Pratyoosh Shukla *Editor*

Microbial Enzymes and Biotechniques

Interdisciplinary Perspectives

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Foreword

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This important and timely book provides an interdisciplinary perspective on microbial enzymes and the latest techniques whereby these enzymes can be studied and utilized. Humans have long relied on microbes and their enzymes in the production of fermented foods and beverages, ever since the Neolithic Age. Our increasingly sophisticated use of microbial enzymes will help us address some of the key challenges related to food, carbon-neutral energy sources, human, animal and ecosystem health. The "green chemistry" enabled by microbial enzymes is rapidly growing in importance in sustainable industrial processes, recycling and bioremediation and this will lead to improved prosperity and welfare.

A striking feature of this book is the broad range of microbes that is considered as sources of enzymes. Bacterial enzymes as well as those from fungi and microalgae are extensively covered. I was particularly interested in the strong chapters on microalgal-derived enzymes and products, a particular interest of my own. In addition, there are chapters that cover approaches and techniques such as inoculant development, protein nanostructures and nanoparticles, and enzyme engineering that will be of broad interest and applicability to all working in the burgeoning field of microbial enzymes.

This substantial volume would not have been possible without the intellectual leadership, persistence and diligence of the Editor of this book, Professor Pratyoosh Shukla. I congratulate my friend and colleague Professor Shukla on this major accomplishment and also for his role as author on several key chapters in this volume. All the authors are thanked for their work in moving forward our understanding of microbial enzymes and biotechniques. All researchers working in the dynamic and interdisciplinary field of microbial enzymes will benefit from this volume.

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Preface

The book *Microbial Enzymes and Biotechniques: Interdisciplinary Perspectives* contains an exceptional collection of several new and eye-catching interdisciplinary aspects of microbiology and biotechnology giving insights on such innovative areas of research. This book enlightens the thought-provoking topics on biotechniques perspectives, i.e. sugar-nucleotide providing enzyme for health benefits, protein nanotechnologies with purpose-designed properties for medicine, the techniques for enzymatic and chemical bleaching in pulp and paper industry, the technological overview of next generation microalgae-based products, and microbial bioinoculant technologies for sustainable agriculture in the initial first chapters.

The latter half of this book covers exciting techniques of biopigments from microalgae, revealing the features of the oxidative enzymes, endophytic bacteria for phytoremediation aspects of organic pollutants, the technological perspectives of arbuscular mycorrhizal fungi (AMF)-based biofertilizers, etc. Finally, the book also deciphers the attractive scope of enzyme engineering techniques for biotechnological applications and the technological advances of emerging areas of probiotics and prebiotics. One interesting aspect of this book is a descriptive overview of various techniques for the evaluation of the chemical and microbiological quality of the coastal environment. This is a high demanding area in terms of sustainable environmental goals. A substantial feature of this book is that it gives the most conversant areas of microbial enzymes and biotechniques with a focused overview on cutting edge description on techniques involved in each of the areas described in each chapter.

This book will be a treasured interdisciplinary resource for senior undergraduate and graduate students, related researchers, professionals involved in biotechniques innovations and other interdisciplinary research groups.

Rohtak, India — Pratyoosh Shukla

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Editor and Contributors

About the Editor

Prof. Pratyoosh Shukla is a Professor and Head of the Department of Microbiology, Maharshi Dayanand University, India. His research interests include enzyme technology, protein bioinformatics and microbial biotechnology. He has more than 20 years of research and 18 years of teaching experience at respected Indian universities of India, and was a postdoctoral researcher at Durban University of Technology, South Africa. He also worked as a Research Professor at the University of Cincinnati under Indo-US Science and Technology Forum (IUSSTF) and American Society of Microbiology (ASM) program. He has written 8 books and 26 book chapters, and published more than 135 peer-reviewed papers in respected national and international journals. He has received several awards, including the Prof. S.B. Saksena Award in Life sciences (1999), Danisco India Award in Probiotics & Enzyme Technology (2010), Indo-US Professorship Award (2014) by the Indo-US Science and Technology Forum (IUSSTF) and American Society of Microbiology (ASM), AMI-Alembic Award (2015), and Faculty Research Award for Top 10 Most outstanding Researchers in the field of Immunology and Microbiology (2018). He is a Fellow of the Academy of Microbiological Sciences, of the Biotech Research Society of India (FBRS), and the National Academy of Agricultural Sciences (FNAAS). In 2011 he was selected as Scientist in the Southern Ocean Expedition.

He also serves as a Guest Professor at the South China University of Technology, Guangzhou, China.

He is Editor of the Indian Journal of Microbiology (Springer), Associate Editor of 3Biotech (Springer), Guest Editor of Current Protein and Peptide Science (Bentham Science), Editor of Protein and Peptide Letters, and Scientific Reports (Nature), Associate Editor of BMC Microbiology, and Academic Editor of PLOS One.

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1 Thermostable Nucleotide-Sugar Synthesizing Enzymes: Opportunity and Future Applications in Therapeutic Antibody and Peptide Area

Mohammad Dadashipour and Yutaka Kawarabayasi

Abstract

Since discovery of the first antibiotic, penicillin, at 1928 by Sir Alexander Fleming from *Penicillium notatum*, many antibiotics were isolated from different types of microorganisms including molds and bacteria, especially actinomycetes. At present, medicines dominantly used are chemical compounds with low molecular weight, including antibiotics, however, it has been often observed that some of them cause serious side effects or damages to patients. Therefore, recently it has been attempted to develop antibody or peptide as medicine, because of their high specificity to target for avoiding unexpected side effect. However, problem to be solved on therapeutic antibody is shortage of its lifetime in the human circulatory system. To overcome this drawback, it is effective to add polysaccharide on these molecules. Conversely, the polymer structure of carbohydrate is known to work as a key molecule in the immune system; e.g. human histo-blood type is determined by only one sugar molecule at the terminal of long polysaccharide. For pharmacological and immunological demands, it is expected to add personal-specific polymer structure of carbohydrate molecules on antibody or peptide molecules. For construction of polysaccharide, many different types of glycosyltransferases were already identified from many different organisms, however, these enzymes can utilize only nucleotide-sugar molecules as substrate. Therefore, efficient and on-time supply of appropriate nucleotide-sugar molecule as substrate should be helpful in development of personalized and optimized

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utilization of therapeutic antibody or peptide. In this chapter, we will overlook enzymes with construction activity of nucleotide-sugar molecules and indicate opportunity on future application of these enzymes.

Keywords

Sugar-metabolising enzymes · Nucleotide-sugar · Sugar metabolism · Personalized medicine · Therapeutic peptide and antibody · Thermostable enzymes · Enzyme engineering

1.1 Introduction

Approximately 100 years ago the first discovery of the antibiotic, penicillin, by Dr. Fleming, drastically changed medical therapy able to overcome many diseases caused by microorganisms. Following the first success of antibiotic molecule, a lot of different kinds of antibiotics were isolated from many organisms, including molds, germs, and fungus, through discovery of organisms producing novel antibiotics. These antibiotics actually decreased some diseases that have been previously afraid as their seriousness. Conversely, too general and huge usage of antibiotics resulted in other serious problems, emerging multi-drug resistant pathogens which can survive in the presence of most antibiotics.

Low molecular weight compounds, which are chemically synthesized, are also generally utilized as medicines. Although they are dominantly prescribed world-wide, side effects are known for some of these medicines.

To decrease side effects of medical treatment, one opportunity is employing antibody or peptide as medicine. Specificity of antibody against antigen is known to be very sharp: one antibody can recognize only one antigen and there is no exception of this specificity. They are known as main molecules functioning in the immune system of higher organisms. Antibody is consisting of two sets of two peptides, two heavy chains (H chain) with constant region and variable region and two light chains (L chain) with only variable region (Fig. 1.1). The specificity and variety of recognition by antibody are introduced through combination of parts for variable region. In immune system, expression level of only demanded antibody is increased to protect against antigens invaded from outside environment. This sharp specificity is convenient for diminishing side effect as medicine. This is also very critical for pharmaceutical companies to decrease expense for compensation against side effect damage on patients. Also for patients, safe treatment will be guaranteed by introduction of therapeutic antibody. Therefore, some of pharmaceutical companies have been attempting to develop therapeutic antibody or peptide to introduce into market. In the present situation, still there are some problems to be solved with these novel medicines; their stability by addition of polymer structure of sugars, expense of production cost, and limitation of target for antibodies.

In this chapter, solution for these disadvantages will be shown using highly stable enzymes with nucleotide-sugar synthesizing activity. These enzymes actually

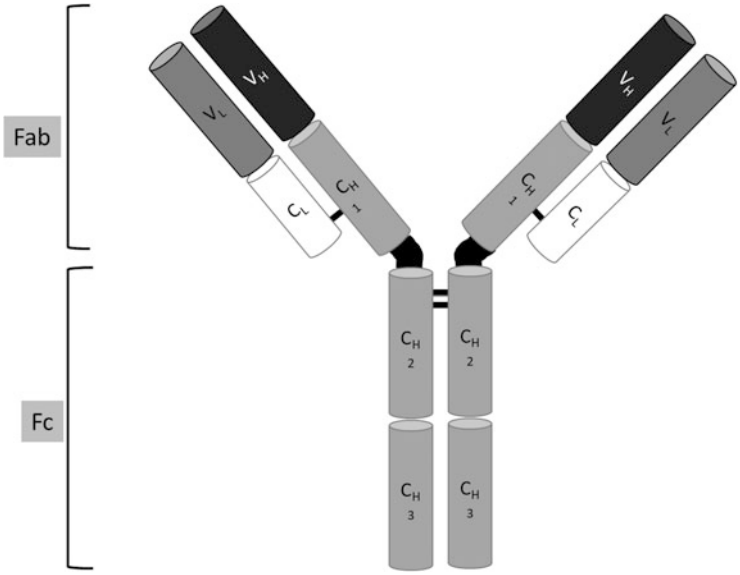

Fig. 1.1 A schematic representation of an IgG molecule. Glycosylation sites are on CH2. *V* variable, *C* constant, *H* heavy, *L* light, *Fc* fragment crystallizable, *Fab* fragment antigen binding

provide very specific substrates for the glycosyltransferases, i.e., the enzymes by which the final step of the process is catalyzed and the phosphorylated sugar nucleotides are transferred onto the proteins and peptides. This chapter will provide valuable information for future precision medicine.

1.2 Importance of Polysaccharide in Medical Field

One of the important roles of polymer structure of carbohydrate molecules is construction of barriers between inner- and outer-environments of living cells, e.g. cellulose, peptidoglycan (murein), and lignin. As these polymer structures of carbohydrate molecules require structure with huge molecular weight, wide network, and high strength, they simply support cell morphology and play a role in separation. In addition to the barrier function of polymer structure of carbohydrate molecules, they are used as energy storage like starch in corn, rice, and potato. Polymer structure of carbohydrate also plays important role for plant's passage period under inappropriate environmental conditions, e.g. low temperature, dry condition, and high temperature. Additionally, these storage molecules assist growth of their host after sprouting as they are used as nourishment.

Moreover, carbohydrate molecules are utilized as important compounds possessing information for distinction between own-selves and others, recognition of specific target for infection or attachment, and transfer of signals. In the human circulatory system, histo-blood type of erythrocyte is determined by only one sugar

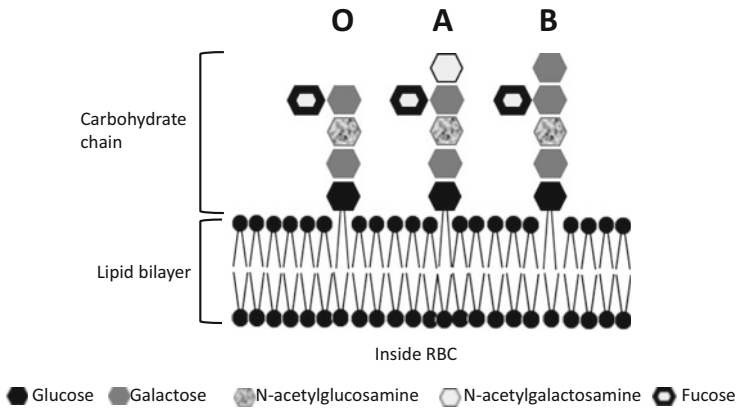

Fig. 1.2 ABO blood group system on red blood cells: how an individual molecule of a sugar can change functionality

molecule attached to the terminal of polymer structure of carbohydrate (Fig. 1.2); when *N*-acetylgalactosamine is attached the histo-blood type is A, when galactose is attached the histo-blood type is B, when both sugars are attached the histo-blood type is AB, and when none of these two sugars are attached the histo-blood type is O. The histo-blood type is the most characterized and famous example of informative role of carbohydrate molecules (Yamamoto et al. 1990).

Similarly most of hormones or transfer carriers in circulatory system are modified by attachment of polymer structure of carbohydrate molecules (glycosylation). For some hormones, peptide, or protein molecules, this modification is essential for expression of their function or functionality. The 165 residues long erythropoietin is known as a hormone, which is produced within the stromal cell of renal tubule in kidney, and plays role to induce proliferation of erythroblast and differentiation into erythrocyte. Attachment of three *N*-linked and one *O*-linked polysaccharide is required for its function (Higuchi et al. 1992). As the erythropoietin is used for treatment of anemia since decades ago, first generation erythropoietin injected into circulatory system was rapidly adsorbed into liver and then degraded. Because of this disadvantage of short lifetime, frequent prescriptions were required for treatment of anemia patients. However, frequent prescription caused financial expenses and physical difficulties for anemia patients, therefore erythropoietin with longer half-life and effective activity was demanded. For this purpose, two additional *N*-linked polymer structure of carbohydrate were introduced to decrease degradation in liver, resulting in second generation erythropoietin indicated longer half-life in circulatory system and stronger hematinic activity.

As described above, polysaccharide molecules play important role within immune system and for exhibiting activity of peptide molecule in circulatory system.

1.3 Nucleotide-Sugar Molecule for Construction of Polymer Structure and Other Modifications

The sugar molecules incorporated into the inner-cellular portion of living cells are usually phosphorylated with sugar-kinases (Figs. 1.3 and 1.4), to give sugar-6-phosphate (Sug-6-P). In the case of glucose, glucose-6-phosphate (Glc-6-P), a product phosphorylated with glucokinase, is usually metabolize through glycolysis pathway to obtain energy. Similarly, fructose-6-phosphate (Frc-6-P) is constructed by the function of fructokinase, which is capable of converting to both Glc-6-P and mannose-6-phosphate (Man-6-P) by the function of specific isomerase enzymes. Meanwhile, Glc-6-P and Man-6-P are converted into glucose-1-phosphate (Glc-1-P) and mannose-1-phosphate (Man-1-P) by the function of phosphoglucomutase and phosphomannomutase enzymes, respectively. These sugar-1-phosphate (Sug-1-P) molecules are utilized as materials for construction of nucleotide-sugar molecules and modified sugar molecules.

