic acid by* Streptococcus zooepidemicus

Streptococcus produces HA as a capsular exopolysaccharide. The capsules are classified as virulence factors that protect the bacteria from being attacked by the complement system and/or phagocytosed by macrophages (Marcellin et al., 2014). Since the prokaryotic polymer is identical to eukaryotes, the synthesis machinery in these microorganisms was probably assimilated from their hosts by horizontal transfer and may be related to the defense against the hosts immune system (Boeriu et al., 2013).

The metabolic pathway of HA biosynthesis by *S. zooepidemicus*, described in Fig. 9.1, showed that HA is obtained from the polymerization of UDP-glucuronic precursor sugars (UDP-GlcUA and UDP-N-acetylglucosamine (UDP-GlcNAc)) by the enzyme HA synthase (HAS) (Tlapak-Simmons, 1999). In *Streptococcus*, the precursor units UDP-GlcUA and UDP-GlcNAc are derived from glucose-6-phosphate and

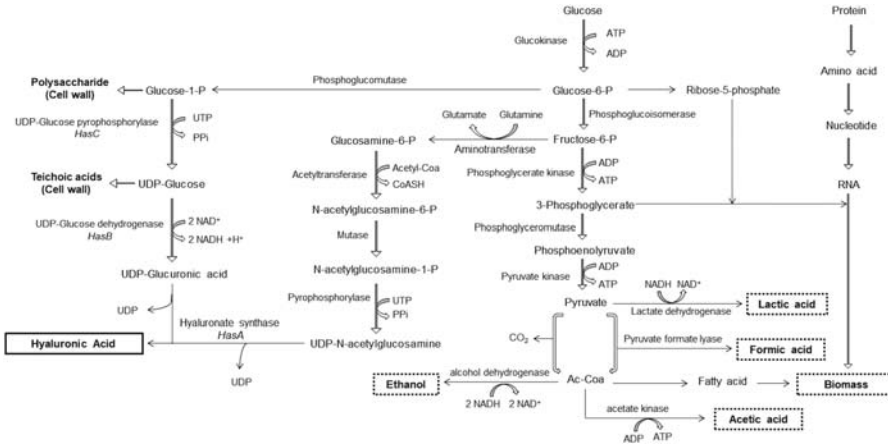

Figure 9.1 Metabolic pathway of hyaluronic acid biosynthesis by *Streptococcus zooepidemicus*.

fructose-6-phosphate, respectively (Fig. 9.1) (Chong and Nielsen, 2003). This process consumes 4 mol of ATP to produce 1 mol of the disaccharide unit. Two moles of ATP are consumed in the glucokinase-mediated reactions to provide the phosphorylated precursor hexoses, and 2 mol of ATP are used to regenerate the UTP donors. The oxidation reaction catalyzed by UDP-glucose dehydrogenase generates 2 mol of NADH for every 1 mol of HA produced (Yamada and Kawasaki, 2005).

Besides the precursors for the synthesis of HA, these pathways also provide the structural components of the bacterial cell wall of the peptidoglycan. Deletion mutants of genes encoding the enzymes responsible for HA synthesis had decreased polymer production and cell growth (Zhang et al., 2016). In this way, the biosynthesis of the polymer competes for carbon sources with cell growth, glycolytic and pentose phosphate pathways (Boeriu et al., 2013; Shah et al., 2013).

The biosynthetic needs of lactic acid bacteria are largely supplied by complex nitrogen sources. Therefore, high amount of the carbon source is converted into the fermentation products, being low levels recovered in the biomass (Chong et al., 2005). Approximately 5%−10% of the metabolized carbon is directed to the production of HA (Chong and Nielsen, 2003), and 90% are converted to lactic acid. However, under certain conditions, homolytic metabolism can be lost and high amounts of formic acid (under anaerobic conditions), acetic acid, and ethanol are produced, characterizing the heterolactic metabolism (Garrigues et al., 2001). The redirection of the carbon flux from lactic acid to acetic acid may favor the production of HA, since the synthesis of acetic acid generates ATP, necessary for the production of the polymer (Chong and Nielsen, 2003).

9.3.2 Fermentation conditions

The fermentation conditions are crucial for the HA production (Shah et al., 2013). Table 9.1 summarizes the different culture conditions for the production of HA by *S. zooepidemicus*. The polymer is usually produced by batch fermentation at temperatures

Table 9.1 Production of hyaluronic acid by different strains of *Streptococcus zooepidemicus*, culture conditions, and fermentation media.

***S. zooepidemicus* ATCC**	**Culture conditions**	**Fermentation medium (g/L)**	**Hyaluronic acid (g/L)**	**References**
39920	1.2 L bioreactor; 37°C; 400 rpm; 1 vvm; pH 7.0	Glucose (30); glutamine (5); sodium iodoacetate (4.6×10^{-3})	5.0	Shah et al. (2013)
39920	3 L bioreactor; 37°C; 150 rpm; 2 vvm, pH 7.0	Cashew juice (2 L) and yeast extract (60)	1.76	Oliveira et al. (2013)
39920	125 mL Erlenmeyer; 37°C; 100 rpm; initial pH 8,0	Sugarcane molasses (30) and yeast extract (30)	0.38	Pan et al. (2015a)
39920	125 mL Erlenmeyer; 37°C; 100 rpm; initial pH l 8,0	Glucose (30) and yeast extract (30)	0.787	Pan et al. (2015b)
35246	2 L bioreactor; 37°C; without aeration; 500 rpm; pH 6.7	Glucose (50), fish viscera peptones (protein 5), and yeast extract (5)	2.32	Vázquez et al. (2015)
35246	5 L bioreactor; 37°C; 1 vvm; 500 rpm; pH 6.7	Glucose (50), lactose (50), cheese whey (protein 5), and yeast extract (5)	4.0	Amado et al. (2016)
39920	4.5 L bioreactor; 37°C; 100 rpm; 0.5 vvm; pH 8.0	Pretreated sugarcane molasses (85.35) and yeast extract (50)	2.83	Pan et al. (2017)

Table 9.1 Production of hyaluronic acid by different strains of *Streptococcus zooepidemicus*, culture conditions, and fermentation media.—cont'd

S. zooepidemicus ATCC	Culture conditions	Fermentation medium (g/L)	Hyaluronic acid (g/L)	References
35246	0.75 L bioreactor; 37°C; 500 rpm; 1 vvm; pH 6.7	Corn steep liquor (10% v/v) and glucose (50)	3.48	Amado et al. (2017)
3523	4.5 L bioreactor; 37°C; 200 rpm; 1 vvm; pH 7.0	Palmyra palm sugar (30) and soya peptone (17.5)	1.22	Ghodke et al. (2018)

near 37°C and pH 7.0 (Liu et al., 2011). The optimum pH of the HAS enzyme has been described in 7.1 (Stoolmiller et al., 1969). But, recent studies show an increase in polymer production when the bacteria is exposed to pH stress conditions in which the cell produces the HA capsule to protect itself in alkaline or acidic media (Liu et al., 2008; Pires and Santana, 2010). Pan et al. (2017) obtained an increase in HA concentration by 2.86 times in fermentation with pH controlled at 8.0 when compared to no pH control (initial pH 8.0). The pH controlled at 8.0 increased the production of lactic acid, acetic acid, and biomass, which are the products of the metabolism of HA production (Fig. 9.1).

Temperature is an important factor in HA production, and 37°C has been reported as the optimal temperature for the production of the polymer by *S. zooepidemicus* (Pan et al., 2015a). This same temperature was defined for HA production by *Streptococcus equi* KFCC 10830. The authors reported that at temperatures below 37°C, the production, and molecular weight of the polymer were reduced (Kim et al., 1996).

The availability of oxygen also influences on the production of the polymer since the carbon flux is directed from the production of lactic acid to acetic acid, leading to an increase in the synthesis of ATP (2 mol of ATP/mol of lactic acid; ATP/mol of acetic acid) (Chong and Nielsen, 2003; Duan et al., 2009). In addition, oxygen generates an oxidative stress on the cell, stimulating the production of HA by the protection mechanism (Hasegawa et al., 1999; Huang et al., 2006). Fermentations with aeration ranging from 1−2 vvm showed higher HA production when compared to anaerobic cultures (Jagannath and Ramachandran, 2010; Oliveira et al., 2013).

Carbon source and the concentration can influence on the HA production. Pan et al. (2015b) observed higher HA production in sucrose (0.488 g/L) compared to glucose-based medium (0.429 g/L). The use of complex sugars, such as starch and sucrose, has been linked to a higher yield of ATP (Jagannath and Ramachandran, 2010). The ATP yield during the catabolism can be increased through carbon limitation. However, glucose limitation reduces the molecular mass of HA because of an inadequate supply of sugar to HAS (Chong and Nielsen, 2003). Patil et al. (2011) evaluated glucose

concentration of 8.8−51.2 g/L, and the authors obtained that the optimum concentration for the production of the polymer was 40.5 g/L. The variation of the glucose concentration of 10−30 g/L had no significant effect on the production of the polymer (Pan et al., 2015a).

The enrichment of the culture medium may also directly influence the metabolism of the bacteria, since *Streptococcus* requires culture medium rich in amino acids, nucleotide bases, and vitamins, necessary for microbial growth and polymer production. These nutrients can be found in complex nitrogen sources such as peptones and yeast extract (Marcellin et al., 2009). Statistical tests showed that yeast extract and soy peptone are significant variables that had positive effect in HA production (Patil et al., 2011; Pan et al., 2015a, 2017).

The addition of glutamine to the culture medium has been described by increasing the production of HA because that amino acid is an amino donor for the synthesis of GlcNAc (Im et al., 2009; Aroskar et al., 2013). Shah et al. (2013) observed that the addition of glutamine led to a carbon flow balance for the UDP-GlcUA and UDP-GlcNAc precursors promoting an increase in the HA production.

Studies indicated a positive correlation between the intracellular concentration of UDP-GlcNAc and the molecular mass of the polymer (Chen et al., 2009, 2014; Badle et al., 2014), which may be justified by the affinity of the precursor for the hyaluronate synthase enzyme. The value of Km, substrate concentration required for the enzyme to reach half its maximum speed, for UDP-GlcNAc is 150−1000 μM, while for UDP-GlcUA, it is significantly smaller, 30−75 μM, showing higher specificity (Itano et al., 1999; Tlapak-Simmons, 1999). Therefore, higher concentrations of UDP-GlcNAc are required to maintain the function of the enzyme hyaluronate synthase (Marcellin et al., 2014).

Im et al. (2009) evaluated the effect of the addition of different ions and amino acids to the culture medium and observed that $MgCl_2$ and K_2HPO_4 are the best mineral and phosphate source, respectively. Mg^{2+} sources were used as cofactors to polymerize UDP-GlcUA and UDP-GlcNAc. Among the amino acids studied, glutamine and glutamate had positive influence in the polymer production.

More recently, Attia et al. (2018) developed a novel HA production method using magnetic nanoparticles and amino acids. The amino acids studied were L-glycine, L-lysine, arginine, aspartic acid, and glutamic acid. Among these amino acids, the glutamic acid showed the highest effect on HA production. Therefore, the production using Fe_3O_4 nanoparticles (20 mg/L) and glutamic acid (0.260 g/L) biostimulated the cells of *Streptococcus equi* to produce the highest dry weight of HA (0.435 g/L) over 600 min of bioprocesses. According to Abdelsalam et al. (2017a, 2017b), the nanoparticles are fastly uptaken by the bacterial cells in shorter time compared to usual bacterial nutrients. Nanoparticles uptake mechanism by *Streptococcus equi,* and its role in HA production coenzymes inside the bacterial cells and plays an important role in the ATP synthesis which biostimulate the bacterial cells and improve the cell activity.

9.3.3 Alternative substrates for production of hyaluronic acid

According to Vázquez et al. (2010), more than 80% of the costs of producing microbial HA are due to the components of the fermentation medium. Thus, several studies have

been proposing the substitution of traditional sources for industrial byproducts. The use of culture media formulated with byproducts can reduce production costs by more than 50% when compared to fermentations using synthetic media (Vázquez et al., 2010; Amado et al., 2016).

The agroindustrial byproducts and residues already studied for the production of HA were: sugarcane broth and molasses (Amado et al., 2017; Pan et al., 2017), cashew juice (Pires et al., 2010; Oliveira et al., 2013), cheese whey protein (Amado et al., 2016), skimmed milk (Izawa et al., 2010), soy protein, soy molasses, and corn steep liquor (Pires et al., 2010; Pan et al., 2015b; Amado et al., 2017). Marine byproducts such as peptones obtained from fish viscera residues and waste water from mussel processing have also been explored (Vázquez et al., 2010, 2015).

Sugarcane molasses was first studied in the production of HA by Pan et al. (2015a). The authors verified a higher yield of HA in medium containing crude sugarcane molasses (without any previous treatment), when compared to the medium based on sucrose or glucose (Pan et al., 2015b). However, Amado et al. (2017) found an inhibition on the growth of *S. zooepidemicus* when 10% (v/v) of raw sugarcane molasses was used. Pan et al. (2017) suggested that the activated charcoal pretreatment decreased the level of inhibition by factors such as excessive metal ions. The authors obtained higher polymer production when crude molasses was replaced by pretreated molasses (Pan et al., 2017).

9.4 Hyaluronic acid function in wound healing

The biological process mediated by HA has a central role in wound healing (Chen and Abatangelo, 1999). The wound healing consists of a perfectly coordinated cascade of cellular, molecular, and biochemical events that culminate in tissue reconstitution. The process is divided into three phases: inflammatory, proliferative, and remodeling (Clark, 1988; Wells et al., 2016).

HA is synthesized on the cell membrane by three HAS proteins and secreted into the extracellular space. These generate predominantly HA with high molecular mass ranging between 200 and 2000 kDa (Chakrabarti et al., 2016). The HA having high molecular mass predominates in noninjured tissue and exhibits antiinflammatory, immunosuppressive, and antiangiogenic properties (Campo et al., 2011; Liang et al., 2011; Papakonstantinou et al., 2012; Caskey et al., 2013). After the tissue injury, the inflammatory phase begins immediately with the release of vasoconstrictive substances in the cell membranes. The injured endothelium and the platelets stimulate the coagulation cascade aimed to restore hemostasis. Next, neutrophils are attracted by chemotactic substances released by platelets producing free radicals that help the bacterial destruction. Neutrophils are gradually replaced by macrophages, which secrete cytokines and growth factors and also contribute with angiogenesis, fibroplasia, and extracellular matrix synthesis (Campo et al., 2007). In this phase, the native HA is degraded due to hyaluronidase enzymatic activity, mechanical forces, and oxidative stress. Then, in the inflammatory phase, the accumulation of low molecular

weight polymer (<200 kDa) and their oligomers (<30 kDa) are observed (Bollyky et al., 2012; Chakrabarti et al., 2016; Liang et al., 2016). HA is also responsible to promote the activation and maturation of dendritic cells, the release of proinflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-12, drive cell migration, promote proliferation by numerous cell types, and promote the matrix metalloproteases activity. Many of these proinflammatory effects of low HA molecular weight are mediated through interactions with the toll-like receptors TLR2 or TLR4 (Bollyky et al., 2012). The healing process is triggered by inflammation response, which needs to be moderate in order to guarantee the normal development of the tissue repair process. HA plays a vital role during the inflammatory response moderation. This is probably mediated through a free-radical scavenging property and through its specific biological interaction with inflammatory response constituents (Chen and Abatangelo, 1999; Brown, 2004).

The proliferative phase is characterized by an intense cell activity and migration of different cell types to the injured region. The major events of this phase is the epidermis restoration (epithelialization), new blood vessels formation (angiogenesis), and granulation tissue (fibroplasia) (Li et al., 2007). During this phase, HA provides a framework for cellular adhesion, migration, and proliferation. Furthermore, HA facilitates the ions and nutrients transport and is responsible to keep the wound moisture (Manuskiatti and Maibach, 1996; Stadelmann et al., 1998; Chen and Abatangelo, 1999). Additionally, it has a protective action on the granulation tissue, avoiding the damaging effects of free radicals and proteases, which preserve the extracellular matrix stability (Chen and Abatangelo, 1999).

The remodeling or restorative phase corresponds to the final stage of healing process, which correspond to the collagen synthesis and its degradation by metalloproteinases (Costa et al., 1999; Singer and Clark, 1999). With the deposition and remodeling of collagen, fibronectin gradually disappears, and HA is replaced by sulphated glycosaminoglycans (chondroitin sulfate) that are subsequently degraded until they reach the concentrations found in normal skin (Deodhar and Rana, 1997; Stadelmann et al., 1998).

9.5 Hyaluronic acid in wound healing therapeutics

The HA role in cell migration and proliferation implies that it might be applied to help stimulate a wound healing (Brown, 2004). Studies in vivo and in vitro showed HA and its derivatives as component of topical formulations or as scaffold provided beneficial healing effects in several types of wounds including burns, venous leg ulcers, and others chronic wounds.

9.5.1 Hyaluronic acid in tissue repair

HA has been studied in topical formulations, and its exogenous application has shown beneficial effects in tissue repair. In clinical studies with HA, no evidence of

immunogenicity, hypersensitivity, or systemic adverse reactions was reported making this biopolymer suitable for wounds treatment (Brown and Jones, 2005; Kogan et al., 2007; Neuman et al., 2015). Hyalgan by Fidia (Abano Terme, Italy) was the first commercial product containing HA and was extensively used in the 1960s as a topical cream for burns and ulcers treatments. Hyaloform (Genzyme) and Restylane (Q-Med) were approved by FDA in 2003 (Marcellin et al., 2009). In 2004, Hyiodine by Contipro Pharma enters in human medicine market becoming one of the most effective products in the market. Composed by HA and low amount of iodine complex (antiseptic), it is mainly indicated to support healing in chronic stagnating wounds. Hyiodine is also used in healing acute wounds, where it accelerates the healing process and offers antimicrobial protection (Hyiodine - About Hyiodine, n.d.).

The literature describes some studies that use Hyiodine in wound treatment. Slavkovsky et al. (2010), studied the Hyiodine effect in rat skin wounds. According to authors, Hyiodine supported wound healing by stimulating wound contraction and epidermal proliferation and by keeping the wound moist through increased exudation. Laznicek and coworkers (2012) also evaluated the effect of HA mixture, iodine complex KI_3, and their combination (Hyiodine) in excision skin wound in rats and concluded that hyaluronan-iodine hydrogel has a great potential for effective wound treatment (Laznicek et al., 2012). In a preclinical study, hyaluronate-iodine complex was applied to patients' wounds from a wound healing center. Hyaluronate-iodine was helpful in the healing of all types of wounds treated in this pilot study. The antiadhesive and antimicrobial properties of hyaluronate-iodine create a desirable environment to wound healing without apparent detrimental effects (Brenes et al., 2011).

HA topical applications to rats' digital shaved skin incision significantly improved the wound healing speed and appearance, acting specifically in the remodeling and fibroplasia phases (Yoshioka et al., 2013). In another study, the increase of keratinocytes migration, formation of granulation tissue and capillaries in wounds treated with gel containing HA are observed (Chang et al., 2015). Tolg and coworkers (2014) compared the effects of native HA and its oligosaccharides. The results showed that HA oligosaccharides mixture promoted fibroblast migration in scratch wound assays in rats while native HA inhibited this migration (Tolg et al., 2014). Gao et al. (2010) reported that HA oligosaccharides (disaccharides 2 to 10) promoted the tissue injuries repair of a dermal excisional wound in mice. Histological analysis revealed an increase of neo-blood and lymph vessels. In addition, treatment with HA oligosaccharides increased production granulation, collagen deposition, fibroblast proliferation, and regulation of certain cytokines and adhesion molecules in treated wounds. The efficacy of formulations containing HA to wound healing has also been demonstrated in clinical trials with cutaneous open wounds dogs (Ferrari et al., 2015).

Diabetic patients have wound healing dysfunction associated with morbidity and mortality. The physiological factors that affect wound healing in diabetic patients are the decreased production of growth factors and vascular insufficiency resulting in decreased local angiogenesis. Due to these factors, there are many studies addressing wound healing in diabetics (Galeano et al., 2011; Mohandas et al., 2015). HA systemic injection (4000 kDa) under skin incisions performed in genetically diabetic mice resulted in a significant improvement in scarring, and benefitted on collagen synthesis

and formation of new vessels at the wound site (Galeano et al., 2011). These results corroborate with findings of Vazquez et al. (2003), who studied the HA application in the chronic wound treatment in diabetic feet, and concluded that HA can enhance granulation tissue formation rate and decrease the fibrotic tissue formation. More recently, topical treatment with liposomes comprising highly skin-permeable growth factors combined with HA was evaluated in a diabetic mouse model and the results suggested an accelerated chronic wound closure through stimulating reepithelialization in association with the regeneration of connective tissue (Choi et al., 2017).

Other studies also report the HA application for the venous leg ulcer treatment. Venous leg ulcers are a common and recurring type of complex wound that affect about 0.10%−0.80% of the general population (Humbert et al., 2013). Clinical study demonstrates that HA local application on leg ulcers of venous or mixed etiology is significantly more effective than a neutral vehicle on wound size reduction, healed ulcers rate, and pain management with a good safety profile (Dereure et al., 2012; Humbert et al., 2013).

9.5.2 Hyaluronic acid scaffolds

HA also has been used in scaffolds development that serve as a three-dimensional template for cell differentiation, adhesion, proliferation, and an extracellular matrix formation, as well as a carrier of the growth factors or other biomolecular signals. Scaffold should be biocompatible and resorbable and not elicit a permanent foreign body reaction and should be eventually reabsorbed and replaced by natural tissue (Collins and Birkinshaw, 2013). A nanofiber scaffold using HA and gelatine was investigated based on a deep second degree burns model for Wistar rats. The research demonstrated the membrane efficacy with a percentage of wound closure of 81.9% compared with 65% of the untreated control (Ebrahimi-Hosseinzadeh et al., 2016). Wound healing rate accelerated was also observed in normal and streptozotocin-diabetic Sprague-Dawley rats by HA/poly(lactic-co-glycolic acid, PLGA) core/shell fiber matrices loaded with epigallocatechin-3-O-gallate (EGCG) (HA/PLGA-E). These authors showed that the wound healing activity of the HA/PLGA-E matrices can be attributed to the synergistic effects of HA and EGCG, which prominently promote the reepithelialization, ECM reorganization, and revascularization in wounds (Shin et al., 2016). A new transdermal device was also successfully fabricated from chitosan and HA to lidocaine anesthetic delivery, and the results showed that it can potentially be applied to human skin with a resistance to microbial activity and no skin irritation (Anirudhan et al., 2016). Mohands et al. (2015) proposed a system of chitosan-HA composite sponge containing vascular endothelial growth factor loaded nanofibrin, and the results suggested that the product induced angiogenesis in wound healing. Enhanced wound healing in the equine distal limb was also observed using thiolated carboxymethyl HA-based film (Dahlgren et al., 2016).

Searching for replacement for the skin transplantation therapy currently held for the healing of chronic wounds, Su et al. (2014) evaluated the HA effect and epidermal growth factor (EGF) applied in decellularized membranes, which were prepared starting from the pig peritoneum. The authors observed that the wounds treated with membranes containing HA and EGF showed 87.41% of repair, while the control exhibits 70.14% repair. Wu et al. (2015) also studied HA effect of EGF-containing membranes

decellularized, and the results showed that decellularized scaffolds loaded with HA and EGF significantly induced the skin wounds recovery when compared with scaffolds alone, suggesting that decellularized scaffolds containing HA and EGF could be a potential treatment for human skin injuries. In another study, HA was incorporated in lyophilized fibrin sheets to improve water retention in the wound bed in rabbits and the increase of water retention, and an improvement in wound healing was detected. After the 14th day, the complete healing was evident in the control wounds, but the treated samples showed better healing due to the newly established collagen matrix (Anilkumar et al., 2011).

Hyalomatrix (Anika Therapeutics) is a commercial scaffold based on a cellular three dimensional matrix made of benzyl ester HA layer and a transparent silicone membrane that has proven to be a good skin substitute in different clinical situations. Six female patients aged from 10 to 60 years were included in a clinical trial to assess the efficacy of Hyalomatrix grafts. Histological observations showed that Hyalomatrix has promoted the growth of a similar normal dermal tissue after 3 weeks of application, showing that Hyalomatrix is a good dermal substitute, enhance dermal regeneration, heal clinically and histologically better the skin, although still unable to control the eventual collagen contraction (Faga et al., 2013). Hyalomatrix has also been demonstrated efficacy as a dermal substitute for use in burns (Gravante et al., 2010), chronic wound (Motolese et al., 2013), and surgical treatment of skin cancer (Dessy et al., 2016).

9.6 Conclusion and perspectives

The production of microbial HA has been preferred since the extraction and purification to animal HA is quite complex and costly, and is not recommended for human therapeutics because of viral contamination risks and other infectious agents. Thus, evaluating the demands of the market, studies has been directed for the production of HA by fermentative processes. During fermentation, a product of desired quality can be obtained for specific applications. HA has a multifaceted role in tissue repair in early inflammatory processes and the formation of granulation tissue and epithelialization. Several studies described its great potential in wound healing treatment, and some formulations composed of HA have been successfully used in clinical practice. Further investigations in tissue engineering and regenerative medicine fields would be helpful to widespread the utilization of HA in tissue and organs reconstructions. Therefore, studies aiming to decrease the production cost of HA such as the use of alternatives substrates by microbial fermentation will contribute to making the product more accessible to the market.

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Recent advancements in fermentation studies for lovastatin biosynthesis

10

R.S. Upendra [1], *Pratima Khandelwal* [2]
[1]Department of Biotechnology, School of Applied Science, REVA University, Bengaluru, Karnataka, India; [2]Teaching-Learning Centre, Global Academy of Technology, Bengaluru, Karnataka, India

10.1 Introduction

As per the World Health Organization (WHO, 2010), hypercholesterolemic patients exhibit elevated levels of blood cholesterol levels and are at high risk of developing cardiovascular diseases (CVDs). The highest percent of global deaths occurring in recent times has been due to the CVDs, and 80% among them have been reported in under-developed countries (WHO, 2011; Prabhakaran et al., 2016). The CVDs affect both male and female population equally and estimate to cause death of 23 million people globally by the year 2030 (Mathers and Loncar, 2006). The condition of hypercholesterolemia can be treated easily with the medication by targeting the reduction of low-density lipoprotein (LDL) levels in the blood (Sánchez et al., 2018). Statins are the drug of choice today for treating CVDs, and they function by reducing LDL cholesterol level in the blood as a result of inhibiting the rate limiting enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) of cholesterol biosynthesis pathway. Among the various statins (Table 10.1), lovastatin is a natural polyketide molecule, discovered in the year 1970, and was clinically proved to lower LDL cholesterol (Goswami et al., 2012; Khandelwal et al., 2013). With successful clinical trials and animal safety studies, Food and Drug Administration (FDA) approved lovastatin as an anticholesterol drug for the treatment of hypercholesterolemia (FDA, 1988). Lovastatin exists in two forms, i.e., a closed, stable lactone, and open and unstable β-hydroxy acid forms (Fig. 10.1) (Lisec et al., 2012). Lactone form of lovastatin consists of β-hydroxy lactone ring attached to hexahydronaphthalene moiety with methyl butyric (R1) and 6-α methyl group (R2) as side chains (Seenivasan et al., 2008).

Statins are typically fungal-derived polyketide molecules and are well known for their inhibitory action on rate limiting enzyme, i.e., HMG-CoA reductase of liver cholesterol biosynthesis pathway; hence, statins are named as HMG CoA reductase inhibitors. Because of their inhibitory activity on HMG CoA reductase enzyme, the statins are used as preventive drugs for CVDs, Coronary Heart Disease (CHDs), and also in conditions such as diabetes and high blood pressure. The HMG CoA reductase is also named as Mevalonate NADP (Nicotinamide Adenine Dinucleotide

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Table 10.1 Statins and their types.