Glc-1-P is converted to dTDP-glucose (dTDP-Glc) and following three-step reactions convert it to dTDP-rhamnose (dTDP-Rha), which is a substrate of incorporation of rhamnose portion into polymer structure of carbohydrate molecules at surface of pathogenic microorganisms. This sugar molecule is found within the arabinogalactan structure of pathogens: therefore, this biosynthetic pathway is one of the targets for development of anti-microbial drug (Li et al. 2006). Genes encoding enzymes consisting of the dTDP-Rha biosynthetic pathway were detected from the genomic data of an acidothermophilic archaeon, *Sulfolobus tokodaii*. The recombinant protein of each gene including this biosynthetic process was successfully produced in *Escherichia coli* as thermostable and soluble protein, and the enzymatic

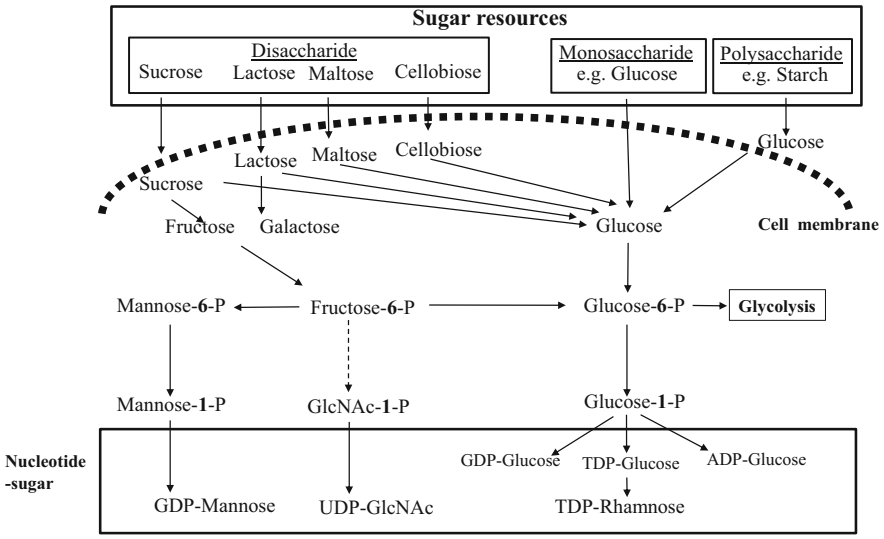

Fig. 1.3 Metabolic pathway of simple, phosphorylated, and nucleotide-sugar molecules

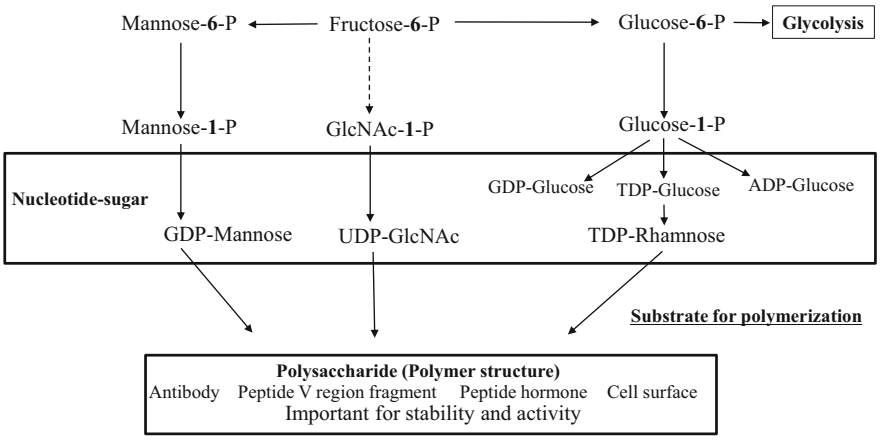

Fig. 1.4 How important nucleotide-sugar constructing enzymes are in medical area

activity of each protein, catalyzing each reaction to produce the final product, dTDP-Rha, was experimentally confirmed (Teramoto et al. 2012). This observation indicates that activated form of Rha, dTDP-Rha, is actually produced within a thermophilic crenarchaeon *S. tokodaii*, which is not a pathogenic microorganism. The exact reason for production of dTDP-Rha within non-pathogenic and thermophilic microorganism is unclear.

Man-1-P is usually combined with GTP to produce guanine diphosphate mannose (GDP-Man), which is widely utilized as substrate for construction of polymer structure of carbohydrate in many organisms (Suzuki et al. 1997). As well as utilization for construction of general polymer structure, Man-1-P is utilized as midway product for construction of mannosylglycerate in *Pyrococcus* species, which is accumulated within the microorganisms exposed by changing of salinity or surrounding water temperature (Martins and Santos 1995). The activated form of Man-1-P, GDP-Man, is combined with 3-phophoglycerate to produce mannosyl-3-phosphoglycerate by mannosyl-3-phosphoglycerate synthase (MPGS), then phosphate is removed from mannosyl-3-phosphoglycerate to give a final product mannosylglycerate by mannosyl-3-phosphoglycerate phosphatase (MPGP). Four proteins including in this catalyzing pathway in *Pyrococcus horikoshii* were already characterized by authors group (Akutsu et al. 2005, 2015) and Empadinhas et al. (2001).

Frc-6-P is used as a start material for construction of UDP-*N*-acetylglucosamine (UDP-GlcNAc) in most organisms catalyzed with the four-step reaction. In this biosynthetic pathway, Frc-6-P is initially converted into glucosamine-6-P (GlcN-6-P) with glutamine:fructose-6-phosphate amidotransferase. As a next step, in microorganisms GlcN-6-P is converted into GlcN-1-P then acetylated into *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P), in eukaryote GlcN-6-P is acetylated into *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) then converted into GlcNAc-1-P, which is combined with UTP to produce the final product, UDP-GlcNAc. As

shown above, sugar-1-P molecules are utilized as substrates for construction of polymer structure of carbohydrate molecules and modified sugar molecules.

For construction of polymer structure of carbohydrate molecules, glycosyltransferase is required. This enzyme family contains many members with different substrate specificity and bonding patters, therefore, this enzyme is classified into 65 classes (Coutinho et al. 2003). The nucleotide-sugar molecule is used as sole substrate for glycosyltransferase. In Eukaryotic cells, polypeptide constructed within the endoplasmic reticulum (ER) is transferred into Golgi body, which is consisted with layers including different types of glycosyltransferase enzymes. In Golgi body an appropriate polymer structure of carbohydrate molecules is added on peptide molecule by function of different kind of glycosyltransferases. On the other hand, actual construction process of polymer structure of carbohydrate in Bacteria and Archaea is unclear. Overall, it is clear that polymer structure of carbohydrate molecules plays important role for stability, activity, and recognition of accepter molecules in their host cells.

1.4 Nucleotide-Sugar Construction Enzymes

As described in the section above, it is well known that nucleotide-sugar molecule is provided as sole substrate for glycosyltransferases. It means that organisms with polymer structure of carbohydrate molecules must possess the nucleotide-sugar construction system within their own cell. Therefore, enzymes with nucleotide-sugar construction activity are present in all organisms, from microorganisms to vertebrates. Variety of enzymes has already been identified with construction activity of NDP-Sug molecules from NTP and Sug-1-P. In this reaction, terminal two phosphate molecules of NTP are released and NMP portion is combined with the phosphate of the sugar-1-P molecule to construct NDP-sugar molecule. As shown in Fig. 1.5, radical has attacked the ion bond of phosphate. We will discuss typical examples of thermostable enzymes with nucleotide-sugar construction activities below.

Enzymes with nucleotide-sugar construction activity include two independent classes; the first class possesses simple and sole sugar-1-phosphate nucleotidylyltransferase (Sug-1-P NTase) activity, the second class includes those with bifunctional enzymatic activities; Sug-1-P NTase and other enzymatic activity correlating with sugar metabolism. From thermophilic archaea some numbers of thermostable and bifunctional enzymes with Sug-1-P NTase activity were identified. Within acidothermophilic archaeon *S. tokodaii,* the ST0452 protein was identified as the bifunctional enzyme with Sug-1-P NTase and amino-sugar-1-phosphate acetyltransferase (amSug-1-P AcTase) activities (Zhang et al. 2005, 2010). The ST0452 protein is stable up to 80 °C, and exhibits its activities over 60 °C (Zhang et al. 2005, 2010). The ST0452 protein possesses multiple substrate specificity in this Sug-1-P NTase activity (Zhang et al. 2005). Reason of multiple substrate acceptability of the ST0452 protein is thought that the genomic size of archaea is smaller than that of bacteria and numbers of genes detected within the archaeal

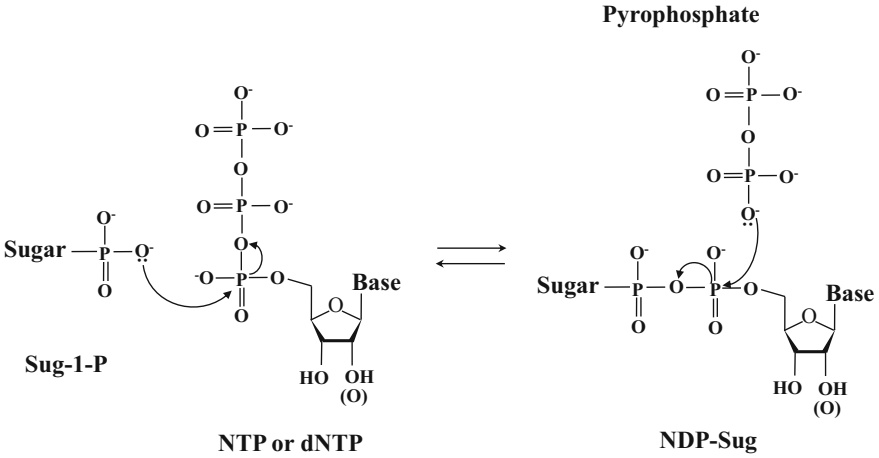

Fig. 1.5 The reaction scheme of the Sug-1-P NTase

genome are limited. Therefore, one protein should express multiple activities for covering all metabolic reactions within archaeal organism.

Similarly thermostable bifunctional enzymes with Sug-1-P NTase activity were identified from anaerobic thermophilic archaeon *P. horikoshii*. The PH0925 protein can catalyze phosphomannose isomerase (which catalyzes a reaction interconverting Frc-6-P to Man-6-P) and Sug-1-P NTase activities, which are utilized for a metabolic pathway producing mannosylglycerate (Empadinhas et al. 2001). Common features of these two bifunctional enzymes with Sug-1-P NTase activity are that they can accept multiple Sug-1-P molecules as substrate, and exhibiting two activities by each enzyme are included within one metabolic pathway, suggesting these features might be appropriate for efficient proceeding of biological metabolic pathway in these thermophilic archaea.

Many simple Sug-1-P NTase enzymes were identified from variety of organisms from microorganisms to mammals. Most characterized Sug-1-P NTase enzymes from bacteria exhibited activity specific to limited substrate molecules, therefore, different enzymes are utilized for metabolism of different substrates. However, sugar-1-P NTase isolated from archaeal species displays multiple substrate acceptability, indicating archaeal enzymes can catalyze multiple reactions. Some of enzymes identified in archaeal species demonstrate stability under high temperature and unusual stress conditions, therefore these are suitable for application in production of useful nucleotide-sugar molecules as well as bifunctional enzymes.

1.5 Engineering Archaeal Enzymes to Enhance Activities for Sugar Metabolism

The ST0452 protein exhibits high stability at high temperature and accepts multiple substrates. However, the k_{cat} value of the ST0452 GlcNAc-1-P UTase activity is smaller than that of the bacterial counterpart enzyme, *E. coli* GlmU. This observation indicates that turnover rate of GlcNAc-1-P UTase activity of the ST0452 protein is slower than that of *E. coli* GlmU. This feature is a drawback for application of this enzyme. It was, therefore, attempted to increase turnover rate of the ST0452 GlcNAc-1-P UTase. To improve GlcNAc-1-P UTase activity, substitution of amino acid residues were carried out within reaction center, which is located as pocket shape at the surface of the ST0452 protein. It was expected that these substitutions may not drastically change overall stability of the protein. At first 11 residues were chosen as candidates of single substitution to Ala. Solubility of 11 single-mutant ST0452 proteins was checked after 20 min treatment at 80 °C. All 11 single-mutant ST0452 proteins indicated the same solubility as the wild-type ST0452 protein, indicating that introduction of single-substitution into amino acid residues located within the reaction center do not affect its solubility (Zhang et al. 2007). Thus Sug-1-P NTase activities of all single-mutants were analyzed. Five of 11 single-mutant ST0452 proteins exhibited higher GlcNAc-1-P UTase activity than that of the wild-type ST0452 protein (open bars in Fig. 1.6). Then single-substitution mutant ST0452 protein with the higher GlcNAc-1-P UTase activity was constructed by introduction of saturation mutagenesis into two different residues, T80 and Y97 (hatched bars in Fig. 1.6) (Honda et al. 2017). Finally the mutant ST0452 protein introduced two-substitution was obtained as the protein with the highest GlcNAc-1-P UTase activity (black bar in Fig. 1.6) (Honda et al. 2018).

As another enzymatic activity, the ST0452 protein displays glucosamine-1-phosphate acetyltransferase (GlcN-1-P AcTase) activity. This enzymatic activity was approximately 1.3-times increased by introduction of single-substitutions into the 311th, 377th, and 340th residues without decreasing their stability (Zhang et al. 2015). From the other side, this activity was increased 16.8-times by truncation of 11 amino acids at the C-terminal (Zhang et al. 2015). A similar increase in enzymatic activity was observed in the case of the PH0925 protein, identified from thermophilic euryarchaeon *P. horikoshii*. The Man-1-P GTase activity of the PH0925 protein was 3.55-times increased by a114-residues-long truncation from the C-terminus. From these observations, it can be concluded that proteins identified from thermophilic archaea are likely able to remain their solubility after treatment at high temperature following truncation or substitutions at their reaction center. Following these successful engineering, these highly stable enzymes are appropriate to be applied in production of valuable nucleotide-sugar molecules, which are employed in medical field.

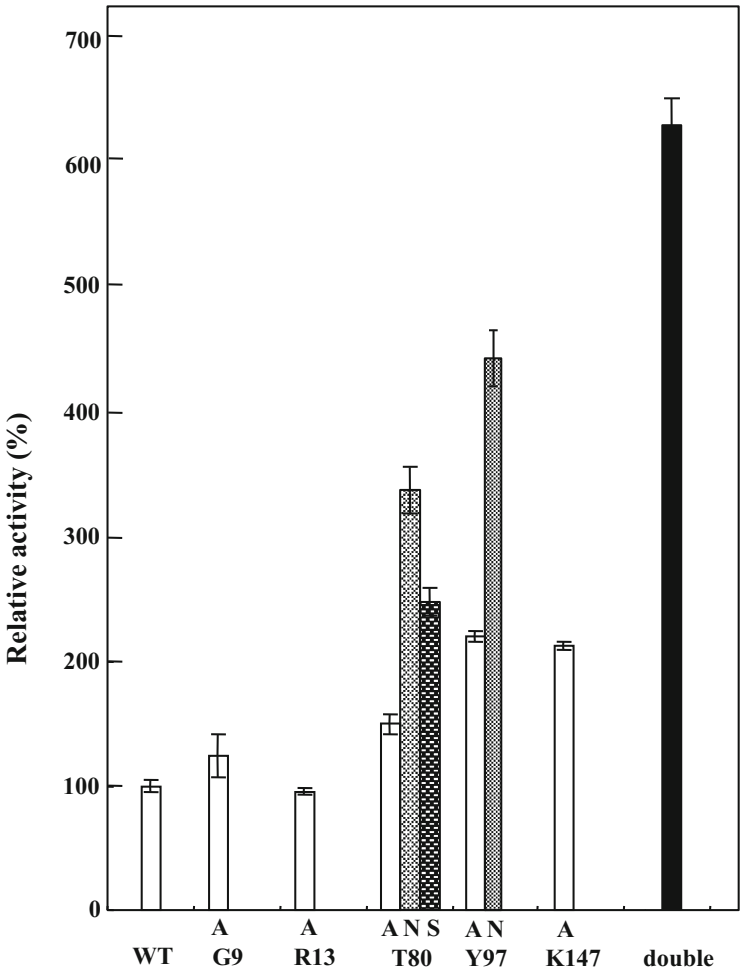

Fig. 1.6 GlcNAc-1-P UTase activity of the wild-type, single mutant, and double mutant of the ST0452 protein. The relative activities are expressed as the ratio of the activity of the mutant to that of the wild-type ST0452 protein. Substitutions are introduced into the 9th Gly (G9), 13th Arg (R13), 80th Thr (T80), 97th Tyr (Y97), and 147th Lys (K147) to Ala (A), Asn (N) or Ser (S). WT: wild-type ST0452 protein, double: mutant protein introduced two substitutions (80th Thr to Ser and 97th Tyr to Asn)

1.6 How to Use Stable Enzymes for Supplying Nucleotide-Sugar Substrates, Contributing to Personalized Therapy

The natural protein and lipid molecules present within or at surface of living cells are usually modified with polymer form of sugar molecules. The attached polysaccharide plays important role for stabilization or activation of the glycosylated proteins

erythropoietin example given earlier in this chapter. At present, antibody or short peptide molecules are expected to work as therapeutic agents in treatment. Antibody molecule requires attachment of polymer structure of carbohydrate molecules to exhibit its activity. For preparation of efficient or person-specific antibody as medicine, attachment of the polymer structure of carbohydrate molecules is required. Attachment of polysaccharide with highly controlled sequence and structure makes the produced antibody more efficient and person-specific molecules, and provides a much longer half-life to these antibodies. For construction of polymer structure of carbohydrates with specific sequence, it is essential and convenient to provide necessary and unique substrate molecules under stringent control of ordering according to the demanded sequences of polysaccharide molecules. Highly stable enzymes from thermophilic archaea with nucleotide-sugar synthesizing activity are expected to be useful for on-time supply of substrates to construct polysaccharide. For this purpose, fixation of enzyme on carrier bead is one solution for continuous and sequential supplying of nucleotide-sugar molecules by step-by-step usage of required enzyme-binding carrier. Also archaeal enzymes with nucleotide-sugar molecule producing activity exhibited wide substrate acceptability; therefore, only one enzyme can be used for providing many different kinds of nucleotide-sugar molecules. Thus providing stable enzymes for antibody production (Dangi et al. 2018; Dubey et al. 2018; Gupta and Shukla 2018; Mandeep and Shukla 2019).

According to these two beneficial features of highly stable enzymes isolated from thermophilic archaea, they are convenient tools in construction of regulated-sequenced polysaccharide molecules for specific protein molecules. Overall, these thermophilic proteins will be utilized in pharmaceutical biotechnology for an ultimate application in medical field.