Sl. No	Derivative type	Statin kind	Chemical formula	Molecular Mass g/mol	Brand name
1.	Fermentation derived	Lovastatin	$C_{24}H_{36}O_5$	404.54	Mevacor Altocor Altoprev
2.		Mevastatin	$C_{24}H_{34}O_5$	390.513	Compactin
3.		Pravastatin	$C_{23}H_{36}O_7$	424.528	Pravachol Selektine Lipostat
4.		Simvastatin	$C_{25}H_{38}O_5$	418.566	Zocor, Lipex
5.	Synthetic	Atorvastatin	$C_{33}H_{35}FN_2O_5$	558.64	Lipitor Torvast
6.		Cerivastatin	$C_{26}H_{34}FNO_5$	459.55	Baycol Lipobay
7.		Fluvastatin	$C_{24}H_{26}FNO_4$	411.466	Lescol Lescol XL
8.		Pitavastatin	$C_{25}H_{24}FNO_4$	421.461	Livalo Pitava
9.		Rosuvastatin	$C_{22}H_{28}FN_3\ O_6S$	481.539	Crestor

Phosphate) 1 oxidoreductase (EC 1.1.1.34) (Nagegowda et al., 2004; Upendra, 2017). Biosynthesis of cholesterol takes place principally in the cytoplasm and membranes of endoplasmic reticulum of all the different tissue types of human body, including liver tissue, intestinal tissue, cortex portion of adrenal gland, and reproductive tissues. In comparison with dietary source, 70% of the total circulating cholesterol in the blood is synthesized internally by the organ liver; hence, inhibiting the rate limiting step of liver cholesterol biosynthesis pathway decreases the overall cholesterol levels in the blood. The mevalonate pathway of cholesterol biosynthesis in the liver and the mode of action of statins on the rate limiting enzyme, i.e., HMG CoA reductase (Mevalonate pathway), is shown in Fig. 10.2 (Egom and Hafeez, 2016).

Lovastatin has been reported to inhibit proliferation of different cell types in human body and control the growth of tumor cells by inducing a process of programmed cell death (apoptosis and necrosis). This has been studied in breast cancer (Klawitter et al., 2010) and colon cancer (Zeidooni et al., 2012), wherein migration of cancer cells was suppressed by inhibiting Ras Farnesylation pathway (Xia et al., 2001). The patients suffering with kidney disease have been treated successfully with lovastatin therapy by promoting creatinine clearance and suppressing the renal abnormalities (Buemi et al., 2002). It is well reported that the lovastatin therapy healed bone fractures by enhancing bone formation rates and has been proved to be safe and effective for bone regeneration (Yoshii et al., 2010). The prevalence and occurrence of Alzheimer's disease in hypercholesterolemia patients was

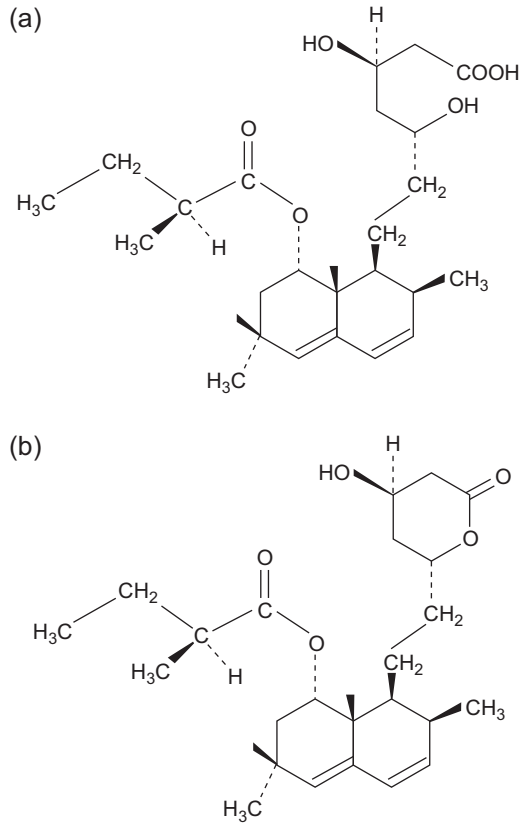

Figure 10.1 Structure of Lovastatin. (a) Structure of β-hydroxy acid forms of lovastatin, (b) Structure of lactone form of lovastatin.
From Lisec, B., Radež, I., & Žilnik, L. F. (2012). Solvent extraction of lovastatin from a fermentation broth. Separation and purification technology, 96, 187−193.

reduced with the treatment of lovastatin (Dolga et al., 2008). The lovastatin inhibits lymphocyte migration in the brain endothelial cells and attenuates autoimmune encephalomyelitis (Greenwood et al., 2003). Lovastatin therapy has been reported to possess many advantages, i.e., enhances the function of endothelial cells, regulates inflammatory response, maintains plaque stability, prevents thrombus formation, and increases thrombocyte counts in dengue hemorrhagic fever (Kozarov et al., 2014). Lovastatin treatment has been found to be safe and effective in children suffering with neurofibromatosis Type 1 (Bruce, 2008; Upendra et al., 2016a) and prevented epileptogenesis by inhibiting the excess protein synthesis in a mouse model of Fragile X syndrome (Flight, 2013). Adverse effects of statin such as muscle ache or pain, diarrhea, weakness, nausea, and indigestions have been reported when it was overprescribed (Golomb and Evans, 2008).

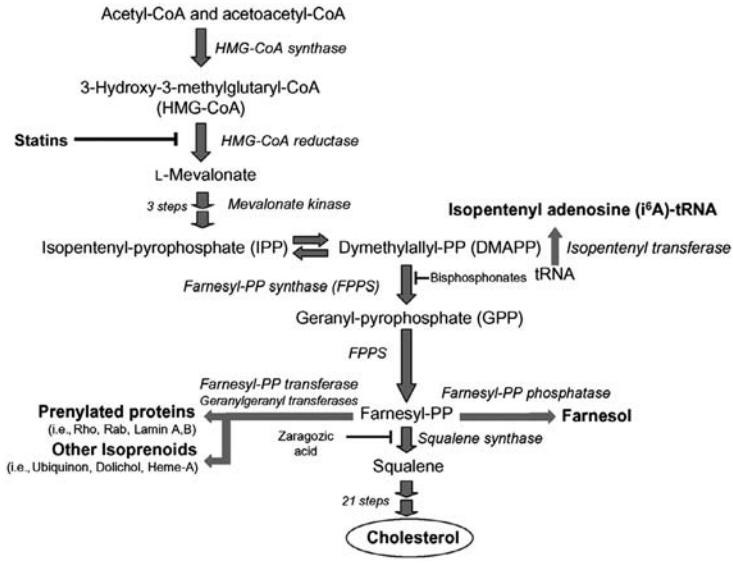

Figure 10.2 Cholesterol biosynthesis (Mevalonate) pathway illustrating the mechanism of action of statin drugs (Egom and Hafeez, 2016).

Aspergillus spp. (Gupta, 2016), *Monascus* sp. (Panda et al., 2010), *Pleurotus* spp., and *Rhizopus oryzae* (Rajkumar and Anusha, 2019) are known to produce lovastatin through polyketide biosynthetic pathway (Upendra et al., 2014a; Mulder et al., 2015; Kamal et al., 2018). The FDA approved *Aspergillus terreus* as the best fungus to produce lovastatin used in the treatment of CVDs (Sreedevi et al., 2011). Liquid surface fermentation (LSF) methodology was employed in earlier years (Seress et al., 2001), but presently Submerged Fermentation (SmF) process is being applied widely for the production of lovastatin (Upendra and Khandelwal, 2016; Ansari et al., 2018). In the recent times, the solid-state fermentation (SSF) process has also been implemented for enhancing lovastatin yield (Balraj et al., 2017; Kamal et al., 2018). Several biomass, e.g., soybean, rice, maize, wheat, and renewable substrates such as wheat bran, black gram husk, etc., have been used successfully as substrates for the synthesis of lovastatin applying SSF process (Shivakumar and Ravuri, 2018). The studies of the published work reveal that there is a good scope of investigating screening of high-lovastatin yielding natural occurring fungal strains, cost-effective fermentation methods, and simplified downstream processing conditions to make the overall lovastatin biosynthesis process more efficient. It also opens the gateway for employing new combination of optimization techniques as shall be discussed in this review.

10.2 Lovastatin producing fungi

10.2.1 *Aspergillus terreus*

A. terreus, a soil fungus, also referred as *A. terrestris*, is found predominantly in the soils all over the world. It was known as a strictly asexual fungus, but recent studies have reported its capability in performing sexual reproduction (Arabatzis and

Velegraki, 2013). *A. terreus* is known to occur in warmer climates like tropical and subtropical regions; soil, compost, and decomposing vegetation. On culture media, the young colonies of the *A. terreus* appears brownish in color and gets in to dark brown color as it grows. The unique cinnamon brown color colonies of the *A. terreus* fungus allow it to be differentiated readily from the other species of the *Aspergillus* genus. The optimal growth temperature for *A. terreus* is reported between 35 and 40°C, and the thermotolerant nature of the species favors the fungus to reach its maximum growth at 45−48°C (Sreelatha et al., 2017). *A. terreus* is known to produce a number of medicinally important secondary metabolites such as lovastatin, terreic acid (Upendra et al., 2016b), terrein, territrem A, citrinin, citreoviridin, gliotoxin, patulin, and terretonin (Stefan et al., 2009). In *A. terreus,* fast and high growth of filamentous hypha result in lower yield of lovastatin. By supplementing the important nutrients during the course of fermentation process, the fungus registers very high yield of lovastatin. The carbon and nitrogen source type and quantity in the fermentation medium influence the yield of lovastatin (Lopez Casas et al., 2003). Apart from lovastatin and different secondary metabolites, *A. terreus* is also known to produce various commercially important organic acids, i.e., *cis*-aconitic acid, itaconic acid, and industrial enzymes such as xylanase (Yang, 2016).

Among the different statins produced by the fungi, lovastatin is found in certain foods such as red yeast rice and oyster mushroom (Upendra et al., 2016a; Pandey et al., 2019). The polyketide pathway of lovastatin biosynthesis was depicted in Fig. 10.3. The enzyme lovastatin nonaketide synthase (LNKS) produced by lovastatin

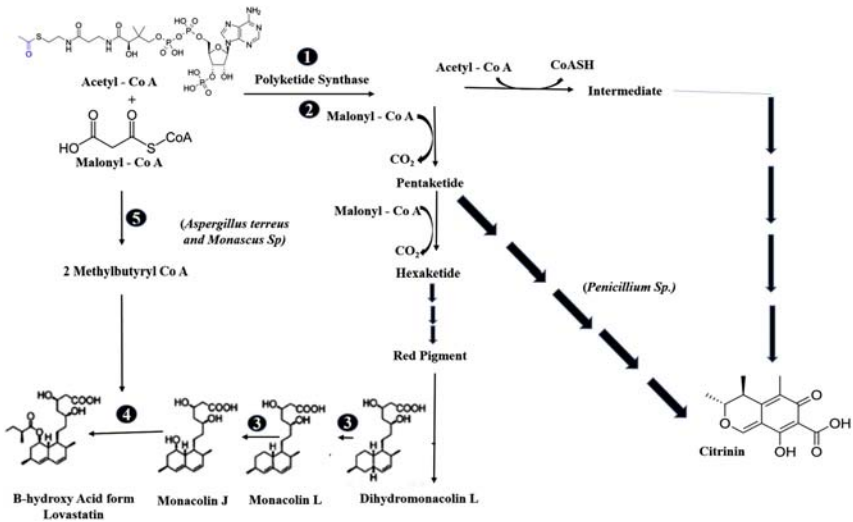

Figure 10.3 Polyketide biosynthesis pathway of secondary metabolite lovastatin in *Aspergillus terreus* and *Penicillium* sp. to citrinin. 1. lovastatin gene (*Lov B*), 2. lovastatin gene (*Lov C*), 3. lovastatin gene (*Lov A*), 4. lovastatin gene (*Lov D*), and 5. lovastatin gene (*Lov F*).

gene (*Lov-B*) and enoyl reductase (ER) derived from *Lov-C* gene synthesize dihydromonacolin L by joining equal amounts of Acetyl CoA and Malonyl CoA. Further, the dihydromonacolin L was oxidized initially to form Monacolin L further to Monacolin J in a two successive steps catalyzed by cytochrome P450 enzyme derived from the gene lovastatin (*Lov-A*). Finally the enzyme transferase, a derivative of lovastatin gene (*Lov-D*), converts Monacolin J to hydroxy acid (open) form of lovastatin (Fig. 10.1). The discussed pathway can be bypassed by the action of *Lov-F* gene, where lovastatin diketide synthase (LDKS) derived from the lovastatin gene (*Lov-F*) coverts equal ratio of Acetyl CoA with Malonyl CoA to 2 Methylbutyryl Co A, which is finally oxidized to from hydroxy acid (open) form of lovastatin (Fig. 10.1) by transferase of lovastatin gene D (*Lov-D*). Further lactonization process forms lactone (closed) form of lovastatin (Fig. 10.1) (Mulder et al., 2015).

10.2.2 *Aspergillus flavus*

A. flavus is a pathogenic fungus with saprotrophic nature and distributed in cosmopolitan conditions. The fungus is well known for its colonization of cereal grains, legumes, and tree nuts. In addition to causing pre-and postharvest infections, the strain is scientifically reported to produce significant quantities of lovastatin and toxic compounds known as mycotoxins. The colonies of *A. flavus* on Potato Dextrose Agar (PDA) medium exhibit powdery masses with yellowish green spores on the upper surface and reddish-gold spores on the reverse surface. In both grains and legumes, the infection is minimized to small areas, and the affected area become dull and discolored. Growth is rapid and colonies appear downy or powdery in texture (Ramírez-Camejo et al., 2012; Kovač et al., 2018).

10.2.3 *Monascus purpureus*

The mold is purple reddish in color, and the most common species is *M. purpureus*. The species is familiar with other names such as rice kernel discoloration mold, *angkak* rice mold, and maize silage mold. The mold is very important and popular in China for its role in the production of certain fermented foods, especially red yeast rice. This species has been reported by many researchers for the production of lovastatin and its analogs such as monoclines (K, L, J) dehydroxymonacolin and compactin (Mevastatin) (Mornar et al., 2013; Suraiya et al., 2018).

10.2.4 *Monascus ruber*

Another species of the genus *Monascus* is *M. ruber* that also produces lovastatin (Panda et al., 2010; Dikshit and Tallapragada, 2015) as well as rice wine, red rice, and food colorants. The young colonies of *M. ruber* on PDA are whitish pink in color and produce reddish to brown pigments that diffuse into the medium.

10.3 Isolation, screening and characterization of lovastatin producing fungi

10.3.1 Isolation and screening of lovastatin positive fungi

Various researchers have attempted to isolate lovastatin positive cultures by screening different sources applying standard microbiological methods such as serial dilution followed by spread plate and pour plate methods on the different fungal selective media i.e., PDA, low carbon agar (LCA), yeast extract mannitol agar (YEMA), and czapek's agar (CZA) media. Lovastatin overproducing strains of *A. terreus* were isolated and further screened, performing bioassay against the sensitive yeast culture (*Candida albicans*). The lovastatin positive culture exhibited zones of inhibition on the lawn cultures of *C. albicans* (Ferron et al., 2005). The different marine *Actinomycetes* cultures, isolated initially from soil samples, were screened to produce lovastatin in a two stage SmF process and evaluated HMG CoA reductase inhibitor activity by agar diffusion method, and reported the isolate (SS16/4) with lovastatin positive strain (Srinu et al., 2010). *A. terreus* strains were isolated from the soils samples collected from different regions of the state Andhra Pradesh, India, and screened for their ability to synthesize lovastatin. The isolate, *A. terreus* (KSVLSUCP-75), was found positive for producing lovastatin (Sreedevi et al., 2011). A total of 40 fungal cultures from Union for International Cancer Control (UICC) were screened for the production of lovastatin using paper disc method through SmF process, and only 18 cultures were shown to synthesize lovastatin (Wibowo et al., 2012). A total of 15 different soil fungi were screened under SmF process to test their ability to produce lovastatin, and *Aspergillus* sp. no.76 was found as the best producer of the drug (Prakash and Srividya, 2014). The yeast (*Saccharomyces cerevisiae*) growth assay method was employed to screen the lovastatin production ability of various isolated strains of filamentous fungi and quantified analytically employing Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and High Performance Thin Layer Chromatography (HPTLC). Among the 36 screened strains, *Cunninghamella blakesleeana* was found to produce 1.4 mg/g Dry Weight of Substrate (DWS) of lovastatin followed by *Aspergillus terreus* (0.83 mg/g DWS) and *A. flavus* (0.3 mg/g DWS) (Balraj et al., 2018).

Hitherto unexplored natural sources of fungi, i.e., soil obtained from the fields of paddy fields, wild oyster mushroom beds obtained from the fields, and compost from the cultivated land were collected in sterile sealed polythene bags from various places of Bengaluru, selected regions in Karnataka and Tamil Nadu of India, and were screened for the presence of lovastatin producing filamentous fungi cultivated on fungal specific media such as PDA, LCA, YEMA, and CZA agar with ammonium nitrate (NH_4NO_3) as the sole nitrogen source, supplemented with 100 μg/mL oxytetracyclin, applying standard microbiological methods (serial dilution and spread plate method). The isolated fungal cultures were morphologically characterized using light microscopy. Further, the fungal isolates belonging to *Aspergillus* sp. and *Rhizopus* sp. were selected and screened through SmF process for their ability to produce lovastatin. Lovastatin content in the extract was determined by Ultraviolet (UV) Spectrophotometer (200−350 nm)

and confirmed through HPLC, ATR (Attenuated Total Reflection)-FTIR (Fourier Transform Infrared) and both ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) studies using the pure lovastatin as standard (Biocon, Bangalore, India). These isolates are also screened for their growth inhibition abilities against *Neurospora crassa* (Microbial Type Culture Collection (MTCC)-790) using bioassay method. High-lovastatin yielding fungal isolates were identified using Scanning Electron Microscope (SEM), and five such isolates were reported (Upendra et al., 2013a).

10.3.2 Molecular characterization methods of lovastatin producing isolates

The identification of *Aspergillus* at species level can be achieved performing a novel characterization method using 18S and 28S Ribosomal Ribonucleic acid (rRNA) genes sequence of Internal Transcribed Spacer (ITS)-1 5.8S−ITS-2 regions compared with referenced strains and clinical isolates of *Aspergillus* in GenBank. The accurate identification of *Aspergillus* at the species level can be explained by both ITS-1 and ITS-2 regions which was demonstrated through the comparison of reference strains and GenBank sequences. Intraspecies variations among clinical isolates and reference strains with respect to ITS region sequence were found to be minimal (Henry et al., 2000). Several sequences for Polymerase Chain Reaction (PCR) primers were tested while developing PCR assays with a wide range of fungal compatibility. A set of four primers group was developed for amplifying ITS regions of fungal rRNA. Primers in the 5.8S sequence were also developed to amplifications of ITS-1 and ITS-2 regions, separately. The complete set of primers developed in the study was highly specific to fungal rRNA and could able to discriminate between plant and fungal sequences successfully (Matrin and Paul, 2005). The differentiation of medically important species of *Aspergillus* from other strains belonging to the same species was successfully achieved through a novel PCR based assay developed by Hinrikson et al. (2005). A set of universal primers was designed to conserve ribosomal genes and species-specific DNA probes directed to the highly variable ITS-1, ITS-2, and D1 and D2 region. These amplified regions were then detected through a simple PCR-based colorimetric Enzyme Immunoassay (EIA). The accurate identification of *Aspergillus* till species level was more reliable with ITS-1 and ITS-2 regions compared to the regions of D1 and D2 (Hinrikson et al., 2005). A polyphasic approach including sequence analysis of parts of the β-tubulin, calmodulin genes, and the ITS region were studied by Samson et al. for the identification of *Aspergillus* species section *terrei*. Seven lineages were observed during the phylogenetic analysis of calmodulin and β-tubulin sequences. It was concluded that the reported isolates of the study were previously been recognized and reported as *Aspergillus terreus* and its subspecies by Raper and Fennell (Raper and Fennell, 1965; Samson et al., 2011).

Upendra et al. (2013a, 2016c) investigated three wild type fungi, i.e., *A. terreus* SSM4, isolated from wild oyster mushroom bed (lovastatin yield of 997 μg/g dry fungal matter (dfm)), *A. terreus* SSM3 from compost source (lovastatin yield of 900 μg/g dfm), and *A. flavus* SSM8 from compost source (lovastatin yield of 643 μg/g dfm). These were initially identified using SEM as shown in Fig. 10.4,

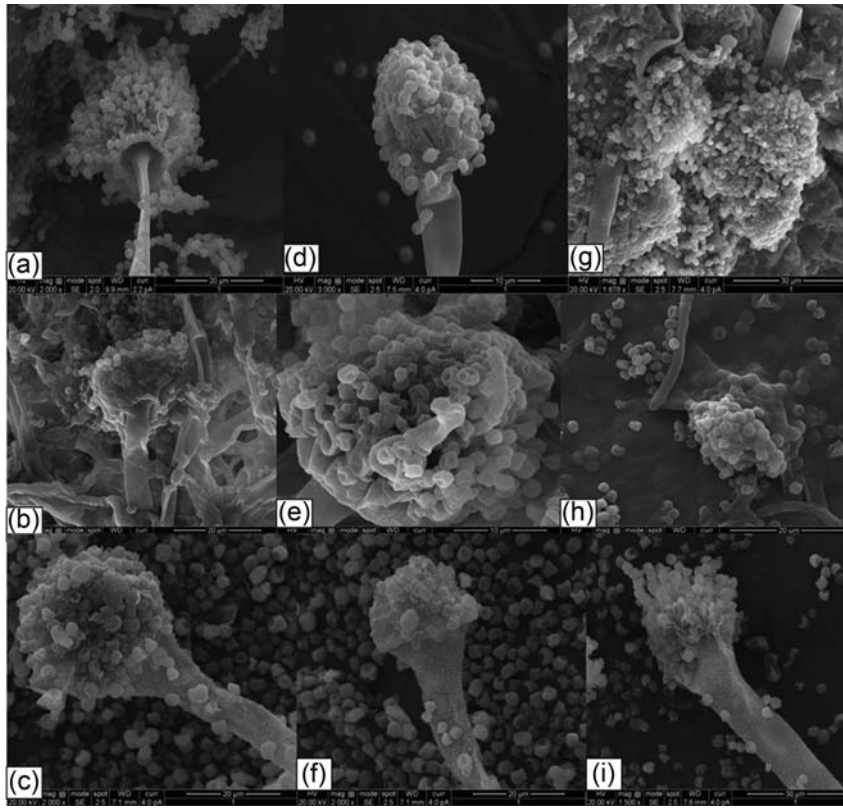

Figure 10.4 SEM Characterization of isolated fungal strains. (a−c). Conidiophores and Conidia of *A. terreus* (SSM4). (d−f). Conidiophores and Conidia of *A.terreus* (SSM3). G-I. Conidiophores and Conidia of *A. flavus* (SSM8).
Adapted from Upendra, R.S., Khandelwal, P., Amiri, Z.R., Swetha, L., Mohammed, A.S., 2013a. Screening and molecular characterization of natural fungal isolates producing lovastatin. Journal of Microbial & Biochemical Technology 5(2), 25−30, Upendra, R.S., *Khandelwal, P.*, Amiri, Z.R., 2016c. Molecular characterization and MTCC submission of lovastatin maximum yielding fungi isolated from natural samples. Proceedings of 3rd Asian Food Safety and Food Security Association Conference on Food Safety and Food Security. *Bhubaneswar,* India, pp. 24−33.

further characterized at molecular level by restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing, and 18s RNA (ITS1-5.8S-ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using MEGA version 5 software (Fig. 10.5). Results confirmed the SSM3 and SSM4 fungal isolates to be *A. terreus*, and also inferred that SSM8 as *A. flavus.* The sequence information of the three isolates was submitted in National Center for Biotechnology Information (NCBI) GenBank with the issued accession numbers: JX419386.1—*A. terreus* (SSM3) (Upendra and Khandelwal 2013b), JQ897354.1—*A. terreus* (SSM4) (Upendra et al., 2013c), and JQ899451.1—*A. flavus* (SSM8) (Upendra and Pratima, 2013a). These were deposited in the MTCC and

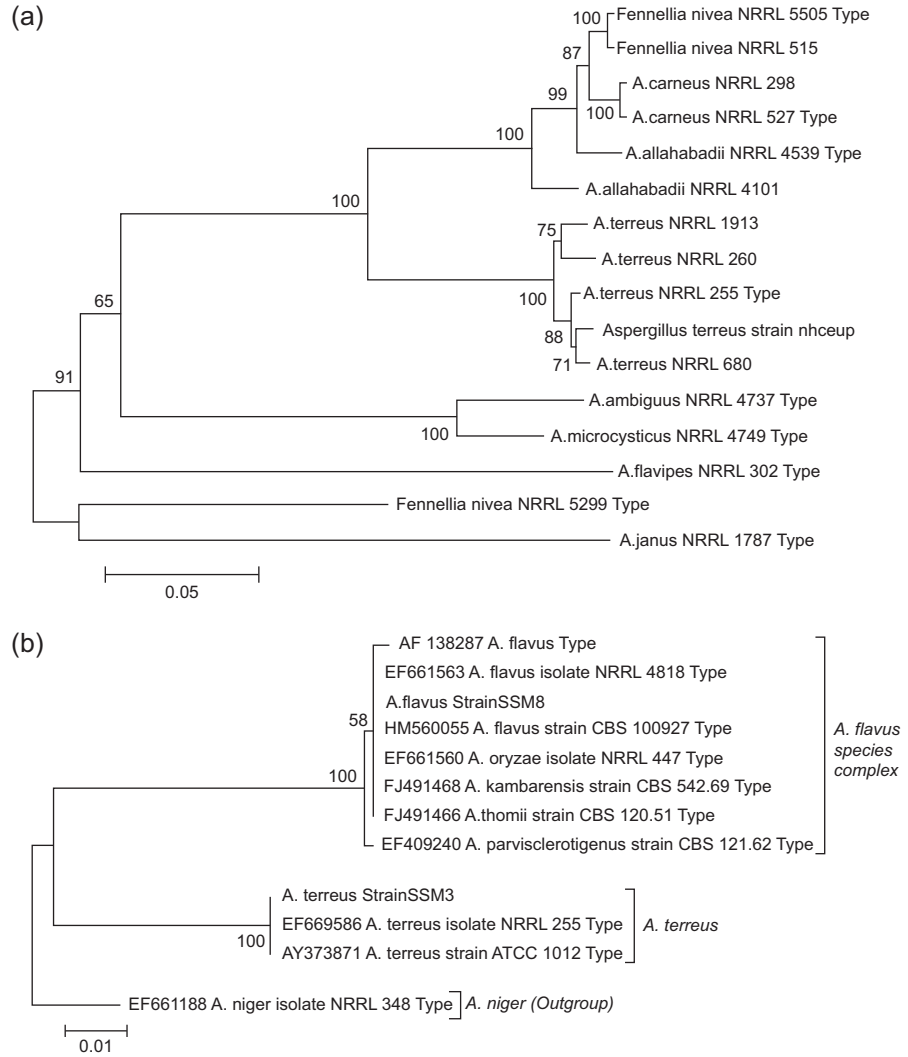

Figure 10.5 Phylogenetic analysis of isolated high yielding fungal strains. 5a. Phylogenetic tree of *Aspergillus terreus* strain nhceup (SSM4), inferred from Neighbor Joining (NJ) analysis of partial β-tubulin gene sequence, 5b. Phylogenetic tree of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) inferred from NJ analysis of ITS1-ITS4 region sequencing. Adapted from Upendra, R.S., Khandelwal, P., Amiri, Z.R., Swetha, L., Mohammed, A.S., 2013a. Screening and molecular characterization of natural fungal isolates producing lovastatin. Journal of Microbial & Biochemical Technology 5(2), 25−30, Upendra, R.S., *Khandelwal, P.*, Amiri, Z.R., 2016c. Molecular characterization and MTCC submission of lovastatin maximum yielding fungi isolated from natural samples. Proceedings of 3rd Asian Food Safety and Food Security Association Conference on Food Safety and Food Security. *Bhubaneswar,* India, pp. 24−33.