1.7 Conclusion

Thermophilic archaea possess highly stable proteins with useful enzymatic activities, enabling them to be applied for production of useful nucleotide-sugar molecule, which can be used for substrate supply of polysaccharides. To this purpose, high stability of protein is very critical and makes the biocatalyst a convenient tool, an important feature observed in many proteins originated from thermophilic microorganisms, especially thermophilic archaea. The k_{cat} values of enzymatic activity of highly stable proteins from thermophilic archaea are usually smaller than those of mesophilic bacterial similar enzymes. However, this limitation is overcome by introduction of mutations, substitutions or truncations. The stable proteins with improved enzymatic activity originally identified from archaea should make medical treatment more personalized and precise in near future.

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The Next-Generation of Microalgae-Based Products

2

Rosangela R. Dias, Ihana A. Severo, Mariany C. Deprá, Mariana M. Maroneze, Leila Q. Zepka, and Eduardo Jacob-Lopes

Abstract

Currently, the main microalgae-based product available in the market is whole dried biomass (single-cell protein), where *Chlorella* and *Spirulina* are the dominant genera. Some speciality chemicals that include beta-carotene, astaxanthin, phycocyanin, eicosapentaenoic acid, and docosahexaenoic acid have already your market share consolidated. All of these bioactive products have applications as a natural colorant, additive, and food supplement. Independent of this, several emerging bioproducts such as lutein, fucoxanthin, phycoerythrin, beta-glucans, exopolysaccharides, arachidonic acid, recombinant proteins, and single-cell protein are in the advanced status of technological development, able to achieve commercial exploitation in the coming years. In this sense, this chapter aims to present status and perspectives on the next-generation of microalgae-based products and their technological advancements.

Keywords

Microalgae-based processes · Biomolecules · Bioproducts · Commercial application

2.1 Introduction

Microalgae, including cyanobacteria, constitute a diversified group of organisms that are mainly unicellular, aquatic, and photosynthetic eukaryotes (Maroneze et al. 2016). They can live in adverse environmental conditions using only light, water, and simple substances. The great diversity of microalgae, still not fully exploited,

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offers a series of biologically active metabolites which are of commercial interest. These bioactive metabolites present antioxidant, antimicrobial, antifungal, antiviral, anti-inflammatory, and anticancer activities (Kothari et al. 2017; Bule et al. 2018; Martínez-Francés and Escudero-Oñate 2018).

The interest in microalgae as promising sources of valuable metabolites recently recovered great importance, driven in part by the focus of microalgae biotechnology to produce renewable and commercially viable biofuels, which can only be possible if higher value products are purposely explored (Dias et al. 2019). Thus, the researchers have been concentrated in the diversity of bioactive molecules that can be obtained from microalgae, and that can be profitable and counterbalance production costs (Deprá et al. 2018; Srivastava et al. 2019; Dixit et al. 2019; Jagadevan et al. 2018; Anand et al. 2017).

Today, the most important products obtained of microalgae are the whole dried biomass (single-cell protein), eicosapentaenoic acid (EPA),docosahexaenoic acid (DHA), β-carotene, astaxanthin, and phycocyanin, established in the market of bioactive compounds for use as natural colorant, supplement, and food additive (Jacob-Lopes et al. 2018). In general, the cost of producing these products is generally higher than of traditional sources. However, some metabolites obtained from microalgae have advantages over their conventional sources. Synthetic molecules are less effective than natural sources, which make their use less competitive industrially (Enzing et al. 2014). Moreover, not always, there is a similar alternative available. Phycocyanin, for example, is the unique natural blue colorant available for use.

Beyond the microalgae products already established, several others are on the way to successful commercialization, utilizing the experience of the products that already reached the food and feed market (Borowitzka 2013; Sathasivam et al. 2017). Therefore, the primary objective of this chapter is to compile information and present status and perspectives of the microalgae-based products will reach to commercial exploitation shortly. These represented the next-generation of microalgae-based products.

2.2 A Brief Overview of the Current Marketed Microalgae Based-Products

In the early 1960s, large-scale commercial *Chlorella* cultures were started, followed by *Spirulina* in the early 1970s. In this period, the main microalgae product that the industry targeted was the single-cell protein, with applications directed to food and prophylactic use. In the 1980s, emerged the production of β-carotene and astaxanthin from *Dunaliella salina* and *Haematococcus pluvialis*, approved as a food coloring and antioxidant. Later, started the production of polyunsaturated fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Table 2.1 summarizes the current market and sales prices of the main commercial products microalgae-based. These bioproducts reached a consolidated food and feed market.

Table 2.1 Commercial products microalgae-based

Bioproducts	Cultivation system	Selling price (USD/kg)	Main product application	Main companies producing
Whole biomass (single-cell protein)				
Spirulina	Raceway ponds	8.0	Food ingredient; dietary supplement	Earthrise; Hainan-DIC Microalgae
Chlorella	Open circular ponds; vertical tubular photobioreactors	19.0	Food ingredient; dietary supplement	Far East Microalgae; Taiwan Chlorella Manufacturing; Algomed
Schizochytrium	Heterotrophic fermenters	5.2	Protein supplement	Alltech Algae
Pigments				
β-Carotene	Open raceways ponds; vertical tubular photobioreactors	790	Food supplement; food colorant	Nikken Sohonsa Co.; AlgaEnergy; Nature Beta Technologies
Astaxanthin	Shallow and open ponds; vertical tubular photobioreactors	2500	Food supplement; feed additive	Algatechnologies; Cyanotech; AlgaEnergy
Phycocyanin	Horizontal tubular photobioreactors	548	Food colorant; fluorescent markers	BlueBio Tech
Fatty acids				
EPA	Hybrid system	100	Food supplement	Cellana
DHA	Heterotrophic fermenters; hybrid system	120	Food supplement	DSM; Alltech Algae; Cellana

Source: Adapted from Jacob-Lopes et al. (2018)

Most of the microalgae-based products on the market are intracellular. Therefore, biomass production represents the primary criterion for technical-economic viability. Moreover, they must comply with many regulations and standards, once are generally destined for human and animal consumption. High value-added products, such as pigments and polyunsaturated fatty acids, have a higher production cost and, consequently, sale higher than whole dried biomass. This can be, partly, explained by the downstream processing of these products. The downstream processing for whole dried biomass production is relatively simple, seeing that only biomass drying is necessary. On the other hand, intracellular products such as pigments and polyunsaturated fatty acid require costly extraction and purification steps. In general, the expenditure involved in downstream processes for whole dried biomass production

is about 2%, while for polyunsaturated fatty acids and pigments are about 70% and 85%, respectively (Jacob-Lopes et al. 2018).

Microalgae products, especially high value-added products, using innovative technologies, have attracted commercial attention, seeing that can be obtained at a reasonable cost. Noteworthy, the market value of these products depends not only on production costs, but also on market conditions, including competition from similar chemicals, supply–demand ratio, and suitability for specific applications (Budzianowski 2017).

Today, in addition to the products mentioned in Table 2.1, there are several other products in different stages of development. Therefore, in this chapter, the status and perspectives of microalgae-based products in advanced development have been reviewed.

2.3 Technological Processes for Manufacturing Microalgae-Based Products

The diversity of microalgae and the capacity of these microorganisms to produce chemical specialities made them a target of research and development. They thrive in adverse environments, and their chemical composition under specific conditions can form and accumulate bioproducts of interest (da Silva Vaz et al. 2016). These products can be allocated in the energy, chemicals and materials, food and feed, and pharmaceuticals and personal cares markets (Barsanti and Gualtieri 2018). Figure 2.1 portrays the schematic of the main steps involved in the industrial process related to the production of microalgae-based products.

The industrialization of microalgae-based products requires large-scale culture systems, which are typically raceway ponds, tubular photobioreactors, or heterotrophic bioreactors (Maroneze and Queiroz 2018). Among these, stands out the raceway ponds systems and tubular photobioreactors. The purchase cost for raceway ponds and tubular photobioreactors is estimated at 4–6 kUSD/m^3 and 40–50 kUSD/m^3, respectively (Ramírez-Mérida et al. 2017; Deprá et al. 2019). The cost of each configuration is related to the quality of the materials required. The magnification of these reaction vessels enables reducing the cost of the process and product. For example, the cost of microalgae biomass (USD/kg dry matter) for raceway ponds and tubular photobioreactors in a scale 1000 m^2 is estimated at US $40.36 and US $21.43 and for a scale of 100 ha is of US $7.04 and US $5.13, respectively (Spruijt et al. 2015).

After the upstream processing, the choice of the downstream process is crucial, once influences the economic viability of microalgae-based products. The downstream processing involves harvesting, pre-treatment, extraction, and purification. Current harvesting strategies include physical and chemical methods (Ummalyma et al. 2017; Zhu et al. 2017; Mathimani and Mallick 2018). Despite the importance of harvesting to the economics and energy balance, there is no universal harvesting technique for microalgae. However, centrifugation is the commonly employed technique (Singh and Patidar 2018).

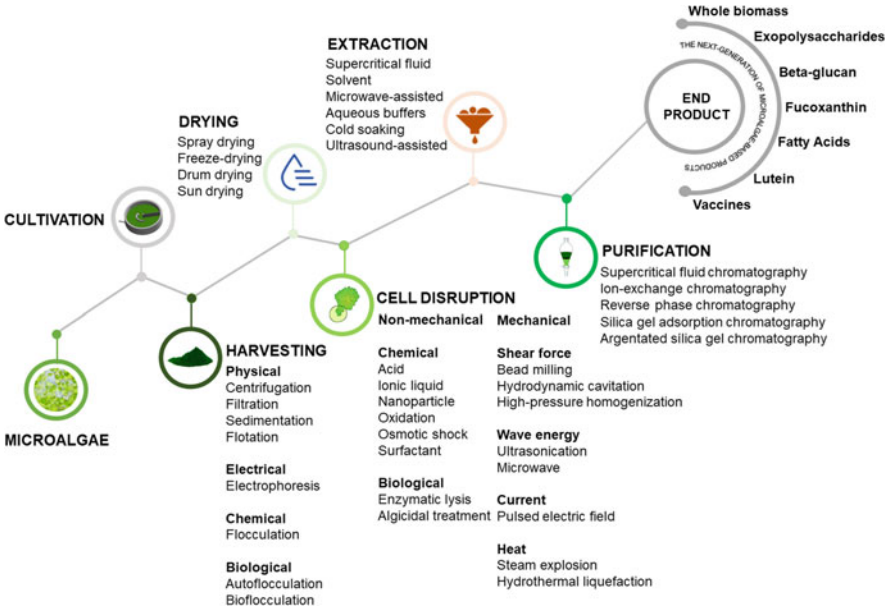

Fig. 2.1 Schematic of the steps involved in the industrial process related to the production of microalgae-based products. Here, the cultivation is considered common for all the bioproducts. Harvesting, pre-treatment, extraction, and purification methods depend on the specific products

After harvest, the drying and cell disruption are most utilized pre-treatment methods and are selected based on extraction procedure and in the desired product (Khanra et al. 2018). The most used drying methods are spray-drying and freeze-drying and cellular rupture can be broadly categorized into two methods, non-mechanical and mechanical. Each method has its advantages and disadvantages (Lee et al. 2017; Dias et al. 2019). In general, the pre-treatment is the key to increase the extraction efficiency of microalgae biocompounds. The extraction follows the process of pre-treatment.

The extraction by supercritical fluids and solvents are widely used to extract biocompounds such as pigments and polyunsaturated fatty acids. Finally, purification is given by several chromatographic methods as supercritical fluids chromatography and ion-exchange chromatography. The purification is done to compounds of microalgae applied to products of high value, to improve its bioavailability, and the method is selected based on the intended product (Voort and VulstekeSaut 2015; Ventura et al. 2018).

2.4 Microalgae-Based Products in Advanced Development

Microalgae offer a chemically diverse and unexplored reservoir of biomolecules that can be harnessed for commercial use. Although many of these compounds have been identified, the metabolic pathways for synthesis are poorly understood for most of them, and extraction and purification methods necessitated to be perfected (Kumar et al. 2019). In this context, independent of the commercially established microalgae products, there are several others at different stages of research and development. The microalgae-based products in advanced development are discussed below.

2.4.1 Single-Cell Protein

The single-cell protein (whole-cell powder) traditionally have been produced as the main microalgae-based product worldwide. This market share is majoritarily represented by the genera *Chlorella* and *Spirulina* as reported early. In consolidated technological routes, the product is sold without any kind of processing except drying. Their application is mainly as dietary supplements for human use (Ramírez-Mérida et al. 2017).

Furthermore, based on diversity of the chemical composition of these microorganisms, other species besides *Spirulina*/*Chlorella* have been considered for production as whole biomass, including mainly *Nannochloropsis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Skeletonema*, *Thalassiosira,* and *Tetraselmis*. These products are presented as live cells, fresh cells, frozen cells, and freeze cells. The applications include starter cultures, concentrates for aquaculture and formulated feeds for aquaculture (Enzing et al. 2014). Besides the unit operations of dewatering, freezing, and drying, some products can include the addition of food-grade preservatives aiming to extend the shelf-life of the product. In terms of safety aspects of these new species, all are classified as "no toxins known (NT)" (Matos 2017).

2.4.2 Recombinant Proteins

Technological advances in the field of recombinant proteins and vaccine delivery systems have driven the use of alternative sources against animal-produced vaccines. Currently, it is estimated that the global market should reach about US $2850.5 million by 2022 (Market and Markets 2017).

In this context, genetically accessible photosynthetic organisms, such as microalgae, the present potential for the generation of high-quality recombinant proteins (Surzycki et al. 2009). Its cellular machinery, mainly involving the presence of chloroplasts, makes these microorganisms excellent sites specific for the double production of complex recombinant proteins. This is because chloroplasts are now known to have peculiar properties, being they the ability to accumulate higher levels of transgenic proteins, since they do not have gene silencing mechanisms. Besides,

they can be supported by multiple genes in a single event due to the availability of numerous insertion sites (Specht et al. 2010).

The mechanism of action for the secretion of proteins is related to the cellular structure of microalgae. The constructs of the genes and the transformation methods depend on the cellular locations of the accumulation of recombinant proteins. It is known that modification of plastid causes accumulation of the transgene material in the chloroplast. However, nuclear genetic materials are accumulated in the cytosol. Thus, appropriate nuclear transformation allows targeting to the endoplasmic and Golgi reticulum for packaging and export to the extracellular medium (Akbari et al. 2014). At the exemplification level, the microalgae *Chlamydomonas reinhardtii* presents the ability to allow adequate folding of proteins strongly bound by disulfide, which is not naturally achieved in other bacterial production platforms.

In addition, among the structural advantages, microalgae are attributed to the potential for rapid cell development, large-scale production and also the non-transmission of animal pathogens (Yan et al. 2016).

The initial studies were supported by hepatitis B antigens of the microalgae *Dunaliella salina* (Siripornadulsil et al. 2007). However, several studies report the use of the microalgae *Chlamydomonas reinhardtii* as the primary potential source in the production of pharmaceutical proteins such as erythropoietin, interferon β insulin, and immunoglobulin A. Recently, this microalga has been used in experimental formulations of vaccines against *Staphylococcus aureus*, while the microalgae *Schizochytrium* sp. has been used to develop novel zika virus vaccines and in both cases the performance of these vaccines presented more satisfactory results in triggering both the humoral mucosal and systemic immune response (Márquez-Escobar et al. 2018; Miquel-Clopés et al. 2019).

Additionally, among the main applications of recombinant proteins, it is assumed that this industrial follow-up may be more economical for vaccines such as HPV, as well as the fundamental element for the development of new vaccines for which there is still no alternative (Specht and Mayfield 2014). This is because, at the comparison level, costs for the production of functional antibodies are estimated at US $150/g in mammalian cells, while in plants the average values estimated are of US $0.05/g. In addition, microalgae are a promising system, and their costs are estimated at US $0.002/g (Potvin and Zhang 2010; Barrera and Mayfield 2013). Therefore, the use of microalgae becomes particularly significant because of the cost-effectiveness of such recombinant proteins in the field of industrial vaccines.

2.4.3 Phycoerythrin

The phycoerythrin (PE) is a photosynthetic pigment belonging to phycobiliproteins, which also include phycocyanin, allophycocyanin, and phycoerythrocyanin. This pigment is considered one of the brightest natural fluorophores identified. The chemical structure of phycoerythrin is shown in Fig. 2.2. According to the light absorption properties, the PE can be classified into two main classes: R-PE (λ_{max} = 565 nm, 499 nm and one shoulder at 545 nm) and B-PE (λ_{max} = 565 nm,

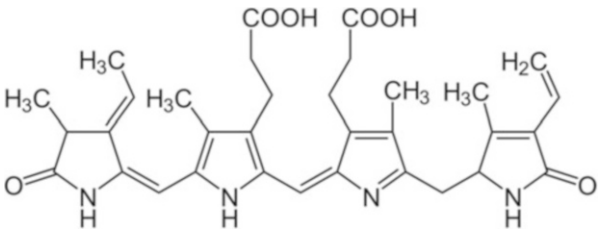

Fig. 2.2 Chemical structure of phycoerythrin. Source: Adapted from Hsieh-Lo et al. (2019)

546 nm and one shoulder at 499 nm). The spectral difference between phycoerythrins is attributed to the presence of prosthetic groups, denominated bilins that covalently bind to cysteine-specific residues on protein subunits. R-PE is commonly extracted and purified from *Porphyra*, *Gastroclonium,* and *Polysiphonia* macroalgae. R-PE is generally used for flow cytometry and other applications that require high sensitivity but not photostability (Gargouch et al. 2018; Leney et al. 2018).