Gene bank at Institute of Microbial Technology (IMTech), Chandigarh, and given accession numbers as *A. terreus* nhceup 11045 (SSM4), *A. terreus* NHCEUPBT 11395 (SSM3), and *A.flavus* NHCEUPBTE 11396 (SSM8).

Morphological methods used alone for species identification of clinically and industrial relevant *Aspergillus* species have limited utility. Use of molecular with traditional phenotype-based methods are stronger, sensitive, and also offer better resolution in the recognition of the novel fungal isolates at species levels particularly with in the *Aspergillus* genus. These studies indicate that for intersection level fungal identification, ITS regions sequencing can be used, and for identification of individual species within the various *Aspergillus* sections, the β-tubulin locus can also be used effectively as shown in Fig. 10.5 (Balajee et al., 2007; Upendra et al., 2013a, 2016c).

10.4 Software tools employed in lovastatin bioprocess optimization

10.4.1 Molecular evolutionary genetics analysis version (5.1)

The molecular evolutionary genetics analysis (MEGA) software is a statistical tool for comparative analysis of the molecular sequence data based on evolutionary principles. It helps in alignment of molecular sequence data, inferring phylogenetic tree and ancestral sequences, calculating divergence times and molecular evolution rate, and also helps in testing evolutionary hypotheses. Many biologists use MEGA for the process of reconstructing the evolutionary histories of species. MEGA software is an effective teaching tool, as this facilitates visualization, interactive exploration of sequence data, phylogenetic trees, and analysis of results with respect to bioinformatics (Tamura et al., 2011).

To exemplify, different species of endozoic fungi present in the sponges isolated from the South China Sea was molecularly catheterized using MEGA v.4.0. ITS-based phylogenetic analysis was carried out to identify the endozoic fungi at molecular level. The study reported 14 various species of endozoic fungi belonging to 14 different genera, i.e., *Aspergillus* sp., *Penicillium* sp., *Scolecobasidium* sp., *Eurotium* sp., *Alternaria* sp, *Fusarium* sp., *Hypocreales* sp., *Yarrowia* sp., *Candida* sp, *Hypoxylon* sp., *Sporidiobolus* sp., *Schizophyllum* sp., *Bjerkandera* sp., and *Trichosporon* sp. (Yu et al., 2013). Upendra (2017) applied MEGA V 5.1 for phylogenetic inference of newly isolated lovastatin-producing fungal species. The study used forward strand partial β 2 tubulin gene sequence (545 nt) of *Aspergillus terreus* SSM4 isolate and aligned with maximum identity score sequence *A. terreus* NRRL 255 strain through Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using NJ method (Fig. 10.5(a)), the optimal tree with sum of branch length = 0.97,315,489 was discussed (Fig. 10.5(a)). *A. terreus* (SSM3) species forward strand 18S rRNA gene sequence (740 nt) was aligned with maximum identity score sequence EF669586-*A. terreus* NRRL 255 strain, and *Aspergillus flavus* (SSM8) forward strand 18S rRNA gene sequence (751 nt) was aligned with maximum identity score sequence EF661563 *A. flavus* NRRL 4818 through BLAST. The evolutionary history was inferred using NJ (Fig. 10.5(b)). (Upendra, 2017)

10.4.2 State Easy version 9.0.0.7

State easy version is a Windows-based optimization program. This consists of several useful statistical tools, i.e., Two-level factorial screening designs, which help in breakthrough improvements in process/product formation by identifying the factors that affects the process or product. It also helps in discovering the best combination of categorical factors, such as source versus type of raw material. The rotatable 3D plots available in the design expert program facilitate the easy view of response surfaces from all angles (Sayyad et al., 2007a; Bizukojc and Gonciarz, 2015).

To illustrate, Lopez Casas et al. (2004) investigated the effect of five parameters, i.e., concentrations of carbon (C) (8−48 g), nitrogen (N) (0.2−0.6 g) and phosphorous (P) (0.5−2.5 g), oxygen content (20%−80% (v/v)) in the gas phase, and fermentation time (7−11 days) on the yield of lovastatin in the SmF cultures of *A.terreus* by applying Response Surface Methodology (RSM) using State Easy V.9 optimization tool. This study revealed that the oxygen content in the gas phase was the principle factor influencing the production of lovastatin and observed that lovastatin yield was significantly reduced by both limited and excess quantities of oxygen. This investigation reported four-fold raise in its yield, compared with the unoptimized process condition (Lopez Casas et al. 2004). Sayyad et al. (2007a) applied Plackett−Burman Design (PBD) to employing State Easy V.9 optimization tool to identify principle nutrients factors in supporting the yield of lovastatin under submerged conditions using cultures of *M. purpureus* MTCC 369 strain. Nine nutrient parameters, i.e., dextrose, peptone, ammonium chloride (NH_4Cl), yeast extract, potassium dihydrogen phosphate (KH_2PO_4), ferrous sulfate hydrated ($FeSO_4$ 7 H_2O), magnesium sulfate hydrated ($MgSO_4$ 7 H_2O), manganese sulfate hydrated ($MnSO_4$ H_2O), and calcium chloride hydrated ($CaCl_2$ $2H_2O$) were screened in 12 factorial design experiments. The results indicated $MnSO_4$ H_2O and dextrose to be the principle factors in enhancing the lovastatin yield. Yeast extract, $FeSO_4$ 7 H_2O, and $CaCl_2$ $2H_2O$ had little impact, while NH_4Cl, KH_2PO_4 and peptone contributed moderately toward its yield (Sayyad et al., 2007a).

10.4.3 Matrix laboratory v. R2011a and 2014

Matrix laboratory (MATLAB) has built-in neural network toolbox that saves from the hassle of coding and setting parameters. Advanced code can also be generated from where a user can change the parameters. MATLAB toolbox is quite easier and self-explanatory on the neural model execution. Neuron, GENESIS, NEST, and Brian were the most generally used biological network simulators. MATLAB R2014a software was used by many researchers for process optimization studies. MATLAB is fourth-generation programming language with multi-paradigm numerical computing environment (Upendra et al., 2014b).

A MATLAB-based fuzzy logic system was employed in the optimization of lovastatin production using airlift bioreactor for the cultures of *A. terreus*. The dilution rate and the biomass concentration were the two parameters selected for the study to understand their impact on the overall lovastatin yield. The study reported approximately 2-fold increase in the lovastatin yield in comparison with

unoptimized process condition (Gupta et al., 2010). Duraklı-Velioğlu et al. (2013) optimized red pigment production for the SmF culture of *Monascus purpureus* using artificial neural network design. The study analyzed the combined influence of different SmF process factors such as temperature, agitation speed, and light on the final yield of red pigment. The ANN (Artificial Neural Network)-optimized process conditions were used for the production of red pigment at higher quantities (Duraklı-Velioğlu et al., 2013).

10.5 Lovastatin production methods

10.5.1 Submerged state fermentation

In the SmF process, organism is grown beneath the surface of the medium. Earlier, lovastatin was produced by LSF technique but presently SmF techniques are being employed throughout the world. Various research groups have screened different carbon employing pure culture and isolated fungal cultures to target enhanced lovastatin yield sources under SmF conditions (Table 10.2).

Table 10.2 Summarized SmF process of lovastatin production.

Sl. No	Substrate used in SmF	Organism/culture used	Yield	References
1.	Glucose	*Aspergillus terreus*	Traces amounts of Monacolin L	Treiber et al. (1989)
2.	Glucose with n-dodecane	*Aspergillus terreus* ATCC 20542	54.9 mg/L	Lai Long- Shan et al. (2002)
3.	Glucose	*Aspergillus terreus* ATCC 20542	230 mg dm−3	Lopez Casas et al. (2004)
4.	Glucose	*Aspergillus terreus* ATCC 20542	80 mg/L	Lopez Casas et al. (2005)
5.	Lactose	*Aspergillus terreus* ATCC 20542	873 mg/L	Lai Long- Shan et al. (2007)
6.	Dextrose	*Monascus purpureus* MTCC 369	Moderate amounts	Sayyad et al. (2007a)
7.	Dextrose	*Monascus purpureus* MTCC 369	Moderate amounts	Sayyad et al. (2007b)
8.	Dox-rice	*Aspergillus terreus* J9	148.6 mg/L	Atalla et al. (2008)

Continued

Table 10.2 Summarized SmF process of lovastatin production.—cont'd

Sl. No	Substrate used in SmF	Organism/culture used	Yield	References
9.	Glucose	*Monascus purpureus*	345 mg/L	Ahmad et al. (2009)
10.	Glucose supplemented with tyrosine	*Aspergillus terreus* LA414	952.7 ± 24.3 mg/L	Jia et al. (2010)
11.	Oat meal	*Aspergillus terreus* TCC 20542	188.3 mg/L	Osman et al. (2011)
12.	Glucose	*Aspergillus terreus* NRRL 265	471.91 mg/L	Ahmed et al. (2013)
13.	Glucose	*Aspergillus terreus* (SSM4)	996.6 mg/L	Upendra et al. (2013a)
14.	Lactose	*Aspergillus terreus* (SSM4)	2990 mg/L	Upendra et al. (2013b)
15.	Lactose	*Aspergillus terreus* ATCC20542	80 mg/L	Bizukojc and Gonciarz (2015)
16.	Glucose	*Aspergillus terreus*	701 mg/L	Syed and Rajasimman. (2015)
17.	Glucose	*Omphalotus olearius*	12.51 mg/L	Burcu et al. (2016)
18.	Date syrup	*Aspergillus terreus*	241.1 mg/L	Ansari et al. (2018)

Conventional fermentation method involves the changing of one independent variable/process at a time while keeping the others as fixed level. Such process is also time-consuming, and, thus, results false impression of data (Lai et al., 2007). The statistical optimization is more reliable and exhibits evident advantages such as screening and short-listing the important fermentation process variables, and access to rapid, reliable, and reproducible results. Further, the optimized fermentation process consorts the interactions of experimental variables and also reduces the total number of experiments at a single point in time (Dhar and Nigam, 2015). The screening of critical factors/variables is initially carried out by PBD studies (Plackett and Burman, 1946). The selected process parameters are then optimized using RSM studies. RSM design has some advantages including fewer experiment numbers, suitability for multiple factor experiments, searching correlation between factors, finding of the most suitable condition, and forecasting response for the process studied (Mouafi et al., 2016; Ansari et al., 2018).

Ahmed et al. (2013) tested *A. terreus* National Regional Research Laboratory (NRRL) 265 strains at lab scale for the production of lovastatin under SmF. The critical fermentation process conditions, i.e., incubation time, temperature, initial pH, and nutritional parameters, i.e., carbon sources, nitrogen sources, age, and volume of inoculum, were optimized to study their impact on the yield of lovastatin. The maximum lovastatin yield of 471.91 mg/L was achieved at the optimized process conditions, i.e., 30°C of incubation temperature, the initial pH of 6.0%, 9% glucose as a carbon source, 2.5% corn steep liquor as nitrogen source, 0.3% ammonium sulfate as inorganic nitrogen source, and 30 h old inoculum at a level of 5% (Ahmed et al., 2013). Syed and Rajasimman (2015) carried out a PBD studies (12 experimental run design) to screen the influence of eight nutrient parameters of SmF process employed with *A. terreus* culture and reported 170.4 mg/L of lovastatin yield. The significant factors influencing the lovastatin yield were identified based on the PBD results and were further optimized applying Central Composite Design (CCD) of RSM. A total of five parameters i.e., glycerol, copper chloride hydrated ($CuCl_2.2H_2O$), $FeSO_4.7H_2O$, KH_2PO_4, and $MgSO_4.7H_2O$ selected for optimization studies employing CCD-RSM. At the optimum condition, a maximum lovastatin production of 701 mg/L was obtained by this research team (Syed and Rajasimman, 2015). Burcu et al. (2016) applied statistical design-based optimization studies such as PBD and Box−Behnken design (BBD) of RSM for the SmF cultures of *Omphalotus olearius* isolate OBCC 2002. Various nutrient and process parameters of the SmF process were screened initially applying 12 factorial design model of PBD. Based on the experimental results, four significant process variables, i.e., glucose, peptone, agitation, and NaCl were selected and further optimized applying BBD of RSM. The optimized combination of medium containing glucose (10 g/L), peptone (5 g/L), thiamine (1 mg/L), and NaCl (0.4 g/L) supported 12.51 mg/L lovastatin yield which was 8-fold higher than that unoptimized process yield (Burcu et al., 2016). The syrup extracted from the dates is a rich source of glucose and fructose, hence supported microbial culture growth and metabolite synthesis. A research group utilized date syrup as a medium for the *A. terreus* culture under SmF conditions to produce lovastatin. RSM design studies were employed to optimize four variables of lovastatin production media, i.e., date syrup as a complex carbon source, yeast extract as nitrogen source, pH, and agitation (rpm). The optimized combination of the studied factors, i.e., concentration of date syrup carbohydrates, 64 g/L; yeast extract concentration, 15 g/L; pH, 6.5; and agitation speed, 150 rpm led to 105.6 mg/L of lovastatin yield. Further batch culture of *A. terreus* isolate with 2.5 L working volume production media maintaining the RSM-optimized combination was tested in the bioreactor. The scale-up process (laboratory scale) reported lovastatin titer of 241.1 mg/L during 12 days of SmF conditions (Ansari et al., 2018).

The RSM design cannot explain the nonlinear relationship between the factors studied, so the results obtained need to be validated applying hybrid Artificial Neural Network and Genetic Algorithm (ANN-GA) studies in order to prove the fitness of the applied RSM design model. ANN is a computerized program designed to simulate the process in the way of Central Nervous System (CNS) function. In recent times, ANN is being increasingly used in biotechnology and pharmaceutical research to predict the nonlinear relationship between casual factors and response variables

(Mohaptra et al., 2016). Hybrid ANN-GA has a remarkable ability to derive meaningful information from the data of independent process variables and predict the genetic fitness of the fermentation process. Optimization of the different parameters of SmF process has been done by the various researchers but limited investigations have been made on optimization of the different nutrients and process parameters together using standard optimization and validation methods such as PBD, CCD-RSM, and hybrid ANN-GA. PBD experimental studies were carried out to screen the effects of five medium constituents, i.e., lactose (40−80g), glycerol (40−80g), honey (40−80g), mycological peptone (5−25g), yeast extract (5−25g), and four process parameters namely pH (6.0−7.6), temperature (24−28°C), agitation (120−200 rpm), and fermentation time (5−13 days) on the yield of lovastatin for the newly isolated fungal strain *A. terreus* MTCC 11045 under SmF condition. The influence of medium variables on lovastatin production in comparison with the lovastatin yield was analyzed. The higher F values and % of contribution identifies the significant factors that contribute for the higher lovastatin yield as shown in Table 10.3. The PBD identified lactose, yeast extract, pH, and fermentation time were the principle factors in reporting the higher lovastatin yield (Upendra et al., 2013b).

The pellet growth of *A. terreus* ATCC 20542 cultures under SmF process in stirred tank bioreactors was studied followed by effects of agitation (impeller tip speed u_t of 1.01−2.71 m/s) and aeration role on the broth rheology, fungal pellet morphology, and subsequently lovastatin yield by Lopez Casas et al. (2005). This study reported approximately four fold (80 mg/L) less of lovastatin yield (Lopez Casas et al., 2005) than the yield (3.453 mg/g dfm). The SmF cultures of *A. terreus* ATCC-20542 strain on LBM was studied by Lai et al. (2007) and reported 873 mg/L of lovastatin (Lai et al., 2007) that was approximately four fold less than the yield (3.453 mg/g dfm). Sayyad et al. (2007b) applied PBD to identify principle nutrients factors in supporting the yield of lovastatin for the submerged cultures of *M. purpureus* MTCC 369 strain. Nine nutrient parameters, i.e., dextrose, peptone, NH_4Cl, yeast extract, KH_2PO_4, $FeSO_4$ 7 H_2O, $MgSO_4$ 7 H_2O, $MnSO_4$ H_2O, and $CaCl_2$ $2H_2O$ were screened in 12 factorial design experiments and reported quite low yield (Sayyad et al., 2007b) of lovastatin as compared to the lovastatin yield of (3.453 mg/g dry matter). The lovastatin yields reported in these studies were much lower to the results reported with *A. terreus* (MTCC 11045) (Upendra, 2017).

The PBD selected significant factors; lactose (40−80 g/L), yeast extract (5−25 g/L), pH (4.0−7.0), and fermentation time (8−14 days) were further optimized applying a full factorial CCD of RSM to achieve the enhanced lovastatin yield. The lovastatin in the spent broth was extracted and quantified by UV spectrophotometrically at 238 nm and confirmed through (^{1}H and ^{13}C) NMR analysis. The goodness of fit values of the studied CCD-RSM model was shown in Fig. 10.6. The goodness of fit values of the studied RSM model explained that the experimental results lied on the 45-degree line implying that the model predicted results were in a high similarity and express close agreement with the experimental data.

The RSM-optimized medium containing 65 g/L of lactose, 20 g/L of yeast extract, 6.8 pH, and 12 days of fermentation time found to support the maximum yield of lovastatin (3.065 mg/g dfm) (Upendra et al., 2014c). RSM result was validated using

Table 10.3 PBD studies depicting the influence of medium variables (5 nutrients + 4 processes + 2 dummy on lovastatin yield).

Designation	Variables	ΣH	ΣL	Mean square	Effects	F-value	% Of contribution
X_1	Glycerol	9.194	10.020	0.062	−0.150	1.016	0.0102
X_2	Lactose	12.261	6.953	2.560	0.965	41.970	0.4252
X_3	Honey	9.196	10.018	0.061	−0.140	1.000	0.0101
X_4	Temperature	10.101	9.013	0.108	0.197	1.770	0.0179
X_5	pH	10.250	9.020	0.136	0.220	2.230	0.0225
D_1	Dummy 1	9.156	10.019	0.016	−0.148	1.000	0.0101
X_6	Agitation speed(rpm)	10.114	9.100	0.093	0.184	1.525	0.0154
X_7	Incubation period	12.260	6.954	2.560	0.964	41.960	0.4251
X_8	Yeast extract	10.412	8.793	0.214	0.296	3.951	0.0400
X_9	Mycological peptone	9.195	10.019	0.061	−0.150	1.000	0.0101
D_2	Dummy 2	9.200	10.120	0.077	−0.148	1.262	0.0127

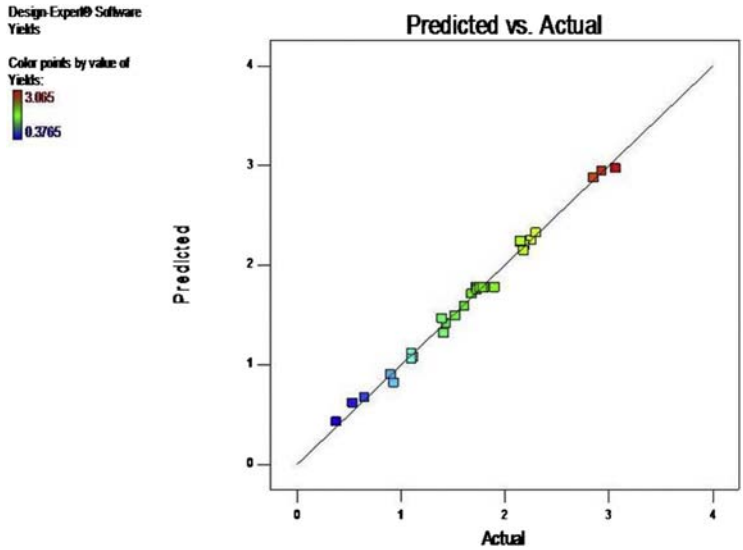

Figure 10.6 Graph representing the actual and CCD RSM predicted values of SmF lovastatin yield.
Source: State Easy Free Trail Version 9.0.0.7.

hybrid ANN-GA design and the topological flow of the model design with input, hidden, and output values as shown in Fig. 10.7. The simulated value of lovastatin yield as predicted by feed forward neural network (FFNN) model (3.064 mg/g dfm) of ANN was in close agreement with the experimental values (3.065 mg/g dfm) and higher than the predicted value of CCD of RSM.

Four *A. terreus* and one *Penicillium patulum* species were screened for their ability to yield lovastatin on different semisynthetic media (Atalla et al., 2008). Among the tested fungal strains, *A. terreus* J9 reported 2-fold less yield (1761.6 mg/L of lovastatin as compared to the yield of lovastatin (3.453 mg/g dfm) reported by *A. terreus* (MTCC 11045) (Upendra, 2017). Ahmad et al. (2009) produced lovastatin using *M. purpureus* strain in SmF process. A synthetic media was employed in the study for the SmF cultivation of *M. purpureus* culture, and a new extraction procedure was applied to purify the lovastatin, which gave yield of 737 mg/L of synthetic medium (Ahmad et al., 2009). That was approximately 5 fold less than the lovastatin yield (3.453 mg/g dfm) reported by *A. terreus* (MTCC 11045) (Upendra, 2017). Jia et al. (2010) studied the effect of supplementation of exogenous lovastatin and different polyketide antibiotic in the fermentation medium on the final yields of lovastatin. A 20%−25% increase in the final yield of lovastatin was observed when SmF process medium was supplemented with any of the five different kinds of polyketide antibiotics such as tyrosin, erythromycin, tetracycline, daunorobicin, and rifamycin separately. The study reported the final lovastatin yield of 952.7 mg/L (Jia et al., 2010) that was approximately 3.5 fold less than the lovastatin yield (3.453 mg/g dry matter) reported by *A. terreus* (MTCC 11,045) (Upendra, 2017).

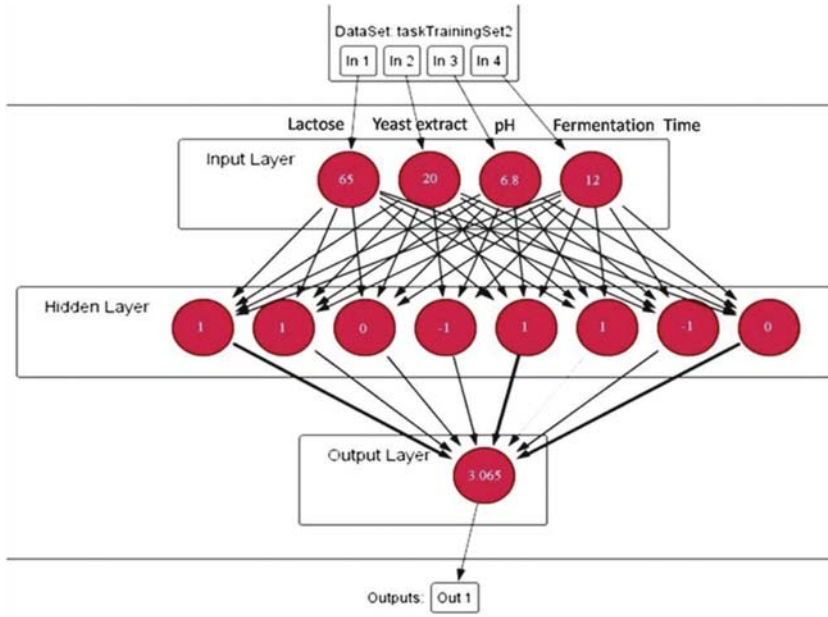

Figure 10.7 Topology of Feed Forward Model of ANN with input layer, hidden layer, and output layer.
Source: State Easy Free Trail Version 9.0.0.7.

GA operates through mechanisms of the population, selection, crossover, mutation, and natural selection process of evolutionary theory proposed by Sir Charles Darwin (Singh and Srivastava, 2013). GA addresses the nonlinear correlation problems of the different process variables and provides an accurate solution. The best fitness plot accessed during the analysis of GA after 90 generations explained the gradual progression of the results with respect to the optimal solution. The amount of mutation decreased along with the average distance between individuals, which was approximately 0 at the final generation (Fig. 10.8). GA reported that the optimal set of factors studied, i.e., lactose (65 g/L), yeast extract (20 g/L), pH (6.8), and fermentation time (12 Days) were found to influence the enhanced yield (3.065 mg/g dry matter) of lovastatin. The yield of lovastatin achieved during SmF process conditions was found to match exactly with the hybrid ANN-GA prediction.

10.5.2 Solid-State fermentation

SmF means of producing lovastatin is being practiced at industrial scale throughout the globe (Lai et al., 2002). Although widespread, this technique possesses certain limitations, i.e., lack of cost-effective production media formulation, high capital investment for the setup, operation and maintenance of the process machinery and high throughput devices, extensive downstream processing methodology, and comparatively less productive yields. In order to overcome these limitations, many researchers in recent years

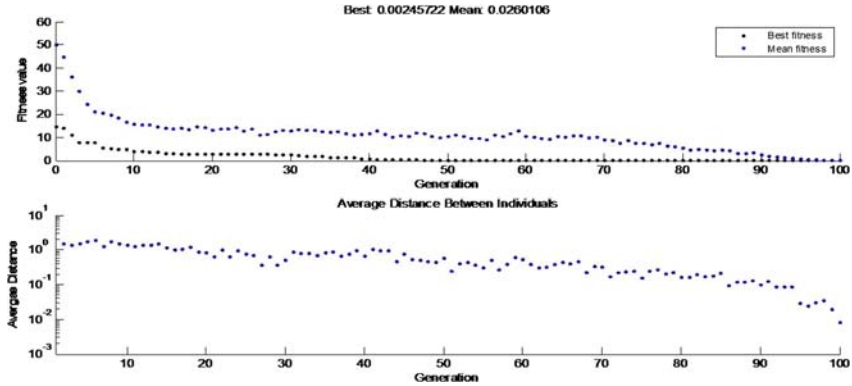

Figure 10.8 GA best fitness graph representing performance progression of *Aspergillus terreus* MTCC 11045.
Source: State Easy Free Trail Version 9.0.0.7.

have attempted SSF process by applying different renewable substrates as the principal carbon source for the production of lovastatin. Compared to SmF, SSF process of lovastatin production exhibits clear advantages in using cost-effective production media (using different renewable sources like agricultural/agro-industrial wastes, whey powder, etc), simpler fermentation machinery, availability of one-step extraction and purification methods, environment friendly process design can be targeted with value addition and zero waste generation along with important feature of higher yield of product (Praveen et al., 2015; Javed et al., 2017). Various researchers have screened different renewable substrates as raw material for the cost-effective synthesis of lovastatin employing different cultures as depicted in Table 10.4.

Lingappa et al. (2005) applied SSF process using the strain of *A. terreus* KLVB 28 on the deseeded carob pod solid medium for the production of lovastatin. The SSF conditions were optimized applying conventional method. The parameters of moisture content, 65%; pH, 4.5; temperature, 35°C; inoculum size, 1×10^8 spores/mL; particle size, 2 mL and bed depth, 2 cm were found to support maximum yield 0.289 mg/g dfm of lovastatin (Lingappa et al., 2005). Another group of researchers employed various agro-wastes such as gram bran, wheat bran, fruits waste, bagasse, barley, soybean meal, and their combinations under SSF process for the production of lovastatin for the cultures of *Aspergillus flavipes* BICC 5174. Among the various substrates tested, wheat bran was reported to support maximum yield in stagnant beds (13.49 mg/g dfm) condition. Further, the group tested the process in aerated stirred beds and reported 16.65 mg/g dfm of lovastatin after 6 days of fermentation. It critically found that supplementation of external carbon sources, i.e., sucrose and lactose, nitrogen sources, i.e., urea, ammonium sulfate, ammonium nitrate to the solid substrate inhibited lovastatin production. Particle size, moisture content, and pH of the substrate also affected the yield of lovastatin. Wheat bran of particle size ranging from 0.3 to 0.5 mm with moisture level of 60% and pH 5.0 supported the maximum yield of lovastatin. The study finally concluded that a yield of 16.78 mg/g dfm of lovastatin was obtained in

Table 10.4 Compiled work of various research groups on lovastatin production using SSF means employing various lovastatin positive isolated and standard cultures.