On the other hand, B-PE is the main phycobiliprotein extracted from red microalgae. The *Porphyridium cruentum* microalgae have been produced for commercialization this pigment. The B-phycoerythrin of *Porphyridium cruentum* is used mainly as an indicator in the quantitative assay for the oxygen-radical absorbing capacity of antioxidants in serum or plasma. B-PE is commonly marketed as a lyophilized powder containing preservatives. The commercial value of B-PE of *Porphyridium cruentum* is about 681.62 USD/mg (Sigma-Aldrich 2019). This high value has encouraged the development of efficient extraction and purification procedures to maximize the purity and commercial yield of B-PE. The complexity of downstream procedures and low yields limit the potential for practical implementation to the commercial level. Noteworthy, under specific conditions, dependent on abiotic and biotic factors, the microalgae may potentiate B-PE accumulation. The B-PE accumulation is desired to optimize its large-scale production (Tang et al. 2016).

In addition, the B-PE, as a natural pigment, exhibits beneficial biological activities for human health. Recent studies have shown that the B-phycoerythrin from *Porphyridium* microalgae species can reduce myeloid tumor cell proliferation in vitro. Treatment with B-phycoerythrin induces apoptosis and can increase glutathione reductase and superoxide dismutase activity. These studies indicate the potential of B-PE as a source of bioactive protein (Minkova et al. 2011; Gargouch et al. 2018).

2.4.4 Fucoxanthin

The fucoxanthin belongs to the class of non-provitamin A carotenoids, which gives brown microalgae their characteristic color. When ingested, the fucoxanthin is metabolized by digestive enzymes in the gastrointestinal tract into fucoxanthinol and then absorbed into intestinal cells. In the liver, the fucoxanthinol is converted to amaruciaxanthin A (Martin 2015). Both structures are shown in Fig. 2.3.

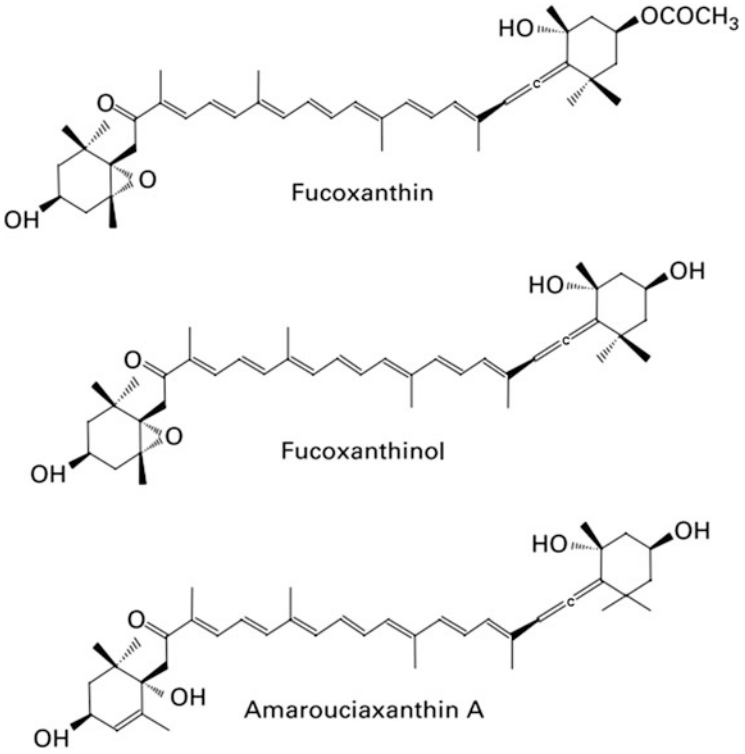

Fig. 2.3 Chemical structure of fucoxanthin, fucoxanthinol, and amaruciaxanthin A. Source: Adapted from Hashimoto et al. (2009)

Experimental evidence reveals that fucoxanthin and its metabolites have present remarkable biological properties, without any evident toxicity (Rokkaku et al. 2013; Asai et al. 2004; Rwigemera et al. 2015, 2014). These metabolites exhibit antioxidant, anti-inflammatory, anticancer, anti-obesity, antidiabetic, hepatoprotective, antimalarial activities (Crupi et al. 2013). In particular, fucoxanthin is a promising nutritional supplement for the treatment of obesity and related pathologies. Nutrigenomic studies reveal that fucoxanthin induces uncoupling protein-1 in abdominal white adipose tissue, conducting to oxidation of fatty acids and heat production in brown adipose tissue(Miyashita et al. 2011; Gammone and D'Orazio 2015; Maeda 2015). The fucoxanthin and its metabolite fucoxanthinol suppress triglyceride absorption. The suppressive effect of fucoxanthinol, however, is more pronounced than that of fucoxanthin (Matsumoto et al. 2010). In contrast, the suppressive effect of amaruciaxanthin A on the differentiation of 3T3-L1 is more pronounced than that of fucoxanthinol (Yim et al. 2011). The fucoxanthin metabolites are considered the active forms that play physiological functions in the human organism. However, both the fucoxanthin as its metabolites has an anti-obesity effect (Hu et al. 2016).

The dietary fucoxanthin preferentially accumulates in the heart and liver as fucoxanthinol and in white adipose tissue as amaruciaxanthin A (Hashimoto et al. 2009; Miyashita and Hosokawa 2018). As amaruciaxanthin A is the dominant metabolite of fucoxanthin that accumulates in white adipose tissue, studies suggest that amaruciaxanthin A may be the molecule responsible for the anti-obesity effect of fucoxanthin in vivo (Yim et al. 2011).

The fucoxanthin has a unique chemical structure, similar to neoxanthin and peridinine, which is different from that of other carotenoids, such as β-carotene and lutein. The alenic linkage, 5,6-mono-epoxide, and nine double conjugated plays an important role within fucoxanthin structure. In part, the biological properties of fucoxanthin and its metabolites are assumed due to the presence of its allenic bond (Fig. 2.3) (Sachindra et al. 2007).

Currently, the commercialization of fucoxanthin is mainly obtained from macroalgae as *Macrocystis*, *Laminaria,* and *Undaria. Phaeodactylum tricornutum* is the unique microalgae produced to obtaining fucoxanthin. Your application is as a dietary supplement for humans, mainly as concentrates or microalgae extracts (Algaech 2019). Beyond the microalgae *Phaeodactylum tricornutum*, there is the opportunity for fucoxanthin production from other microalgae species such as *Odontella aurita*, *Cyclotella cryptica*, *Chaetoceros calcitrans,* and *Isochrysis galbana* (Foo et al. 2015; Kim et al. 2012a, b). Typical concentrations found in microalgae are at least an order of magnitude greater than those found in macroalgae (McClure et al. 2018). The microalgae *Isochrysis galbana*, for example, is capable of producing 18.2 mg of fucoxanthin per g DW, while species of macroalgae (*E. bicyclis*, *K. crassifolia*, *A. crassifolia*, *S. horneri*, and *C. hakodatensis*) produce only 0.04–1.52 mg of fucoxanthin per g DW (Airanthi et al. 2011; Gong and Bassi 2016). In addition, the microalgae *Odontella aurita* presents concentrations up to 18.47 mg/g DW, can accumulate, under specific growth conditions, concentrations superiors than 20 mg/g DW (Xia et al. 2013). Considering the high content of fucoxanthin in *Isochrysis* sp. and *Odontella aurita*, these microalgae could be proposed as a source of this compound for nutraceutical and pharmaceutical applications. Noteworthy, although the content of fucoxanthin in microalgae is more significant than the found in macroalgae, this pigment continues to be produced mainly by macroalgae, because its cultivation is cheaper. In contrast, an advantage of cultivating microalgae in closed systems and controlled is the availability of a continuous supply of biomass throughout the year (Crupi et al. 2013).

2.4.5 Lutein

The lutein is a carotenoid of lemon-yellow tint considered essential for human health. This compound is not produced by humans and should be ingested through diet. Lutein contains a characteristic structure composed of 10 conjugated double bonds (Fig. 2.4). Its primary biological functions are determined by the extended system of conjugated double bonds, which is also responsible for its color (Stringheta et al. 2009). In the diet, free or esterified lutein is absorbed from the

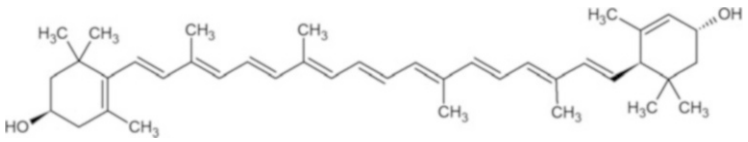

Fig. 2.4 Chemical structure of the lutein. Source: Adapted from Bernstein et al. (2016)

gastrointestinal tract via uptake by enterocytes. This carotenoid is predominantly found in the esterified form. However, for commercialization, it is normally saponified for esters removal (Kijlstra et al. 2012). Scientific information about its bioavailability demonstrates that free lutein is more bioavailable than lutein in the esterified form (Norkus et al. 2010).

The lutein exhibits antioxidant and anti-inflammatory properties and has demonstrated, from several studies, to be useful in protecting against atherosclerosis and in preventing macular degeneration and cardiovascular diseases (Chung et al. 2017; Gong and Bassi 2016). In particular, numerous studies emphasize a relationship between lutein consumption and a reduction in the incidence of eye diseases (Johnson 2014; Bernstein et al. 2016). Studies in patients with age-related macular degeneration (AMD) show that lutein-containing supplementation may impede the progression of AMD and improve visual function (Weigert et al. 2011; Ma et al. 2012a, b). Moreover, studies have associated lutein supplementation with the prevention of cataract development and progression (Arnal et al. 2009; Karppi et al. 2012). Despite the scientific evidence presented between lutein consumption and the reduction in the incidence of eye diseases, requests for health claims have not yet obtained ubiquitously successful (Nwachukwu et al. 2016).

The world market for lutein as from different sources was estimated at USD 308 million in 2018 and is expected to reach US $396.4 million by 2024. This increase is a projection based on demand for lutein for various applications. The lutein is especially used as dye, food additive, and as an eye health supplement (Lin et al. 2015; Global 2019).

Currently, most of the lutein is produced from marigold flowers. The disadvantage of calendula cultivation as a source of lutein is the demand for land and manpower intensive. In this context, the microalgae represent an alternative to traditional sources, with a sufficiently high content of lutein. The market estimate for lutein obtained from microalgae is US $3.14 million (Hu 2019). When compared to calendula cultivation, microalgae present advantages such as higher growth rate, a predominance of lutein in free form, annual productivity regardless of seasons, and coproduction of value-added compounds (Fernández-Sevilla et al. 2010; Araya et al. 2014).

Some strains of microalgae such as *Chlorella vulgaris*, *Muriellopsis* sp., *Scenedesmus* sp., *Desmodesmus* sp., *C. zofingensis,* and *C. protothecoides* are recognized as lutein producers (Chen et al. 2017; Xie et al. 2017). The quantity of lutein generated by microalgae depends on some environmental conditions, such as light intensity and temperature (Bhalamurugan et al. 2018). Based on studies, a

lutein content in the microalgae biomass of 5 g/kg is estimated. Although they have a relatively high content of lutein and advantages when compared to marigold flowers, there are still no lutein products obtained from commercially established microalgae. There is only a small market participation represented by the genera *Chlorella* and *Scenedesmus*, turned for application as food supplement and supplement feed (Camacho et al. 2019; A4F 2019).

Some technical inconvenient, such as elevated harvest costs and high energy demand for the pre-treatment and lutein extraction, persist. Therefore, strategies to reduce the cost of production and the search for strains with higher lutein content are being evaluated to make microalgae economically viable sources of lutein (Acién et al. 2012).

2.4.6 Beta-Glucans

The β-glucans are heterogeneous polysaccharides united by β-(1,3); (1,4) or (1,6) glycosidic bonds, found in plants, fungi, bacteria, and microalgae (Fig. 2.5). The β-(1,3)-glucan is the simple of all. A well-known example of this structure is paramylon found in microalgae such as *Euglena* sp. and *Astasia longa*. The β-(1,3;1,4)-glucan can be found in the cell wall of microalgae as *Micrasterias* sp. and *Monodus subterraneus*, and the branched β-(1,3;1,6)-glucan is found in diatoms and chrysophytes (Barsanti et al. 2011). These polysaccharides have been extensively studied and are considered safe for use. Based on clinical evidence, the

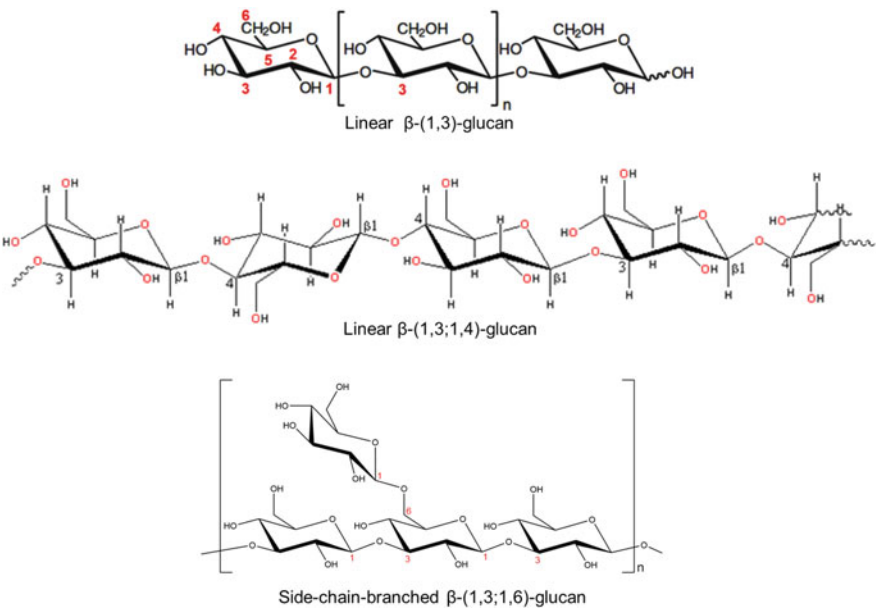

Fig. 2.5 β-glucan structures identified in microalgae. Source: Adapted from Barsanti et al. (2011)

β-glucans have been established as beneficial natural polysaccharides in the treatment of multiple infirmities. The β-glucans exhibit antitumor, anti-inflammatory, anti-osteoporotic, and immunomodulating activities (Jayachandran et al. 2018).

The β-glucan structures vary according to the different sources, and their physiological functions and biological activity vary each other. The mechanism of action of β-glucans in the human biological system depends on its molecular weight and solubility in water, which depends on its molecular structure. The higher the molecular weight, the higher the viscosity and the greater the health benefits (Maheshwari et al. 2019). With the remarkable range of benefits offered by these polysaccharides, it is expected, stimulation scientific efforts to understand better the structure/function relationships and the mechanisms involved behind their biological activity (Izydorczyk 2016).

β-glucans can be ingested as a dietary supplement or as part of a diet. Currently, the most commercially available β-glucans are in the great part native of fungi; however, there are clear opportunities for successful commercialization of β-glucans derived from microalgae (Kim et al. 2019). The microalgae cultivation is more straightforward, the costs are lower, and the productivity of β-glucan is higher than, for example, in the cultivation of mushrooms and yeasts. Several species of mushrooms cannot be cultivated and are collected from nature. The microalgae, on the other hand, are easily cultivable and represent a more interesting alternative (Schulze et al. 2016). Moreover, the microalgae-based processes do not involve costly extraction steps and, therefore, their cost is substantially smaller. Another advantage of microalgae compared to traditional sources of β-glucan is its bioavailability. In microalgae, the β-glucan is mainly β-(1,3)-glucan, while yeast-derived products are mostly a combination with β-(1,6)-glucan, which affects their bioavailability (Stephen 2016).

The microalgae *Euglena gracilis* is a rich source of β-(1,3)-glucan and has been exploited for the production of this polysaccharide (Russo et al. 2017). Their application is as immune-support ingredient. The products are presented mainly as concentrates or whole-cell of *Euglena gracilis* (Gissibl et al. 2019). Other microalgae species source of β-glucan include *Scenedesmus obtusiusculus*, *Porphyridium purpureum*, *Pavlova mesolychnon*, *Phaeodactylum tricornutum*, *Odontella aurita,* and *Chaetoceros muelleri* (Bashir and Choi 2017). It is expected that the increasing use of this ingredient mainly in the pharmaceutical and nutraceutical industry boost its development and production from microalgae (Barsanti and Gualtieri 2018).