Sl.No.	Substrate used in SSF	Organism employed	Yield of lovastatin mg/g of dry fungal matter (dfm)	References
1.	Sweet sorghum/ Whey powder	*Aspergillus terreus*	1.50 mg/g	Szakács et al. (1998)
2.	Carob pod	*Aspergillus terreus* KLVB 28	0.289 mg/g	Lingappa et al. (2005)
3.	Wheat bran	*Aspergillus flavipes* BICC 5174	16.65 mg/g	Valera et al. (2005)
4.	Rice	*Aspergillus terreus* ATCC 20542	2.90 mg/g	Lian et al. (2007)
5.	Dox-rice	*Pleurotus ostreatus*	0.055 mg/g	El-Shami and Hameed (2007)
6.	Rice	*Monascus purpureus*	High conc. of red pigment consists lovastatin	Hasimdanuri (2008)
7.	Rice	Co-culture of *Monascus purpureus* MTCC 369 and *M. ruber* MTCC 1880	2.83 mg/g	Panda et al. (2008)
8.	Cooked nonglutinous rice	*Monascus species.*	3.42 mg/g	Panda et al. (2009)
9.	Polyurethane foam	*Aspergillus terreus*	19.95 mg/g	Baños et al. (2009)
10.	Wheat bran	*Aspergillus terreus* UV 1617	1.72 mg/g	Pansuriya and Singhal (2010)
11.	Wheat bran	*Aspergillus terreus* JPM3	0.98 mg/g	Jaivel and Marimuthu (2010)
12.	Wheat bran supplemented with mycological peptone	*Aspergillus terreus* UV1718	3.72 mg/g	Pansuriya and Singhal (2010)

Continued

Table 10.4 Compiled work of various research groups on lovastatin production using SSF means employing various lovastatin positive isolated and standard cultures.—cont'd

Sl.No.	Substrate used in SSF	Organism employed	Yield of lovastatin mg/g of dry fungal matter (dfm)	References
13.	Koji rice	*Aspergillus terreus*	11.46 mg/g	Triana et al. (2011)
14.	Black gram Husk	*Aspergillus fischeri*	12.63 mg/g	Pallem et al. (2011)
15.	Pomegranates seeds	*Aspergillus terreus*	12.5 mg/g	Naik and Lele (2012)
16.	Wheat bran	*Aspergillus terreus* KLVB28mu21	1.110 mg/g	Prabhakar et al. (2012)
17.	Wheat bran	*Aspergillus terreus*	3.27 mg/g	Praveen and Savitha (2012)
18.	Rice straw	*Aspergillus terreus* ATCC 74135	0.22 mg/g	Faseleh Jahromi et al. (2012)
19.	Wheat bran	*Aspergillus terreus* 4	9.70 mg/g	Gulyamova et al. (2013)
20.	Wheat bran	*Aspergillus* sp. no.76	18.75 mg/g	Prakash and Srividya (2014)
21.	Wheat bran	*Fusarium pseudocircinatum* IBRL B3-4	2.27 mg/g	Syarifah et al. (2014)
22.	Waste tofu, gadung (*Dioscorea hispida*), and rice	*Monascus purpureus* HD001	7.00 mg/g	Priatni et al. (2014)
23.	Wheat bran	*Aspergillus terreus*	1.31 mg/g	Praveen et al. (2015)
24.	Wheat bran	*Aspergillus terreus* (KM017963)	1.00 mg/g	Kamath et al. (2015)
25.	Soybean	*Monascus sanguineus*	20.04 mg/g	Dikshit and Tallapragada (2016)

Table 10.4 Compiled work of various research groups on lovastatin production using SSF means employing various lovastatin positive isolated and standard cultures.—cont'd

Sl.No.	Substrate used in SSF	Organism employed	Yield of lovastatin mg/g of dry fungal matter (dfm)	References
26.	Wheat bran	*Aspergillus fumigatus*	3.35 mg/g	Mouafi et al. (2016)
27.	Wheat bran	*Aspergillus flavus* GCBL-60	0.28 mg/g	Javed et al. (2017)
28.	Whey Powder	*Aspergillus terreus* 11045	21.84 mg/g	Upendra (2017)
29.	Wheat bran	*Meyerozyma guilliermondii,*	1.65 mg/g	Shivakumar and Ravuri (2018)
30.	Rice straw	*Aspergillus terreus* FFCBP-1053	2. 14 mg/g	Kamal et al. (2018)
31.	*Saccharina japonica*	*Monascus purpureus*	13.98 mg/g	Suraiya et al. (2018)

the reactor under optimized conditions after 6 days of fermentation (Valera et al., 2005). *Pleurotus ostreatus* culture was screened on different SSF media for their ability to produce lovastatin and the process was optimized with conventional optimization method to identify the optimal condition to support enhanced yield. The optimum conditions representing 10 days of incubation time, 180 rpm aeration speed, 28°C temperature, pH 6.0, and dox rice as substrate were found to support maximum yield of 0.055 mg/g dfm of lovastatin (El-Shami and Hameed, 2007). Various agro-based products, biomass, and waste were screened under SSF employing *A. terreus* American Type Culture Collection (ATCC) 20542 isolate to produce lovastatin. Among the screened biowastes, rice bran and wheat bran were found to be suitable solid substrates to achieve maximum lovastatin yield. The study reported that 50%−60% of moisture content, 5.5 pH, and 28°C incubation temperature for 11 days of fermentation using rice bran as a substrate reported maximum yield 2.90 mg/g dfm of lovastatin by the SSF cultures of *A. terreus* ATCC 20542 isolate (Lian et al., 2007).

Coculture of *M. purpureus* MTCC 369 and *M. ruber* MTCC 1880 using rice bran as the solid medium under SSF process was tested to prepare red mold rice containing lovastatin. Selected parameters of the SSF process, i.e., temperature, fermentation time, inoculum volume, and pH were optimized applying RSM to maximize lovastatin concentration in red mold rice. RSM-optimized process condition for the said cocultures reported 2.83 mg/g dfm of lovastatin yield after 14 days of fermentation

(Panda et al., 2008). Three strains of fungus *M. purpureus* AKI, *M. purpureus* AKII, and 915 were screened for their ability to produce lovastatin in PDA medium. Based on the reported red pigment (lovastatin) yield, *M. purpureus* AKII strain was selected using different solid substrates, i.e., white rice of IR-42 variety, red rice of BP-1804-IF-9 variety, and a 1:1 (w/w) combination of both the rice varieties as a solid substrate for SSF process to improve lovastatin yield. This investigation concluded that the combination media tested (combination of rice White IR-42 and red rice BP-1804-IF-9) supported for the higher concentration of red pigment containing lovastatin after 16 days of fermentation time (Danuri, 2008). *M. purpureus* strain MTCC 369, 410, 1090 from MTCC-IMTech, Chandigarh, India, were selected for optimization studies under SSF conditions. Initially PBD was employed to screen the effects of four nutrient medium parameters, i.e., NH_4Cl, $MgSO_4$, NaCl and $CaCl_2$. Based on the results obtained, these PBD studied four medium variables (i.e., NH_4Cl, $MgSO_4$, NaCl, and $CaCl_2$) were optimized using BBD of RSM design implemented through Point Prediction Tool of Design Expert Ver. 7.1 software. The researchers concluded that a solid medium containing 20g rice and 40 mL liquid nutrient (combination of NH_4Cl, 14.32 g/L; $MgSO_4$, 0.76 g/L; NaCl 14.65, g/L; and $CaCl_2$, 0.54 g/L) at 14th day of fermentation resulted in 3.420 mg/g dfm of lovastatin (Panda et al., 2009).

Baños et al. (2009) developed a novel SSF process for the production of lovastatin using high density polyurethane foam (PUF) as an inert support resulted in 7.50 mg/g dfm lovastatin. They further recommended that forced aeration to the process could not support higher yield since it reduced moisture content of the media. Higher lovastatin yield was obtained on PUF at a density of 17 or 20 kg/m, which revealed that the density of culture support was a key parameter in determining lovastatin production. The investigators submitted that as compared to SmF process (0.57 lovastatin mg/g dfm), SSF process resulted in 35-fold higher yield (19.95 lovastatin mg/g dfm) (Baños et al., 2009). Jaivel and Marimuthu (2010) studied the ability of the fungus *A. terreus* to produce lovastatin in different solid substrates, i.e., wheat bran, rice bran, maize flour, and sorghum grain under SSF and reported that the lovastatin yield was higher in case of wheat bran (0.98 mg/g dfm) followed by sorghum grains (0.85 mg/g dfm (Jaivel and Marimuthu, 2010). *A. terreus* strain UV 1718 was cultured on different solid substrates, and various combinations thereof were evaluated for lovastatin production. Among the different substrates tested, wheat bran reported maximum lovastatin yield (1.45 mg/g dfm). Further a 24 full-factorial CCD of RSM design was employed to study the combined effects of four selected medium constituents, i.e.,. moisture content, particle size of the substrate, di-potassium hydrogen phosphate, and trace ion solution concentration. The optimized condition supported 2.969 mg/g dfm of lovastatin yield. They further reported that supplementation of mycological peptone to the RSM optimized medium resulted in 2.6-fold enhanced lovastatin yield 3.72 mg/g dfm in comparison with unoptimized process conditions (Pansuriya and Singhal, 2010).

Lovastatin and sulochrin production pattern was evaluated in 11 days SSF of *A. terreus* cultures, and their yields were measured using Liquid Chromatography—Mass Spectrometry (LC-MS) (404.5 m/z and 332.3 m/z, respectively). The study critically observed that lovastatin production started on the Day 2 (0.04 mg/g dfm) and achieved its peak on Day 7 (11.46 mg/g). It was observed that sulochirin production

commenced on Day 4 (0.60 mg/g) and continued until the end of fermentation period at Day 11 (3.11 mg/g dfm). This investigation concluded that SSF using *A. terreus* has potential to be used for production of lovastatin (Triana et al., 2011). Black gram husk as the solid substrate was screened under SSF cultures of *Aspergillus fischeri*. The different variables of SSF process including both nutrient and process variable were optimized applying RSM design. It was reported that black gram husk as solid medium with the optimized process conditions of 7 days of fermentation time, 60% v/w of initial moisture content, 2 mL of inoculum volume, initial pH of 5.0, 30°C of incubation temperature, 1% w/v lactose as carbon source, and malt extract at 1% w/v were found to support lovastatin yield of 12.63 mg/g dfm (Pallem et al., 2011). Another research group studied both wild-type and mutated strain of *A. terreus* culture in SSF using pomegranate seed powder as a solid substrate to produce lovastatin. The different process parameters were optimized employing CCD of RSM design studies in order to improve lovastatin production abilities of the employed wild type and mutated *A. terreus* strains. CCD-RSM design suggested optimized combination of the significant factors, pomegranate seed powder (5g), KH_2PO_4 (0.1% w/v), glucose (5% w/v), moisture (60% w/w), pH 5 in 15 days resulted in 4.20 mg/g dfm of lovastatin for the wild type *A. terreus* cultures. The yield was further increased to 6.50 mg/g dfm using mutated strain of *A. terreus* developed applying systemic chemical mutation method (Naik and Lele, 2012). The wheat bran was screened as solid substrates under SSF using *A. terreus* KLVB28mu21 strain for the production of lovastatin. The fermentation process parameters of SSF were optimized: moisture content, 65%; pH, 5.5; temperature, 30°C; inoculums size, 1×10^8 spores/mL and bed depth, 2 cm were found to support lovastatin yield (1110 μg/g DWS) (Prabhakar et al., 2012).

Two strains of *A. terreus* (ATCC 74135 and ATCC 20542) were screened for their potential to produce lovastatin on solid substrates such as rice straw and oil palm frond medium under SSF process. Further various SSF process parameters i.e., incubation temperature, moisture content, particle size, inoculums size, and pH were optimized to improve lovastatin yield. The optimization study results showed that pH, 6; incubation temperature, 25°C; particle size, 1.4−2 mm; initial moisture content, 50%; and fermentation time 8 days were found to support the maximum yield of lovastatin 175.85 and 260.85 mg/kg dfm for *A. terreus* ATCC 20542 and ATCC 74135 cultures, respectively, using rice straw as the solid substrate (Faseleh et al., 2012). Gulyamova et al. (2013) studied the ability of lovastatin production by indigenous strains (*A. terreus* 4 and *A. terreus* 20) in SSF process using various solid substrates, i.e., oat bran, rice bran, wheat bran, maize bran, and mix of wheat and peanut bran. It was observed that fermentation of *A. terreus* 4 on wheat brawn medium and *A. terreus* 20 on oat bran medium reported the maximum lovastatin yield of 9.7 and 9.56 mg/g, respectively (Gulyamova et al., 2013). Both perceptible anticholesterol substrates, rice bran and brown rice were grown with locally isolated *Fusarium pseudocircinatum* B3-4 to obtain the best lovastatin activity. Lovastatin production at different substrate thickness ranges of 0.25−1.5 cm in a static tray system ($20 \times 20 \times 6$ cm^3) along with impact of various process conditions was evaluated. The results revealed that thickness of 0.5 cm and original substrate size of 0.5 cm, 60% (v/w) moisture content, and temperature of 30 ± 2°C at 12th day of SSF were

found as the most suitable conditions to generate 2.27 mg/g of lovastatin yield (Syarifah et al., 2014). Prakash and Srividya (2014) studied different soil fungi for their ability to produce lovastatin under SSF process using wheat bran as support. HPLC analysis confirmed lovastatin in the extract of *Aspergillus* sp. no. 76 SSF culture by representing the same retention time with respect to the standard (12.4 min) lovastatin drug. It concluded that the said fungi led to 18.75 mg/g of lovastatin yield under SSF using wheat bran as a solid substrate (Prakash and Srividya, 2014).