2.4.7 Exopolysaccharides

Among the polysaccharides and their derivatives, the sulphated exopolysaccharides (sPS) of microalgae are in focus. The sPS of microalgae are complex polymers composed of numerous monosaccharides, the most abundant being xylose, glucose, and galactose (Marcati et al. 2014). The structure of the sPS and its complexity varies according to the microalgae species. Therefore, each new purified sPS is a new

compound with unique structures and, consequently, with unclear biological activities. Thereby, the precise knowledge of the structural relationship with its biological activities remains under-revealed for most sPS structures (Pandeirada et al. 2019). However, it is reported that the bioactivity of the sPS is related to its molecular weight, monosaccharide composition, sulfate content and the position of the sulfate ester group (Hahn et al. 2012).

Several studies have already highlighted the pharmacological activities of the sPS of microalgae (de Jesus Raposo et al. 2014; Sun et al. 2012; Challouf et al. 2011; Liu et al. 2016). They perform, at the cellular level, different functions. The bioactive properties of these biomolecules have been associated with anti-inflammatory, immunomodulatory, anti-tumor, antiviral, antioxidant, antibacterial, anti-lipidemic, and anti-glycemic activities (de Jesus Raposo et al. 2013; Sanjeewa et al. 2018).

Microalgae with potential for production of sPS include *Chlorella stigmatophora*, *Chlorella capsulata*, *Tetraselmis* sp., *Nannochloropsis oculata*, *Botryococcus sudeticus*, *Neochloris oleoabundans*, *Gyrodinium impudicum*, *Dunaliella tertiolecta*, *Isochrysis* sp., *Phaeodactylum tricornutum*, *Porphyridium marinum*, *P. cruentum*, *P. purpureum*, *Cylindrotheca closterium*, and *Spirulina platensis* (Pletikapić et al. 2011; Guzman et al. 2003; Guzman-Murillo and Ascencio 2000; Soanen et al. 2016; Balti et al. 2018; Goo et al. 2013). Unlike macroalgae, the microalgae do not have proper names for their sPS, except spirulan, produced by *Spirulina platensis* (Raposo and Morais 2015). Data demonstrated that spirulan isolated from *Spirulina platensis* is a potent antiviral agent (Lee et al. 2000).

In sPS it was verified that higher sulfate content induces an increased in the antiviral activity (de Jesus Raposo et al. 2014). In general, the content and position of the sulfated groups may differ in the various sPS, which is dependent on the strain, culture conditions, and extraction procedures (de Jesus Raposo et al. 2015).

Sulphated exopolysaccharides can find applications in functional foods, cosmetics, and pharmaceuticals. The commercial exploitation of these polymers from microalgae is limited basically by their low concentrations (Patel et al. 2013). Information regarding the estimated price range and market of these biomolecules remains unclear.

2.4.8 Arachidonic Acid

The arachidonic acid (AA) is considered a constituent of biomembranes, essential for cell function, especially in the nervous system, skeletal muscle, and the immune system. Besides that, it is a precursor of prostaglandins and eicosanoids. Due to its importance in the development and function of the central nervous system and retina, AA has been recommended for infant formula supplementation (Tallima and El Ridi 2018).

The AA is obtained from the diet or through converting linoleic acid by desaturation and elongation processes. The biosynthesis of AA from linoleic acid is shown in Fig. 2.6 (Hanna and Hafez 2018). It is noteworthy that only a small portion of linoleic acid is converted to AA in the body. The production of AA on an

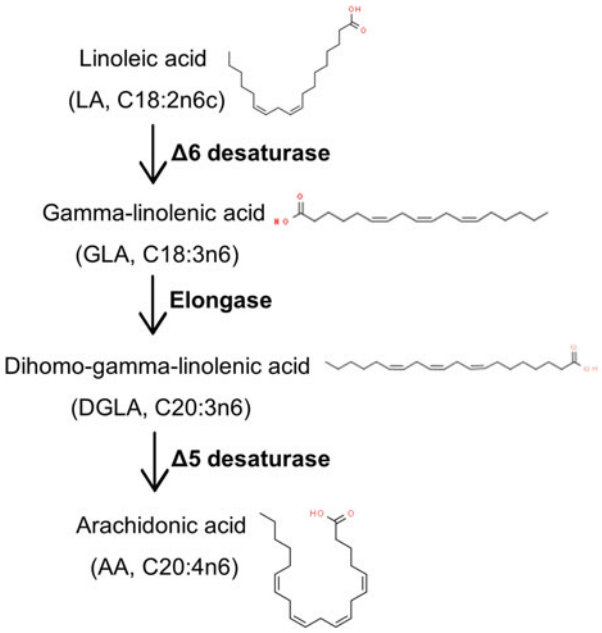

Fig. 2.6 Biosynthesis of AA from linoleic acid. Source: Adapted from Shanab et al. (2018)

industrial scale originates mainly from fungal strains belonging to the genus *Mortierella* sp., of which the *M. alpina* species is considered the predominant source with a content of up to 70% of the total lipids (Dediukhina et al. 2011). The *Lobosphaera incisa* microalgae are also capable of producing a high AA content, capable of reaching up to 77% of total fatty acids. These have been exploited for AA production as a food supplement for infants (Camacho et al. 2019). The laboratory-scale production has already been enlarged and is found currently on pilot-scale. Open culture systems, such as cascade raceways and raceway ponds, and closed systems, such as tubular and flat-plate photobioreactors, have been used in the cultivation of *Lobosphaera incisa*. Typically, an induction phase is required for AA accumulation. Other microalgae species, such as *Porphyridium purpureum*, *P. cruentum*, *Myrmecia incisa*, *Gracilaria* sp., and *Rodomella subfusca*, have an AA content of 40–60% of total fatty acids. These represent a promising alternative source for AA production (Shanab et al. 2018).

2.5 Microalgae-Based Products in Early Development

Regardless of commercially established microalgae-based products and those in advanced development, there are others in early development with potential for commercial exploitation that includes biofuels, polyhydroxyalkanoates, enzymes, violaxanthin, zeaxanthin, prebiotics, phenolics, glutathione, and sterols.

Among biofuels, it is possible to mainly produce biodiesel and biohydrogen from microalgae (Shuba and Kifle 2018). However, economic aspects are the barrier to the final commercialization of these biofuels (Su et al. 2017). A critical analysis of the current status of microalgae biofuels indicates the unfeasibility of their production, which is amplified by the low price of fossil resources (Deprá et al. 2018). Despite this, there is optimism based on the sustainability of microalgae as feedstock and the technological advance already achieved to make them competitive with petroleum (Adeniyi et al. 2018).

Recently, microalgae have been proposed for the production of polyhydroxyalkanoates (PHA). This polymer is produced from microorganisms and considered as a substitute for petroleum-based plastics (Cassuriaga et al. 2018). Currently, the production of PHAs by bacteria is limited by the cost of production (Rahman and Miller 2017). Given this scenario, microalgae were suggested for the production of PHAs at a relatively minor cost. Attributed to the minimum nutrient requirements needed for cultivation. Numerous studies, under specific growth conditions, evaluated PHA production during the cultivation of different strains of microalgae. Concentrations of 5–55% of PHA (dry cell weight) were reported (Nishioka et al. 2001; Kavitha et al. 2016; Kovalcik et al. 2017; Toh et al. 2008; Coelho et al. 2015).

Commercially important enzymes can be produced from microalgae and can be used as biocatalysts in a range of industrial applications. Previously neglected, the microalgae have now been proposed as enzymatic factories. Enzymes derived from microalgae include cellulases, galactosidases, proteases, lipases, phytases, laccases, and amylases (Brasil et al. 2017). To date, there have been no reports of commercial production of microalgae-based enzymes, but laboratory studies have demonstrated the potential of these microorganisms to synthesize these substances (Yong et al. 2016). In this context, strategies to optimize enzyme concentration and reduce operating costs are underway. The concept of biorefinery has been approached as an efficient strategy to overcome the existing bottlenecks and make possible the production of enzymes in the future.

Microalgae compounds with promising biological activities are being evaluated with a focus on human health and disease prevention (Dewi et al. 2018). In vitro and animal studies support the potential of microalgae and their isolated bioproducts as an innovative strategy for the treatment of various diseases (Talero et al. 2015; El-Hack et al. 2019). The carotenoid violaxanthin isolated from microalgae strains, for example, demonstrated in vitro to have antiproliferative effects against the growth of cancer cells (Cha et al. 2008; Pasquet et al. 2011; Soontornchaiboon and Kim 2011; Amaro et al. 2013). Microalgae such as *Chlorella protothecoides*, *Chlorella vulgaris,* and *Scenedesmus obliquus* produce this carotenoid (Grudzinski et al. 2016; Patias et al. 2017).

The zeaxanthin commonly referred to as macular pigment such as lutein can be synthesized by microalgae as *Dunaliella*, *Spirulina*, *Synechococcus*, *Chlorella*, *Prochlorococcus*, and *Prochlorothrix*. Zeaxanthin currently available in synthetic form has a complex production process and low biological activity. On the other hand, the production from vegetables and fruits presents low extraction and

production rate. In this context, the production of zeaxanthin from microalgae can be seen as a promising alternative for applications as dye and food supplement. From genetic engineering and the use of new technologies, it is possible to increase the accumulation of zeaxanthin in microalgae, which can make them commercially viable sources (Sajilata et al. 2008; Zhang et al. 2018).

Active compounds such as prebiotics found in microalgae include galacto-oligosaccharides, xylooligosaccharides, oligosaccharides derived from agarose and alginate, galactans and arabinoxylans, although the criteria for classification as prebiotics have not been validated for some of them (de Jesus Raposo et al. 2016). This is a promising area of research, capable of providing biocompounds that are essential for the prevention of various human diseases.

Microalgae can also produce phenolics and glutathione (GSH) with antioxidant properties applicable in the food, pharmaceutical, and cosmetic industries (Maadane et al. 2015; Goiris et al. 2015; do Nascimento et al. 2019; Sathasivam et al. 2017; Choochote et al. 2014). Currently, synthetic antioxidants are the most utilized in industrial applications. However, concerns regarding its safety and toxicity led to the search for natural antioxidants (Morowvat and Ghasemi 2016).

Due to the content of sterols of some microalgae, these are commonly used to promote the growth of juveniles. Beyond cholesterol, unusual sterols like brassicasterol, campesterol, stigmasterol, and sitosterol are reported. Noteworthy, microalgae sterols seem to present hypocholesterolemic properties (de Jesus Raposo et al. 2013; Fagundes et al. 2019).

2.6 Conclusion and Way Forward

With the emergence of new techniques and improving them for the study of microalgae-based products, especially those with bioactive potential, this group of microorganisms gains a closer look as a source of natural products. Given their extraordinary biological availability and structural variety, the screening of microalgae bioactive compounds will be certainly successful in the global market. However, there are many obstacles related to this that need to be overcome to make microalgae-based products an industrial reality. Some recommendations of the way forward are: (1) select microalgae strains suitable to be controlled at the industrial level. The use of biotechnology techniques, including the genetic engineering tools, could be a major leap toward the search for compounds with unique and multifunctional activities; (2) culture conditions should be improved to raise the amount of the microalgae bioactive compounds of particular interest inside their chemical composition; (3) bioreactors engineering must be enhanced to achieve higher workloads and then scale-up. These advances will impact the process design; (4) implement approaches that maximize the technical, economic, and environmental performance of microalgae-based processes and products. We can cite three main strategies for this purpose: the process integration, the process intensification, and the integrated biorefinery model; (5) perform assays that allow determining the biological effects, such as antiviral, anticancer, antibacterial, and antioxidant, associated with robust

extraction methods; and (6) establish duly safety and regulatory issues for the microalgae bioproducts, since many of these bioproducts are intended for human or animal consumption. Finally, since all these concepts are demonstrated, the scientific communities must carefully elucidate the aspects related to the scaling up.

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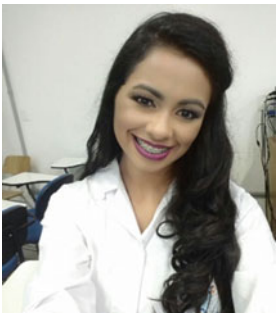

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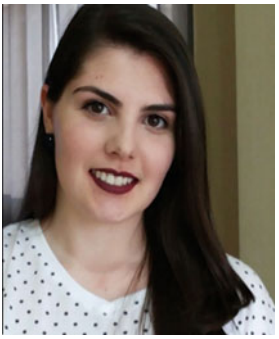

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Advanced Techniques for Enzymatic and Chemical Bleaching for Pulp and Paper Industries

3

Guddu Kumar Gupta, Rajeev Kumar Kapoor, and Pratyoosh Shukla

Abstract

The use of chemicals in the bleaching process is the traditional bleaching process and generated vast amounts of toxic materials in the form of bleach effluents of chemicals used for bleaching. Therefore, the generation of high lignin content incorporation with chlorine-based oxides is responsible for the environmental issue as reproductive damages in humans and aquatic animals. In this chapter have been highlighted some modified bleaching techniques for the mitigation of toxic chemicals released from traditional bleaching process. Hence, usage of enzymatic bleaching method instead of conventional bleaching method is a revolutionary bleaching concept in paper mill. This chapter emphasizes the sustainable utilization of bio enzymes in the pulp industry, along with its efficiency and future challenges. Thus, enzymatic bleaching technique is most significant and eco-friendly as well as cost effective than traditional technique. The enzymes xylanases and laccases are showed the most efficient and cost effective process either alone or in combination with each other. Therefore, laccases play a significant role in the presence of mediators such as ATBS and HBT, etc.

Keywords

Pulp and paper · Bleaching · Xylanases · Laccases · Waste papers · Chemical bleaching

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3.1 Introduction

In the pulp and paper industries, either virgin raw materials (wood) or wastepapers (newsprint and mixed office wastepaper) are used for the making of paper. Nowadays, the making of paper uses recycled paper is one of the most prominent methods and also reduces using virgin raw material, which mitigation on environmental impact. One study reported that using recycled paper in Europe is a vital part of the paper production, with predictable utilization of about 72% today (Cepi 2013). Another report demonstrated that recycling of paper might play a significant role in environmental benefits (Laurijssen et al. 2010). Thus, recycled paper used for the papermaking process has been reported as beneficial from the environmental perspective; it should be promoted as much as possible (Biedermann and Grob 2010). In the paper mill, the manufacturing and production of paper consist of the two phases one is paper pulp production by the paper industries. At the same time, another one is paper product manufacturing by separate sectors. The current report has established that paper and its products may have large chemical particulates (Bradley et al. 2008), out of these associated with the printing industries, where approximately 7000 or above chemicals used in ink production alone. However, very few studies have been reported the identification and challenges (BMELV 2012) of specific substances released from paper waste (Geens et al. 2012). The papermaking industry has an effect on the environment in both ways, like upstream as well as downstream. Nowadays, approximately 90% of paper mills used wood as raw materials for the making of pulp and accounts for 1.2% of the world's total economic output. However, to minimize the use of wood, the pulp paper mill used recycled newsprint as well as printing or copier paper as raw materials (waste paper) instead of wood, which saves tons of tree wood (Counsell and Allwood 2006).

In addition to these, the necessity of cellulose component for paper making is available in bamboo (approx. 56–65%) in comparison to that of hardwoods (53–59%) and softwood (51–60%). According to Singh et al. (2019), the replacement of the chemical bleaching process with the bio-bleaching process is eco-friendly as well as cost effective techniques (Trier et al. 2011). At the industrial scale, uses of eco-friendly bleaching enzymes such as xylanases (Kumar et al. 2019a) and laccases are most significant for the biobleaching against agro-waste residues and wood pulps. Another study reported that the enzymatic pre-bleaching study in India is still under investigation (Singh et al. 2019). After the pulping and deinking of waste papers, the bleaching process of the pulp in the paper industries is the most crucial process and notable in the accounts of environmental perspective. The removal of lignin from the pulp is known as bleaching, which is about 5% per unit of weight in the unbleached pulp and due to it is a very dark color in nature. The presence of lignin in virgin pulp can be treated with bleaching agents (Song et al. 2000).

Therefore, bleaching can be done by chemically and enzymatic methods. In the traditional bleaching (also known as chemical bleaching) of pulp, the hazardous pollutants released when chlorine-based bleaching process is used. These toxic chemicals mainly come into adsorbable organic halides (AOX), which generated

during the elemental chlorine-based chemicals used in bleaching (Liao and Kannan 2011). To mitigation of these hazardous chemicals disposed of directly or indirectly into the environment in the form of waste, waters are becoming rigorous, thus necessitating the legislative and restrictions on the papermaking industries. Among these a modifying as well as treatment methods employing into the pulp mills. Therefore, eco-friendly techniques such as the use of enzymatic bleaching process instead of traditional techniques applied in the paper industries (Kumar et al. 2019b). However, because of their texture the usage of non-wood fibers as raw materials for the making of paper reduces also the usage of virgin wood. In recent years, agro-waste residues used as a source of material because of their viability, economic feasibility, as well as sustainability. Moreover, there is a vast range of agro residues used such as wheat straw, bamboo and bagasse are the furthermost noteworthy ones (Bhardwaj et al. 2019).

3.2 Pulping and Deinking of Waste Papers

Over the past decade, wood was extensively used as raw material by the paper mills, so there is huge demand by the paper industries for the tree planting (Mohandass and Raghukumar 2005). Pulping can be done by chemically as well as enzymatic pulping. The chemical pulping can be achieved by the kraft sulfate process, soda process, and chemi-mechanical pulping process. In the kraft sulfate process, the pulp cooked at a very high temperature (165–170 °C) in the presence of sodium sulfide and sodium hydroxide. In this process, the recovery of chemicals is approximately 91–94% and reused for the further next cycle (Pivnenko et al. 2013). In the soda pulping process, caustic soda is being used at high temperature (150–160 °C) for the pulping of agro-waste residues. On the other hand, chemi-mechanical pulping process treated the pulp with mild soda derivative reagents to separate lignin and resin from cellulose and then treated with the mechanical method for the refining of pulp (Boruah et al. 2016).

Making of paper commonly involves the main three steps, such as collecting wood or waste paper, sorting out them based on their types, and processing them for papermaking (Maity et al. 2012). For the making of paper pulp, the sorted raw materials mainly soaked in a pulper, which contains water and chemicals. However, there are generally three types of methods used for the making of pulp such as mechanical, chemicals, and enzymatic methods. In mechanical methods, the sorted waste papers are soaked for some time or sometime overnight (depends on the experiment) in water and making pulp with the help of a pulper machine (Ibarra et al. 2012). In chemical pulping method, chemicals such as sodium oxide and chlorine-based agents (chlorine dioxide, organochlorine) are used for the pulping process (Call and Strettmatter 1992). Besides the above methods nowadays, most of the paper mills used enzymes for the making of pulp instead of chemicals.

Due to the generation of toxic and environmental issues, while using chemicals for pulping, many more pulp industries use the enzymatic process for the pulping of waste papers (Xu et al. 2009). During the washing process of the pulping, there are

lots of chemicals and tinny fibers (cellulose fibers) are released, which are causes of environmental as well as the loss of fibers yield at the end of the papermaking process. However, to minimize these problems pulp mill has been applied to the enzymatic pulping techniques for the pulping. After the making of pulp, it passes through the screens containing different sizes, holes, and slots (Virk et al. 2011). It removes small contaminants such as bits of plastics and stickies by the mechanism called screening. In addition, then pulp is also passed through large cone-shaped cylinders, heavy metals like staples thrown out and lighter contaminate collected into the center of the cone. In the deinking process, removal of ink particles from the pulp fibers can be done by mechanical, chemical, and enzymatic processes (Singh et al. 2008). For recycling of waste papers, deinking is the primary process and involve the removal of ink from the fiber by flotation and washing process. There are two types of ink present in the secondary tissues, one is impact ink present in newsprint, which is easier to remove as compared to other non-impact ink present in xeroxed or laser printed papers (Eugenio et al. 2010).

The traditional deinking technique uses chemicals like sodium hydroxide, chlorine-based chemicals, hydrogen peroxide, sodium silicate, and other chelating agents, which reported as hazardous reagents and the causative agent for the environmental health issue (Leduc et al. 2011). Therefore, many researchers said that enzymatic deinking has massive attention by pulp industries due to a decrease in the utilization of chemicals and released its derivatives into the environment, also its effective cast process and making it eco-friendly. The usage of enzymes can be done either on the ink or on the pulp fibers in the deinking process (Dixit et al. 2019). The following enzymes (cellulase, hemicellulase, pectinase, lipase, xylanase, amylase, laccase) are used for the deinking purpose. Most of these enzymes are produced from the thermophilic fungus. In the deinking process, enzymes such as cellulases and hemicellulases, have been used in combination, which showed maximum efficacy (Woldesenbet et al. 2012).

3.3 Techniques Used for Enzymatic and Chemical Bleaching

The bleaching process in pulp and paper mill achieved by either chemical bleaching or enzymatic bleaching process.

3.3.1 Enzymatic Treatments for Pulp Bleaching

The enzymatic bleaching of pulp is the most prominent and cost effective as well as the eco-friendly process in the accounting of the release of toxic chemicals in comparison to the traditional bleaching process. Nowadays, several types of commercially available enzymes are used as alone and as cocktails for the bio-bleaching process (Bajpai 2012). Different kinds of combinations are used based on paper making process in emerging as well as in India. Besides, the uses and production of enzymes from local isolated cultures and compared with commercial enzymes with

their characterized pre-bleaching process set a remarkable interest in Indian pulp mills (Dutt et al. 2009). Many enzymes such as xylanases, cellulases, amylases, lipases, and laccases are used in the pulp bio-bleaching process. Mostly used enzymes are xylanases and laccases either alone or in combination depends on the bleaching process and due to its efficiency. The study reported that before the twentieth century biobleaching agent xylanase used as commercial trademarks like Novozyme 473 and Cartazyme HS-10. It reduces the consumption of chlorine by 31% and also enhanced the brightness of pulp by 2.1–4.9 points. One another study has reported that xylanase (thermostable cellulase free) produces by *Streptomyces* sp. QG-11-3 and used it against eucalyptus kraft pulp at for 2 h at 50 °C and pH 8.5. Enzymatic treatment of pulp enhances brightness and decreases the kappa number of pulp by 20–25% (Beg et al. 2000). The study has reported that xylanases produced from *Bacillus megaterium* has potential activity on pulp making it increase 13% brightness and 8.1% reduction in kappa number, respectively. Xylanase produced from mutant *Thermomyces lanuginous* MC 134 strain showed a decrease in kappa number after the treatment of 3 h (Adhyaru et al. 2017). Laccase is also known as polyphenol oxidase(Yang et al. 2017), which has a broad substrate range such as amino phenols, methoxy phenols, mono-, di-, and polyphenols, ascorbate and aromatic amines. It has redox metalloenzymes from the Multi Copper Oxidase (MCO) (Chandra and Chowdhary 2015). The usage of laccases as bleaching agents and mode of action on pulp is highly impressive when it used in combinatory with some radical mediator species such as 1-hydroxybenzotriazole (HBT), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and Syrsyringaldehyde or many more (Falade et al. 2017). According to Bourbonnais and Paice (1990), the laccase produces from *Coriolus versicolor* has the catalytic property of non-phenolic benzyl alcohols with oxidation and converted it into its aldehydes derivatives with the help of ABTS (Singh et al. 2019). However, the application part of laccases is in the many more industries such as food and beverage processing, bioethanol production, and in organic synthesis as a green catalyst (Zerva et al. 2019) along with pulp industries. Although, the laccase produce from thermophilic fungi *Trametes versicolor* (Atalah et al. 2018) and it has a high stability, lower redox potential with high catalytic efficiency (Zhou et al. 2018). The details of enzymes and characterization for use in the bio-bleaching process are described in Table 3.1. Nowadays, cellulase is the most abundant enzyme used in the pulp mill, which is produced by the aerobic cellulolytic fungi. Cellulases, a group of enzymes that comprehends *endo*glucanases, cellobiohydrolases, and β-glucosidases (Binod et al. 2013), and its mode of action on cellulose and hemicellulose pulp fibers. It breakdowns internal and external cellulose fibers bond, increases brightness, and reduces kappa number. Therefore, the cellulase used in the paper industry is the thermophilic origin. It has the strength and durability of cellulases in the high thermal industrial processes (Wakai et al. 2019). The strategies behind using enzymes for pulp bleaching it enhances the removal of lignin from pulp with the help of Xylanase (Kumar et al. 2017). While another one is that using of laccases or ligninases in the direct delignification of pulp. However, xylanases have recognized widely as the most prominent and successful in commercialization in the global

Table 3.1 Thermophilic enzymes and their characterization for the usage of the deinking and bleaching of different pulp in paper manufacturing industries

S. No.	Types of enzymes	Organism	Type of pulp/ consistency (%)	Redox mediator (concentration mM or %)	Properties of enzymes			References
					Optimal pH	Optimal temp.	Stability	
1	Xylanase	*Nonomuraea flexuosa*	Wheat straw/10	NA	80 °C	8.0	4.5 h at 80 °C	Hakulinen et al. (2003)
		Caldicellulosiruptor owensensis	Eucalyptus kraft/10		90 °C	7.0	1.0 h at 80 °C	Liu et al. (2018b)
2	Laccase	*Aquifex aeolicus*	Eucalyptus kraft/10	ABTS (2) and HBT (1.5) Acetosyringone (1.5) Syringaldehyde (3.0)	75 °C	7.0	1.0 h at 80 °C	Fernandes et al. (2007)
		Bacillus sp. *FNT*	Pine kraft/10 Soft and hardwood pulp/10		70 °C	7.0	3.0 h at 70 °C	Sharma et al. (2019)
3	Lipase	*Pyrococcus furiosus*	Softwood kraft ulp/10	NA	80 °C	7.0	6 h at 75 °C	Alquéres et al. (2011)
		Ureibacillus thermosphaericus	Hardwood kraft pulp/3.5		80 °C	8.0	>6 h at 70 °C	Samoylova et al. (2018)
4	Cellulase	*Fusarium oxysporum*	Mixed wood/10	NA	70 °C	5.5	3.0 h at 65 °C	Olajuyigbe et al. (2016)
		Thermotoga naphthophila RKU-10	Sisal or soda pulp/ 5.0		95 °C	7.0	6.0 h at 80 °C	Akram et al. (2016)
5	Amylase	*Thermotoga petrophila*	Wheat straw/8.0	NA	98 °C	8.4	4.5 h at 80 °C	Hameed et al. (2017)
		Aspergillus sp.	Soda pulp/5.0		60 °C	8.4	1.0 h at 55 °C	He et al. (2017)

market. At the same time, laccases are still under investigation for their efficiency and bleaching property (Verenich et al. 2008). Using cloning expression technique, for the first time the thermotolerant beta enzyme was produced from *Thermotoga naphthophila* RKU-10. Therefore, β-glucosidase gene encoding protein (BglA) was cloned with *Thermotoga naphthophila* RKU-10 and overexpressed in *E. coli* BL21 codon Plus. Thus, this enzyme acted upon at 95 °C and pH 7.0 optimized condition. Cellulases are used in the bio-bleaching process, which enhances pulp brightness and reduces the utilization of alkali compounds in the bleaching process. Another study reported that cellulase is used in combination with xylanase for the pulp bio-bleaching, which shows significant bio-bleaching activity (Akram et al. 2016).

3.3.2 Bleaching/Delignification of Pulp with Chemicals

In pulp and paper industries, delignification of the pulp can be done by using the conventional bleaching agents such as chlorine and chlorine-based reagents for removal of lignin and improving the brightness of pulp. However, the researchers have reported that the usage of these compounds in excessive and releases of toxic bleach effluents are causing environmental issues (Andreola et al. 2011). Thus, to overcome all these problems, most pulp industries have been used chlorine dioxide (ClO_2) as the elemental chlorine-free (ECF) compound for the generation of excellent quality of the paper. A few studies reported that the chlorine dioxide is used at the last stage of bleaching, which mitigates the impact of calcium hypochlorite and bleach effluent load in the environment (Bucher et al. 2009). Most of the study revealed that the sequencing of beaching processes like CEPHH and CEPHD had selected, where is C-chlorine, EP-extraction using hydrogen peroxide, H-calcium hypochlorite, and D is the chlorine dioxide. The generation of organic agents and interacted with ClO_2, results in the production of organochlorine compounds (measured as adsorbable organic halogens, AOX), and released as toxic pollutants in the aquatic ecosystems (Saini et al. 2020).

Moreover, to mitigate the generation of hazardous materials, the pulp mill with some modifications has employed an alternative method (i.e., cooking or oxygen, zonation, and hydrogen peroxide). Another study has demonstrated that oxygen delignification (O stage) prior to bleaching can done to the reduction of wastewater load in the aquatic ecosystem (Bajpai 2012). For this mechanism, the pulp treated with oxygen at 5 kg/cm^2 pressure in air heated digester. Some researchers have reported that the pulp after the o stage bleaching was again bleached with the following optimized steps such as OC_DED, OC_DE_PD. In this sequences, 50–50% of chlorine dioxide and elemental chlorine are used (Sharma et al. 2019). In addition, soda is the most important component for the bleaching of pulp, which reacts with hydrogen peroxide and forms perhydroxyl (HCOO–) anion to get better results of hydrogen peroxide (Liang and Wang 2015). It is more competent in the presence of heavy metals, i.e., copper, manganese, and iron, as well as catalase enzyme and high pH and temperature. A recent study reported that the decomposition of peroxide in the bleaching process shows harmful effect on pulp fibers and brightness reduction

of pulp (Liang et al. 2019). Hence, chelating agents such as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) react with the metal ions to help in the decomposition of hydrogen peroxide (Wei et al. 2018). According to International Standard Organization (ISO) sodium hydrosulfite (also known as dithionite, a strong reducing agent) has preferred at Y stage bleaching over the hydrogen peroxide to increases brightness (70%) and reduction of effluent load (Kaur et al. 2019). There are different types of chemicals used at different bleaching stages, which are described in the given table (Table 3.2). In the paper mill, the bleaching of pulp by the following consecutive steps such as C stage or chlorine stage, CD Stage, E Stage, H Stage, D Stage, P Stage. Chlorine is used as the bleaching agent in the c stage, which increases pulp brightness (Carre and Wennerstrom 2005). CD stage is the part of modified c stage, which uses chlorine dioxide instead of chlorine and also reduces the formation of free chlorine radicals in the bleaching process (García et al. 2010). In the E stage, lignin is extracted from pulp during the bleaching process, which has toxic effluents (Hostachy and Serfass 2010). The diagrammatic representation of chemically and enzymatic bleaching of pulp is shown in Fig. 3.1. The first study of ClO was carried out by Schmidt in 1921 and its commercialization was in the mid-1940s as a bleaching agent. In the H and P phases of bleaching process, hypochlorite solution and hydrogen peroxide are used for better brightness and reduction in pulp kappa number (Chirat et al. 2005).

Due to the utilization of bleaching chemicals in intensive in nature and generation of hazardous effluents, most of the paper mills used modern technology such as a totally chlorine-Free (TCF) process with few modifications in their operational mode (Fukushima et al. 2002). Oxygen-based compounds such as dimethyldioxirane (DMD), peroxy acids, ozone, and hydrogen peroxide are used in the Total Chlorine Free (TCF) process (Sirviö and Visanko 2017). TCF process is the most prominent and eco-friendly bleaching process, which mitigates the biological and chemical oxygen demand in the water body (Afsahi et al. 2019).

3.4 Future Perspective and Challenges

Based on paper demand, paper mill is the most auspicious industry in worldwide. There are lots of toxic effluents produced from the pulping, deinking, and bleaching process of wastepapers (Bajpai 2012). However, the mitigation of effluents from the bleaching process, especially chemical bleaching has to be noteworthy. There are still challenges to reduce the consumption of hazardous chemicals for the bleaching process (Adhyaru et al. 2017). Nowadays, the significance of enzymatic bio-bleaching process is much better than the traditional bleaching process in terms of release of hazardous materials. There are lots of commercial bio enzymes available in the market and can be used by many paper making industries to resolve these issues. Xylanases enzyme used for the enzymatic bleaching either in alone or in combinatory with laccases and showed greater effect on pulp other than traditional process (Wakai et al. 2019). The hazardous effluents released from traditional bleaching have an immoral impact on the environment as well as human health.