Effect of different culture settings on lovastatin biosynthesis was also investigated employing *A. terreus* (KM017963) isolate in SSF using wheat bran. It was reported that the optimal combination of pH of 6.0, temperature of 28°C, and inoculum size of 108 spores/mL supported higher lovastatin yield. Wheat bran supplemented with 3% (w/w) of glucose or dextrin reported 5-fold increase in lovastatin yield (Kamath et al., 2015). Dikshit and Tallapragada (2016) tested *Monascus sanguineus* culture for its ability to produce lovastatin under SSF applying RSM. Soybean powder, $CaCl_2$, acetic acid, and inoculum size were the four variables selected for the optimization applying RSM design studies. The RSM design identified optimal combination of 20 g/L of soybean, 2.5 g/L of Calcium chloride $CaCl_2$, 25 mL of acetic, and 3.4 mL of inoculum volume to support 20.04 mg/g (dry substrate) of lovastatin yield.

The RSM-optimized design methods fails to address nonlinear relationship between the measured factors, so the yield achieved may not be sustained when the process is scaled up to the higher volumes. Thus, it is recommended to validate the results obtained under RSM by applying hybrid ANN-GA in order to test the fitness and the authenticity before applying process scale-up. Upendra (2017) screened various agro-industrial by-products/waste-wheat bran, whey powder, fermented ragi/finger millet (*Eleusine coracana*) residue containing dead yeast, groundnut oil cake, black gram husk, and jackfruit seed powder as renewable substrates for the production of lovastatin under SSF process using selected *Aspergillus* strains. Among the four strains studied, *A. terreus* 11045 resulted in highest yield of lovastatin using whey powder (6.5 mg/g), followed by wheat bran (5.00 mg/g) used as a solid substrate. Further PB design study was employed to screen the effects of six medium constituents i.e., wheat bran, malt extract, whey powder, dextrose, sodium acetate, and linoleic acid, and three process parameters i.e., moisture content, pH, inoculums volume, on the yield of lovastatin under SSF condition employing said culture. PBD identified whey powder, linoleic acid, moisture content, and pH as principle factors influencing the yield of lovastatin. PBD optimized selected factors were further optimized using CCD of RSM. RSM optimized conditions of a medium containing 40 g whey powder, 6 g of linoleic acid, 65% moisture content, and 6.5 pH resulted in high yield 21.8475 mg/g of lovastatin under SSF process. The experimental results and the RSM design predicted values obtained from the model equation were compared and found that goodness of fit function Fig. 10.9. The employed RSM design goodness of fit values explained that experimental results lied on the 45-degree line, thus predicted results were in close agreement with the experimental data. The optimized trail values with respect to lovastatin yield were further validated using FFNN-ANN and found that the simulated value of lovastatin yield (21.84749 mg/g) of ANN design was in very close agreement with the experimental values (21.8475 mg/g). A

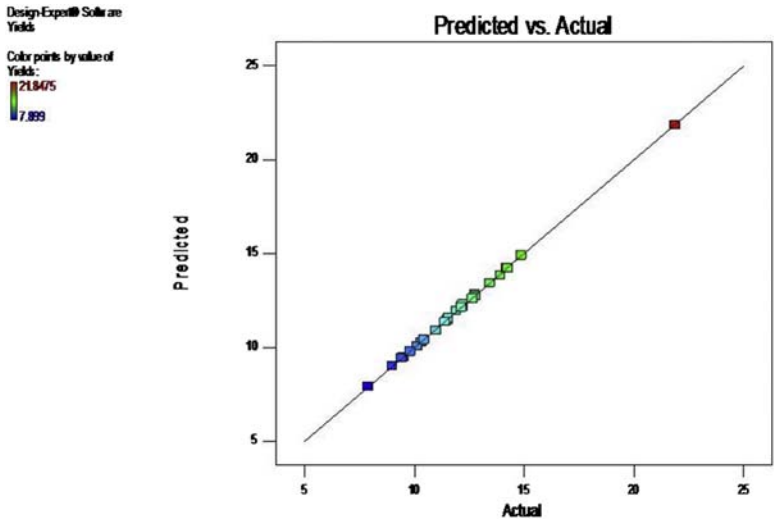

Figure 10.9 Graph representing the actual and CCD predicted values of lovastatin yield under SSF for the cultures of *Aspergillus terreus* 11045.
Source: State Easy Free Trail Version 9.0.0.7.

regression analysis between ANN outputs and the experimental data was carried out. This ANN model indicated a precise and effective prediction of the experimental data with a correlation coefficient (rr) of 0.999, 0.995, 0.999, and 0.996 for training, validation, testing and all data, respectively, as indicated in Fig. 10.10.

The percentage of contribution of RSM optimized and ANN validated parameters on the lovastatin yield was calculated. Among the four significant parameters optimized, moisture content contributed 55%, followed by whey powder as solid substrate contributing 34%, pH with 6%, and finally linoleic acid used as an elicitor contributed 5% in the final lovastatin yield under SSF condition for the cultures of *A. terreus* 11,045 (Upendra, 2017).

10.6 Genetic engineering of microorganism for industrial production of lovastatin

Filamentous fungi are used in bioprocess technology as cell workshops for biosynthesis of various chemicals, polyketide secondary metabolites such as lovastatin, pharmaceuticals products, and industrial enzymes. Natural cultures employed in the bioprocess of these industrially important compounds will register low yield; hence the market sustainability of these important compounds become obsolete. In order to make them available at affordable price one must achieve the higher yields of the compound. This can be achieved by employing powerful approaches such as the genetic engineering. Different transformation methods available along with nucleic acid (DNA and RNA) based methodologies as well as metabolic engineering can help in boosting the desired compound in many fold improved yield

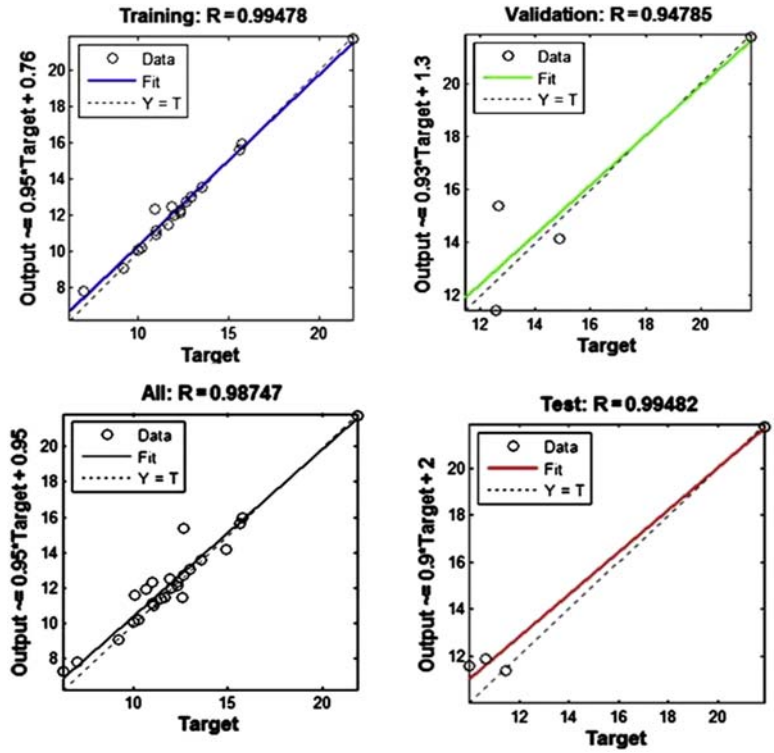

Figure 10.10 ANN Regression Plots for SSF process representing Training, Validation, Test, and All (Training + Validation + Test).
Source: State Easy Free Trail Version 9.0.0.7.

(Meyer, 2008). In the postgenomic era, variable novel promising approaches are available to discover natural products in the fungal species such as mining techniques of the entire genome, activation of gene cluster responsible for biosynthesis of molecules of interest, and heterologous pattern of gene expression and interchange of enzyme segments as well as restructuring of metabolic pathway that leads to the enhanced biosynthesis of industrially important metabolites such as lovastatin, terreic acid, itaconic acid, etc. (Scharf and Brakhage (2013). The success of metabolic engineering in developing high-yielding fungal varieties depends solely on the in-depth understanding of the interrelationship between the metabolite production and the corresponding gene expression pattern. Correlation studies at gene level allow the researchers to precisely engineer the genes, thus the organism can be tailored to produce metabolites of interest. Lovastatin can be produced at higher rates while stopping the synthesis of unwanted metabolites such as citrinin. Genomic fragment microarray is one such study where the entire genome of *A. terreus* fungal species transcriptional profile is generated from the genetically engineered strain in order to produce the enhanced amounts of lovastatin. For example, to analyze the pathway lead to the production of lovastatin and the other

metabolite, (+)-geodin. The metabolic detection methods need to be employed to understand the metabolite profile of the fermented broth. Along with, assessment of transcriptional and metabolic comparison and correlational work give insights into the genetic and process control mechanism of lovastatin and (+)-geodin biosynthesis. These studies are important in constructing promoters for reporter-based selection systems to improve overall lovastatin production by the fungus *A. terreus* (Askenazi et al., 2003). Bizukojc and Ledakowicz (2007) studied the simultaneous production of lovastatin and (+)-geodin for the *A. terreus* ATCC 20542 SmF cultures. The study screened different carbon source, i.e., glucose, fructose, galactose, and lactose, to understand their impact on the metabolic profile of the fungus *A. terreus*. The result showed that the higher concentration of lovastatin with the depleted concentration of nitrogen source (yeast extract) directs the synthesis of (+)-geodin, and the balance between carbon and nitrogen source enhanced the production of lovastatin while on the other hand (+)-geodin yield reported at very trace amount. It is evident from the study that the ration of C/N plays a predominant role in metabolite profile of the *A. terreus* cultures in producing the lovastatin at industrial scale (Bizukojc and Ledakowicz, 2007).

Filamentous fungi are the source of abundant range of structurally different polyketide metabolites with noticeable medicinal potentials named as lovastatin and paclitaxel. These are US FDA approved drugs used in the treatment CVDs as anticholesterol agent. When we look into the genome of the fungus *A. terreus*, it encompasses gene clusters which principally encode lovastatin and paclitaxel metabolites. The expression pattern of these genes might be different in response to the variable nutritional and process conditions. Different methods such as genome editing and the molecular regulatory mechanisms, and prospective of clustered regulatory interspaced short palindromic repeat/Cas9 system are applied to engineer the gene cluster and promoters to improve the yield of lovastatin from *A. terreus* at industrial scale (El-Sayed et al., 2017).

10.7 Conclusion

This chapter discusses the recent approaches in biosynthesis of lovastatin employing various fungal sources. It has critically presented and analyzed the contribution of various researchers who have investigated isolation and characterization of lovastatin producing fungal cultures, and has provided an insight into applying statistical optimization strategies over conventional practices for much controlled, enhanced, and validated output in terms of lovastatin biosynthesis. It further encompasses the current research status of the lovastatin fermentation by analyzing various fermentation processes employed such as LSF, SmF, SSF, and summarizes the associated advantages and further opportunities.

10.8 Future prospects

High-lovastatin yielding fungal isolates as discussed in the chapter can be further subjected to strain improvement studies for both random mutations applying UV

radiation or chemical mediated mutation and site directed mutagenesis for enhancing the yield efficiencies of the fungi. Different industrial waste can be further screened as substrates for the production of lovastatin. The ANN-validated optimized production media calls for industrial scale tests under both SmF and SSF conditions.

Acronyms

ANN	Artificial Neural Network
ATCC	American Type Culture Collection
ATR	Attenuated Total Reflection
BBD	Box−Behnken Design
BLAST	Basic Local Alignment Search Tool
CCD	Central Composite Design
CNS	Central Nervous System
CVDs	Cardiovascular diseases
CZA	Czapek's Agar
DFM	Dry Fungal Matter
DMAPP	Dimethyllallyl pyrophosphate
DWS	Dry Weight to Substrate
EC number	Enzyme Commission number
EIA	Enzyme Immunoassay
FDA	Food and Drug Administration
FFNN	Feed Forward Neural Network
FPPS	Farnesyl Pyrophosphate Synthase
FTIR	Fourier Transform Infrared
GA	Genetic Algorithm
GPP	Geranyl Pyrophosphate
HMG-Co A	3-Hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
I^6A	Isopentenyl Adenosine
IMTech	Institute of Microbial Technology
IPP	Isopentenyl Pyrophosphate
ITS	Internal Transcribed Spacer

LC-MS	Liquid Chromatography−Mass Spectrometry
LCA	Low Carbon Agar
LDL	Low-Density Lipoprotein
LSF	Liquid Surface Fermentation
MATLAB	Matrix Laboratory
MEGA	Molecular Evolutionary Genetics Analysis
MTCC	Microbial Type Culture Collection
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
NMR	Nuclear Magnetic Resonance
NRRL	National Regional Research Laboratory
PBD	Plackett−Burman Design
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PUF	Polyurethane Foam
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
RSM	Response Surface Methodology
S	Svedberg unit
SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
SSF	Solid-State Fermentation
TLC	Thin Layer Chromatography
UICC	Union for International Cancer Control
UV	Ultraviolet
WHO	World Health Organization
YEMA	Yeast Extract Mannitol Agar

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About the Authors

Dr. Ramesh C. Ray is a Former Principal Scientist (Microbiology) and Head of the ICAR-Central Tuber Crops Research Institute (Regional Center), Bhubaneswar, India. He has 35 years of research experiences in agriculture and food microbiology, published 140 research and review papers in international journals and 63 books chapters, edited 14 books, and authored 3 books. He is a distinguished fellow of the prestigious National Academy of Agricultural Sciences, New Delhi, India, and 10 other scientific societies. Currently, he is the Director of Centre for Food Biology and Environmental Studies, Bhubaneswar, India.

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