Table 3.2 Different chemicals used with their characterization for the pulp bleaching in the paper mill

S. No.	Stages/ symbol	Types of bleached chemicals	Pulp consistency (%)	Optimal temp. and duration	Mode of action	References
1	C stages	Elemental Chlorine (Cl_2), calcium hypochlorite and hypochlorous acid (HCIO)	3–4%	At ambient temp. for 0.3–1.0 h	oxidation of lignin moieties	Andreola et al. (2011)
2	CD stage	Modified part of C Stage, Elemental Chlorine Free (ECF) and chlorine dioxide (ClO_2)	–	–	scavenger of chlorine radicals and mitigated to chlorination	Bucher et al. (2009)
3	H stage	Hypochlorite solution usually as the sodium salt NaClO	4–18%	35–45 °C for 1–1.5 h	Formation of HCIO, oxidant of carbohydrates	García et al. (2010)
4	D stage	chlorine dioxide (ClO_2) with caustic (NaOH) solution	10–12%	60–80 °C for 2 h	ClO_2 is formed, decreases the accumulation of gas	Kaur et al. (2019)
5	P stage	Hydrogen peroxide	10%	60–70 °C for 2–4 h	Act as oxidant on carbonyl groups of carbohydrates	Wei et al. (2018)
6	O stage	NaOH in the presence of oxygen pressure (550–700 kPa or 80–100 psi)	3–4%	90–13 °C for 0.2–1 h	Act as oxygenation on lignin derivatives	Chirat et al. (2005)
7	Modified bleaching process	5% dimethyldioxirane (DMD) and Ozonation of pulp treated with mild oxidative chemicals	5%	60–70 °C for 2–4 h	Act as active oxygen and Reduction of adsorbable organic halides (AOX)	Sirviö and Visanko (2017)
8	New technique	Totally chlorine-Free (TCF) used oxygen derivatives molecules such as peroxy acids and hydrogen peroxide with enhanced additives, i.e. cyanide, etc.	8–10%	70–80 °C for 1–3 h	The formation of peroxymonosulfuric acid acts as an oxidizing agent	Fukushima et al. (2002)

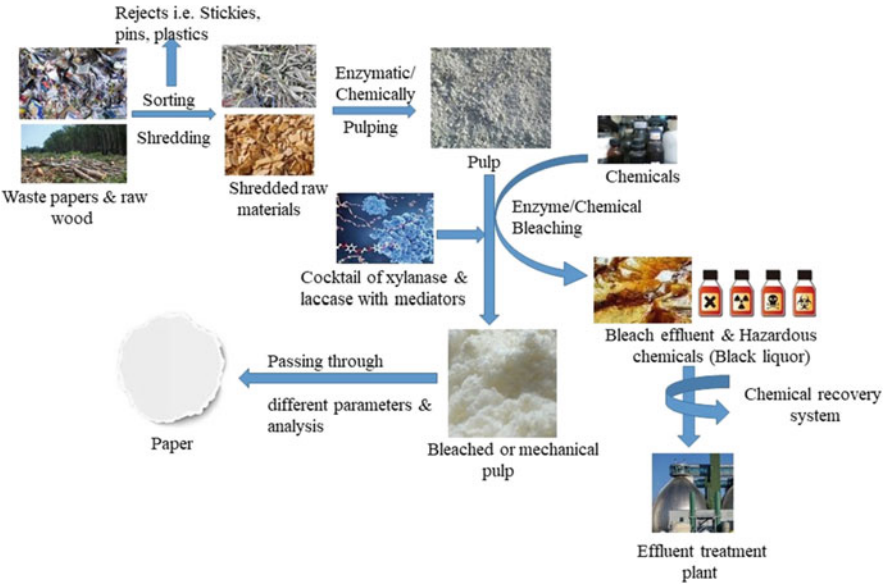

Fig. 3.1 Enzymatic and chemicals treatment (Bleaching) of pulp produces from waste papers

The usage of laccases in the presence of mediators is the most prominent and cost effective process over the traditional methods. In the enzymatic bleaching, the quality and brightness of the pulp have enhanced and reduced in the kappa number in comparison to chemical bleaching. In most of the developing countries, the bleaching techniques used in the paper mill need some modification for more and efficient results. The pulp produced from enzymatic bleaching treatment shows comparable strength and, in some cases, has maximum strength. The use of enzymatic bleaching leads to environmental sustainability. So, the commercialization of bio enzymes incorporation with pulp and paper mill is a way forward (Liu et al. 2018a).

3.5 Conclusion

The mitigation of chemicals used in the bleaching process can be achieved by using enzymatic bio-bleaching techniques. The generation of hazardous effluents has been reduced by the use of modern and cost effective techniques such as xylanase or laccases based process. However, the treated pulp with chlorine and its derivatives is most toxic to the aquatic ecosystem as well as terrestrial animals. The use of enzymatic bleaching will be most prominent and eco-friendly in comparison to chemical bleaching of pulp. The quality of pulp will increase higher with enzymatic treatment.

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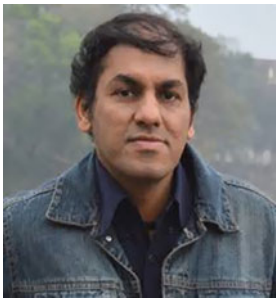

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Commercial Bioinoculant Development: Techniques and Challenges

4

Twinkle Chaudhary and Pratyoosh Shukla

Abstract

Bioinoculants or biofertilizers are well known for the enhancement and improvement in agricultural practices. Due to its eco-friendly nature, microbial inoculants are utilized on a large scale. A wide range of microbial flora supports the relationship between soil and plant by several mechanisms. Therefore, various bioformulation are formed by mixing microbes with their suitable carrier and applied in fields. Nowadays, along with solid and liquid formulation, a well developed encapsulation technology is used with a controlled microbial discharge into the soil and longer shelf life. This chapter demonstrates the various techniques of bioinoculant development like liquid, polymeric, encapsulation with their outcomes and also focuses on how this bioinoculant formulation can be a tool for the enhancement of plant yield and growth.

Keywords

Bioinoculants · Soil microbial communities · Carrier · Polymeric substance · Bioencapsulation

4.1 Introduction

Bioinoculants or microbial inoculants are the soil amendment that helps in the promotion of plant growth and health. Endophytic or rhizospheric bacteria are used as bioinoculants that show symbiotic relationship with the host plant by various methods (Malusa et al. 2016). Bioinoculant involves both plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF). Those

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bioinoculants that act as PGPR affect plant health by reducing and lessen plant diseases. A large number of bioinoculants like *Pseudomonas*, *Bacillus*, *Azotobacter*, *Bradyrhizobium*, *Azospirillum,* etc. are broadly used as biofertilizers (Mahanty et al. 2017). They are eco-friendly and provide nutritional requirements by direct or indirect methods to the host plants. By the indirect method, bioinoculant inhibits the growth of pathogenic microbes. It includes induced systemic resistance, siderophore production, hydrogen cyanide production, and cell wall degrading enzymes. Direct method helps in the direct promotion of plant growth (Imam et al. 2017; Olanrewaju et al. 2017). It includes nitrogen fixation, production of cytokinin, ACC Deaminase, auxin, gibberellin, and phosphorous solubilization. The most widely used microbe as bioinoculant are the diazotropic bacteria, i.e. rhizobia and *Azospirillum* for the growth promotion. They help in the induction of ISR (intrinsic systemic resistance) mechanism along with the production of Phytohormones (Chaudhary and Shukla 2019a, b). Depending on their utilization, bioinoculants are divided into different categories. Phytostimulators is that type of growth promoting bacteria that helps in the root elongation by the production of auxin (Bharti et al. 2017). Among all bioinoculants, the rhizobial inoculants are used as worldwide for commercial purpose. Along with rhizobial inoculant, some other beneficial bioinoculants such as AMF (arbuscular mycorrhizal fungi), PGPR (Plant growth-promoting rhizobacteria), (PGPF) Plant growth-promoting fungi and nitrogen fixers are applied on plant surfaces and seeds (Mader et al. 2011; Navarro et al. 2019). They promote the plant growth by providing the nutrients to the host plant and colonization process. This inoculation process is used where native population of microbes is low or no native microbe is found (Ali et al. 2019). Other important aspect of using microbial formulation is to diminish the effect of harmful chemical fertilizers. Some low quality of bioinoculants are produced by various techniques of formulation (de Bashan 2012). To overcome this problem, some new techniques of formulation are used with an efficient shelf life of microbes (Chaudhary et al. 2020). The primary aim of bioformulation is to find out the best microbial consortium that positively affects the growth of plants. There are various techniques of formulation by which beneficial microbes are used as inoculant. In formulation methods, bacterial strains are mixed with uniform mixture of the carrier that is used in the field (Vassilev et al. 2020). The quality of bioinoculants depends on the effective and viable cell count that is used for commercial purpose. Apart from this, there are various features of a good bioinoculant development are described as:

1. Eco-friendly: Inoculant should be biodegradable, non-polluting, non-toxic and without any carbon particle. This type of application can reduce environmental risk.
2. Storage and shelf life: The shelf life of a bioinoculant should be sufficient for two or more years for better flourishing integration into the agricultural system.
3. Physical and chemical nature: The carrier of an inoculant should be easily sterilized with high water holding capacity.
4. Handling and manufacturing process: It should be easily handled and manufactured for the controlled discharge of microbe in the field. The raw

materials of a bioinoculants formulation must have simply changeable pH during the addition of nutrients (Raghunandan et al. 2019).

Apparently, a bioinoculant cannot have all this type of characteristics for a better approach. A bioinoculant should have a realistic quality at a reasonable cost. The application of better inoculants in various fields like nanotechnology and pharmaceuticals is more expensive due to polymer use (Bhau et al. 2016). Due to non-affordable cost, very few bioinoculants and their carrier are formed for environmental and agricultural purposes.

4.2 Techniques of Bioinoculants Development

4.2.1 Liquid Bioinoculant Development Technique

In this technique, microbial count cells are mixed with some liquid substances that act as surfactant and improve the adhesiveness and stabilization ability of strain (Zayed 2016). It also provides an adequate amount of inducers, nutrients, and protectants of cells that help in the improvement of cyst and spore formation. One another advantage of the liquid formulation is the longer shelf life of microbial cells in adverse environmental conditions without any contamination (Suman et al. 2016). Apart from this, there are some disadvantages: (1) low temperature is required for their storage process, (2) high cost that is not affordable in developing countries, (3) in several cases, there is a limited shelf life of microbial cells. In this technique, broth suspensions and liquid cultures are used in minerals, water and organic substances. Before the sowing process, seeds are mixed with bioinoculants with the spraying process. After the spraying process, seeds are placed for drying and sown into the fields. This type of product is commercial in many countries like Canada, Brazil, and USA. A very common and universal preservative is sucrose that helps in the survival rate of PSB (phosphate-solubilizing bacteria), rhizobia and *Pantoea* species. In this technique, glycerol also acts as a preservative for the long-lasting process of storage in *Rhizobium* and *Pseudomonas fluorescens*. Glycerol is also used as an amendment because it helps in the protection of cells from dehydration (Dai 2018). Other additives like PVP (polyvinylpyrrolidone) and CMC (carboxymethyl cellulose) are used that are readily available and polymeric in nature. It is used in very minute concentration, i.e., (1/6; w/v). Some biocontrol agent like *Pantoea aeruginosa* is also used in liquid medium that are enriched with 0.1% CMC and sprayed on the ryegrass for the repression of gray leaf patches. In the same way, many bioinoculants like *Acinetobacter calcoaceticus*, *Brevibacillus brevis,* and Micrococcus sp. are used for the growth of *Jatropha curcas* in adverse condition (Jha and Saraf 2012). For the improvement of adhesiveness in rhizobial strain, gum Arabic is also inoculated with metal tolerant PGPR like *Sinorhizobium* and *Bradyrhizobium* sp. Along with gum Arabic, PVP is also used for the survival of rhizobial species (Sehrawat et al. 2017). It protects from desiccation and many inhibitors that are harmful to rhizobial species. In short, after knowing the advantage

and disadvantages of liquid bioinoculant, this type of formulation fits for commercial purposes in the industrial area (Bashan et al. 2014).

4.2.2 Inoculants Development by Using Solid Carrier

Bioinoculants development by using solid carriers is the most prominent and oldest method of formulation. In solid carrier, both types of inoculants, i.e., organic and inorganic carrier are used. In solid carrier mainly peat is used as the chief component of inoculants that act as organic carrier. Peat is applicable for all type of growth-promoting bacteria but infrequently available in some countries. Among all promoting bacteria, rhizobial species fits better with peat carrier. Mainly, Australia, Europe, and North America used peat as the main component and sold on a large scale. The main factors that are kept in mind during its formulation are quality of inoculants, pH, grain size, and humidity (Lobo et al. 2019). The physical condition of peat (slurry or powder) makes a variation in the inoculation process. In *Pisum sativum*, when peat is inoculated with *Rhizobium leguminosarum* in granular form, the seed production was too high as compared to liquid formulation at moderate temperature (Dubey et al. 2019). Among all the physical form of peat, granular form is the best one followed by peat powder and liquid formulation (Clayton et al. 2004). But an exception was reported in Australia fields where slurry inoculants show better results than granular form (Denton et al. 2009). A large number of ingredients are added with the microbes to improve the formulations of granular peat. The chief ingredients are vermiculite, *Agaricus bisporus* and chitin that are used for the enhancement of microbial activity. Some fungus that is used as amendment is also work as bio control against various pathogens. Sometimes the combination of *Aspergillus niger* and *Fusarium udum* for groundnut and pigeon pea crop, respectively, show high biomass of plant growth with positive biological control (Amer and Utkhede 2000; Sangma 2020). The *Trichoderma virens* cultures were added to silicate act as a supplement with peat and used for the reduction of *Meloidogyne* (nematode). Other than peat, some other carriers are charcoal, bagasses, vermiculite, and lignite are also used (Sahai et al. 2019). In brief, some organic carriers are evenly well or superior to peat for field practices. But these carriers except peat cannot form a base for large scale use in industry. Other than organic inoculants, inorganic inoculants prepared from synthetic and natural inorganic polymers. In comparison between polymeric and inorganic inoculants, polymeric preparation is the latest one method of bioformulation preparation. But both types of these inoculants are used as a small scale for agricultural practices and known for the experimental use in laboratories.

4.2.3 Polymeric Bioinoculant Formulations

In polymeric formulation, mainly synthetic carriers are used that provide a generous advantage than coal and peat carriers. It comprises high cell density, more shelf life, and a sufficient survival rate that provides better performance in the field (Bartakke

2018). In this formulation, chitosan, agar, pectin, bean gum, and carrageenan are used as polymers. These types of polymers are eco-friendly in nature and used for agricultural purposes. Some basic necessities of these polymers are: (1) without any harmful preservatives that can affect both inoculated plants and bacterial cells, (2) gradually degradable by the microbial community in the soil during seedling germination and its emergence, (3) gives protection and act as defenders against stress conditions and soil competitors (Dangi et al. 2018; Bartakke 2018), (4) should contain adequate supply of water for the better survival of bacteria (Covarrubias et al. 2012), (5) dissolvable in water for the ease bacterial movement from polymer carrier to plants. An illustration of different formulation types by various bioinoculants is described in Table 4.1. Other than this, some more beneficial features of a potent bioinoculant are: (1) it can be dried up and store at normal temperature for longer time period, (2) it should be able to change according to the requirements of microbial species, (3) provide a reliable and quality of batch environment to microbes, (4) able to improve all nutritional requirement for the better survival rate of microbes. These features help in the critical associative competition between the native microbial species and plant growth-promoting bacteria. Apart from the beneficial effects, there are some drawbacks of using polymeric bioinoculants like their handing procedure and expensive cost. Due to non-affordable cost, polymeric formulations are less used and not presently accessible at commercial scale as compare to solid formulation (Costales et al. 2019). But the positive feature of this formulation is that they are available in the research laboratories for the scientific literature.

4.2.4 Encapsulation Technologies

In the encapsulation technique, microbial cells are covered by a protective and delicate capsule or shell. This process is of two types, viz. micro and macroencapsulation. In microencapsulation, cells are enveloped by another substance on a small scale, i.e., less than hundred microns. The materials inside capsule released either by an external force or slowly by the diffusion process (Madhumita and Prabhakar 2019). In macroencapsulation process, cells are entrapped in large sized polymeric substances such as resins or plastics. The main side effect of macro encapsulation is the less uniformity of bioinoculants when mixed to the seeds. Hence, the loss of viable cells in microencapsulation is less than the macroencapsulation. In this technology, microbeads are formed and coated with the hydrogels capsule. The texture and shape of beads vary according to the microbial cells and coated substances (Kabir et al. 2018). The shape may be irregular, globular or oval with one or more microbial cells. In multilayered microbeads, cells are more long-lasting as compared to single layered cells. In the single layered process, microbial cells are more uniformly mixed with the coated materials. Figure 4.1 showing a schematic description of different types of formulations with their carriers. Generally, the materials for encapsulation are synthetic and polysaccharides polymers. These polymers may be homo or

Table 4.1 An illustration of different formulation types by various bioinoculants

Microbial species	Substrate	Formulation type	Treatment	References
Klebsiella Pneumonia and *Bacillus subtilis*	Pigeon pea	Peat	*Aspergillus niger* and Chitin	Sangma (2020)
Pantoea Agglomerans, *Klebsiella Pneumonia* and *Herbaspirillum seropedicae*	Maize	Peat	Coal	Morales-Garcia et al. (2019)
Bradyrhizobium	Soybean and *Hedysarum*	Peat	Arabic gum	Vassilev et al. (2020)
Azotobacter vinelandii	Corn	Lignin	Corn straw	Chen and Sun (2007)
Pseudomonas putida	Lettuce	Wheat	CMC (carboxymethyl cellulose)	Bartakke (2018)
Bacillus megaterium and *Bradyrhizobium japonicum*	Soybean	Cork compost	CMC (carboxymethyl cellulose) and gum Arabic	Brahmaprakash et al. (2020)
Pseudomonas fluorescens	*Trifolium repense*	Sawdust	Gum Arabic	Vassilev et al. (2020)
Rhodobacter capsulatus and *Bradyrhizobium*	*Leucaena leucocephala* and Greengram	Liquid	Gum Arabic	Gamal-Eldin and Elbanna (2011)
Bacillus licheniformis, *Acinetobacter calcoaceticus*, and *Micrococcus*	*Jatropha curcas*	Liquid	Carboxymethyl cellulose	Jha and Saraf (2012)
AMF and *Pseudomonas jessenii*	Wheat and rice	Charcoal	Carboxymethyl cellulose	Bartakke (2018)
Co-inoculation of and *Thiobacillus Rhizobium*	Groundnut	Clay soil	Sulfur	Thomas and Singh (2019)
Bacillus megaterium and *Bradyrhizobium japonicum*	Soybean	Perlite	CMC	Brahmaprakash et al. (2020)
Coniochaeta Ligniaria	Tomato	Grass fibers	Gum Arabic	Sharma et al. (2019)
Pseudomonas fluorescens	Mango, tea, rice, sugarcane, mungbean	Talc	CMC	Bashan et al. 2014)

(continued)

Table 4.1 (continued)

Microbial species	Substrate	Formulation type	Treatment	References
Pantoea dispersa	*Cistus albidus*	Clay pellets	Gum Arabic	Schoebitz et al. (2014)
Azospirillum brasilense	Several desert trees	Alginate	None	Bashan et al. (2014)
Azospirillum brasilense and *Raoultella terrigena*	None	Alginate	Starch	Schoebitz et al. (2014)
Burkholderia tropica, *Herbaspirillum seropedicae* and *Gluconacetobacter diazotrophicus*	Sugarcane and Cowpea	Corn starch	MgO	Scheidt et al. (2019)
Bacillus megaterium and *Azotobacter chroococcum*	None	Vermicompost	Lignite	Soumare et al. (2019)

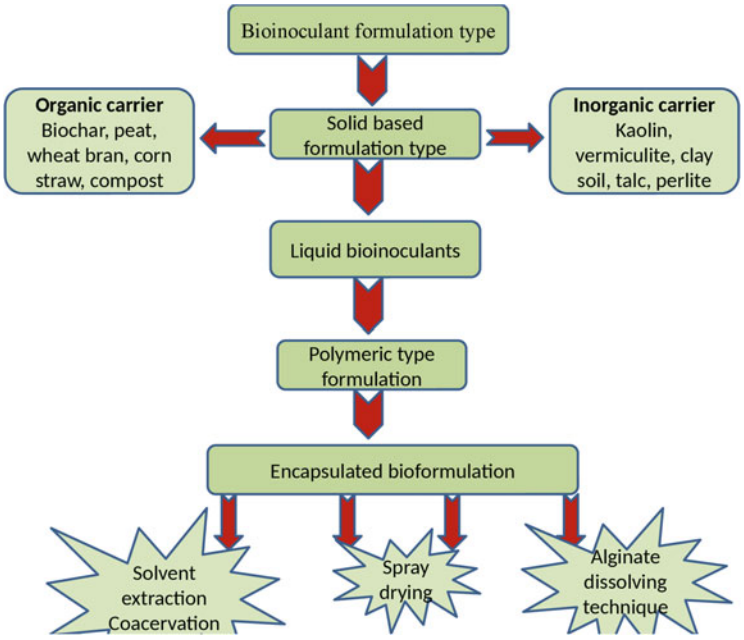

Fig. 4.1 A schematic description of different types of formulations with their carriers

co-polymers depend on their chemical composition. Several interactions like hydrophobic interactions, hydrogen bonding, and intermolecular interaction exist in these monomer units. The molecular weight and chemical composition both play an

important role in the encapsulation process. Due to high molecular weight, the application of PEG (polyethylene glycol) has become reduced for the encapsulation process (Milian et al. 2017). There are a lot of microencapsulation techniques like spray drying, coacervation, gel dissolving technique, etc.

4.2.4.1 Spray Drying

In all types of microencapsulation techniques, spray drying is a well known process because a large scale production of material is possible by this process. But due to the high mortality rate of microbes, its demand is reduced (Picot and Lacroix 2003). It is observed that high temperature and dehydration cause inactivation of microbial cells. By the process of emulsification and spray drying fine powder particles are formed in the grinding system. The particle size of cultures powder is lessening by the process of micronization for the adequate death rate of heat susceptible microbes (Picot and Lacroix 2003). It is reported that broth culture can be sprayed after dried in a polymer mixture with hot compartment (Ghosh 2006). This type of hot compartment is mostly kept close for the more viability of non-sporulating microorganisms such as rhizobium. But other sporulating bacteria and fungus can tolerate high temperature and dry conditions. Spray nozzle with high pressure can make very fine particles and also helps in lessen the time period of heat-sensitive microbes. The sizes of microencapsulated bacteria possibly will a limited range when compared with the conventional type of formulation (Borges et al. 2018). The small size of beads has much better performance than large size beads. But the major drawback of using pressure nozzle is the high rate of mortality of microbes that can harm the shell that leads to the breakdown of the capsule. The cost of these types of apparatus, pricey carriers, and power expenditure is too high. It is reported that in the microencapsulation of *P. fluorescens* by the spray drying process, none of the microbes were in live conditions at 80 °C. Although at 60 °C, the bacterial colony was found nearly 10^8 CFU/g count with a high survival rate. When the temperature increased from 65 to 80 °C the moisture content was reduced in the microcapsule at the initial time period. Therefore, it is understandable that the drought condition harm the outer protective and microbial cells. Hence, many careful precautions have to be taken for their survival. Figure 4.2 showing a well defined encapsulation technique by spray drying process. This type of experiment was reported in the encapsulation of *Beauveria brongniartii* (biocontrol fungal), when the temperature was increased from 54 °C ± 3 °C (Acheampong et al. 2019). The nourishment rate also affects the survival rate of microbial cells through the spray drying process. It was observed that the less feed rate lessens the survival rate of microbial cells. At the rate of 4–5 mL/min, no microbial cell was present at the initial time, but when the rate increase their survival rate becomes ~10^5 CFU/g. The survival rate also double when the moisture content increases during spray drying (Amiet-Charpentier 1999)

4.2.4.2 Alginate Dissolving Technique

Pregel or alginate dissolving technique was described by Sun and Lim in 1980 (Lim and Sun 1980). The alginate beads are formed by conventional method by mixing the cells of sodium alginate with calcium chloride solution (Sharma 2017). After this, the carboxyl group of the alginate beads is permitted to react with the imine

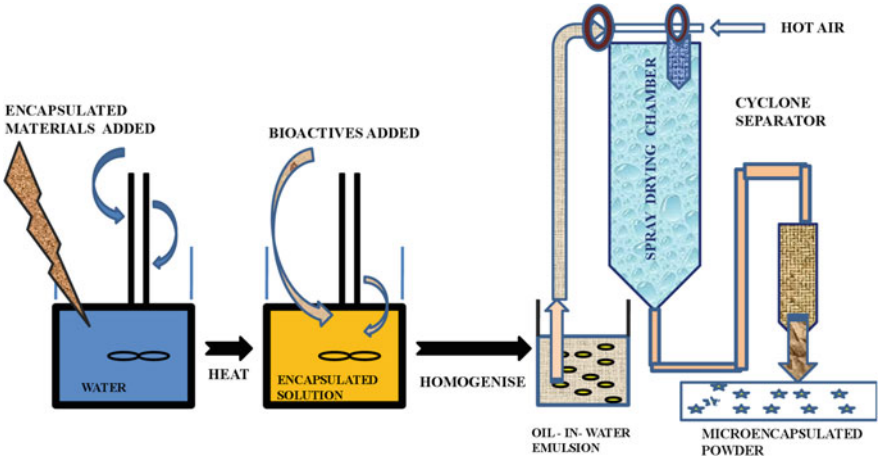

Fig. 4.2 A snapshot of encapsulation technique by spray drying process

group like polyethyleneimine. By this reaction, a multifarious membrane is made on the exterior face of calcium alginate beads and retreated with the solution of sodium alginate. This reaction helps in the crosslink of remaining polyethyleneimine on the bead surface. The liquid central portion of microbial cells is formed by mixing the capsule part of calcium alginate with the sodium alginate solution (Alehosseini et al. 2019). As a result of more stability of these beads, there is a big problem in the release of microbial cells; hence this technique is of less use in agricultural practices on a large scale. But it can be used for the industrial propose in the production of many compounds like organic acid via micro-aerophilic bacteria.

4.2.4.3 Phase Separation or Coacervation

This type of encapsulation is used for drug release, and this method depends upon the less solubility of the polymer due to the addition of different compounds with different temperature and pH (Gutierrez 2018). This method is mainly used for industrial purposes. The basis of this technique is the phase separation of polymer solution in which one is less in polymer content but other as high polymer content (Alehosseini et al. 2019). This is done by the separation of the coating polymer mixture. This technique may be of the simple type or complex depends upon the polymer used. Simple separation involves the use of one polymer by the addition of non-solvents and inorganic salts. Alternatively, complex phase separation involves the opposite charge type of rich or dense polymer. This method based on the various factors like pH value and electrostatic interactions. The polymer rich phase surrounds the liquid central part as a result of which the supernatant separates and leads to solidification of particles. This phase separation technique is not useful for the small size of microspheres due to the production of harmful residues. Therefore, by the use of supercool gases these harmful compounds can be minimized (Freitas et al. 2005).

4.2.4.4 Evaporation/Solvent Extraction

It is a simple method of microencapsulation to attain a particular particle size. There is no need of any inducer and high temperature for the phase separation. For the particular size of microparticle less amount of residual solvent is used during preparation (Freitas et al. 2005). By the less amount of residual content nano to micrometer size of particle can be attained. It is reported that in solvent extraction process there are mainly four steps: (1) Diffusion of the microbial cells with the energetic ingredients in a mixture; (2) Emulsification of this mixture with a succeeding phase; (3) Extraction of the solvent after globules formation and convert them into concrete microspheres; (4) Aeration and harvesting of microspheres particle (Freitas et al. 2005). As described earlier, high temperature is not used in this process and is bearable to the microbial cells. Other than, the active ingredients used in this process may be lethal for them and cause a high mortality rate (Ozkan et al. 2019). In most of the cases, too much concentration of the solvent dry out the cells for the long time period and influence the feasibility of microbial cells. Therefore, this type of encapsulation not is feasible technique for the biocontrol.

4.3 Conclusion and Challenges

A better perception of the relationship between the microbe-soil-plant helps in the improvement of growth-promoting bacterial potential towards field application (Imam et al. 2016). There are a lot of factors like pH, humidity, stress condition and available nutrients in soil that affect the rhizocompetency of microbes (Chaudhary and Shukla 2019a, b). But all the familiarity of microbial behavior at root zone is still not known. Therefore, the beneficial effect of bioinoculant by mix with different carriers facilitates a good approach for understanding their behavior (Santos et al. 2019). A large number of formulations like encapsulated, liquid, polymeric, inorganic and organic are formed by mixing different carriers with bioinoculants. Most of the bioinoculants are designed according to the crop season. On the other hand, there is a rising demand of bioinoculants rather than synthetic fertilizers for the agricultural practices. Though, the improvement in the technologies helps in the enhancement of growth-promoting bacterial applications. The future challenges are associated with the selection of best bioinoculant under several stress conditions (Dubey et al. 2019). Another aspect is the shelf life of culture, bulk production, and moisture content that influence the microbial selection.

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5 Protein Nanostructures with Purpose-Designed Properties in Biotechnology and Medicine

Evangelia G. Chronopoulou, Elisavet Ioannou, Fereniki Perperopoulou, and Nikolaos E. Labrou

Abstract

Protein nanotechnology aims to harness different protein architectures for developing well-defined nanostructures with expanded functional features. Protein assemblies is an emerging area in biotechnology that contributes to both basic and applied research. It can unveil the mysteries of protein folding and creates new protein structures, with higher-order complexity, chemical and thermal stability, as well as allosteric regulation. Despite the difficulty of this field—as the design is a complex approach and, in some cases, unpredictable—numerous protein nanostructures have been created with precisely defined geometries. Studies on protein nanostructures have already reinforced our knowledge regarding protein structure as well as function and have opened new pathways in medicine and biotechnology. In this chapter, different design strategies of proteinaceous nanostructures will be presented and discussed, along with their potential applications in a biotechnological and biomedical setting.

Keywords

Protein nanostructures · Protein assembling strategies · Purpose-designed scaffolds · Natural protein assemblies

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5.1 Introduction

Protein nanostructure research is inspired by natural biomolecules, such as collagen, actin, DNA clamps, Hcp1, ferritin (Fig. 5.1) (Kuan et al. 2018; Glover and Clark 2016; Pieters et al. 2016) and has attracted significant scientific interest, as it focuses on nanostructure fabrication with customized characteristics and properties (Kuan et al. 2018; Luo et al. 2016). Therefore, natural nanostructures can be considered promising scaffolds for artificial architectures. Ferritin and heat shock proteins have already been used for the construction of protein-based nanocontainers in drug delivery applications (Schoonen and van Hest 2014). Natural protein assemblies are characterized by a diverse functional repertoire (e.g., control cellular morphology, transport cargo, compartmentalization within cells) (Jiang et al. 2018; Gerrard 2013), that is related to their ultrastructural shape (fibers, sheets, hollow shells) (Pugh et al. 2018). For example, in nature shapes like cylinders are used for intracellular and intercellular transport, while protein shells are used for protection of genetic material or cell surface recognition (Pugh et al. 2018).

One of the goals of protein assembling is to endow proteins with new properties and expand their potential applications. Taking into account the link of function and shape that nature supports, engineered nanostructures focus on shapes (e.g., lines, rings, tubes, and cages) (Gerrard 2013; Gradišar and Jerala 2014) whose function can be predicted. For instance, hollow assemblies can afford encapsulation, compartmentalization, and protection from the environment; cage-like assemblies can protect and deliver a genome, while planar assemblies can be used in molecular filtration and immobilization of enzymes (Li et al. 2017; Howorka 2011). Apart from expanding protein function, protein nanomachines aim to improve the physicochemical properties and overcome limitations of localization and solubility (Jang and Champion 2016). Significant advantages have already been described regarding stability at high temperatures and organic solvents (Brodin et al. 2014; Bai et al. 2019; Yang and Song 2019). Therefore, they present great potential for being used in valuable applications ranging from drug delivery vehicles to sensing, catalysis, or signal transduction and vaccine development (Bai et al. 2019; Pugh et al. 2018; Sunita et al. 2020; Sinha et al. 2019; Dubey et al. 2018; Dangi et al. 2018). However, this chapter will focus on specific applications in biotechnology and medicine.

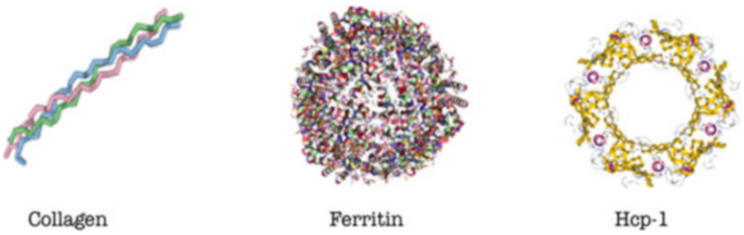


Fig. 5.1 Natures' protein nanostructures: collagen (PDB: 1BKV), ferritin (PDB: 2FHA), and Hcp-1 (PDB: 4W64). Protein images were created using the NGL viewer (Rose et al. 2018)

The design and creation of tailor-made protein nanostructures is considered a challenging research field since proteins possess vast structural diversity and multifunctionality (Kuan et al. 2018; Shen et al. 2018; Lai et al. 2012). However, several design strategies have been described for the construction of protein nanostructures—mainly using bottom-up approaches—with precisely defined symmetries, geometries ranging from 2D arrays to 3D tetrahedral, icosahedral with sizes span in the level of nanometers (Bai et al. 2019). Protein assembly is a process where an ordered supramolecular structure is generated through noncovalent or covalent intermolecular and intramolecular interactions by homo- or hetero-assemblies (Tramontano 2017; Bai et al. 2019; Yang and Song 2019; Bailey et al. 2016). Symmetry is considered one of the principles that simplifies the construction of higher-order assemblies, as few interactions in geometric arrangements are required (Bailey et al. 2016).

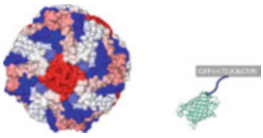
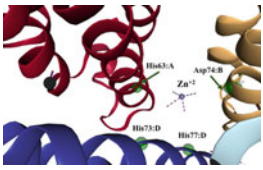
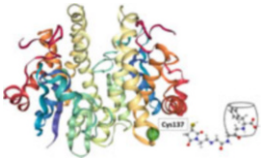
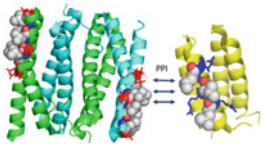
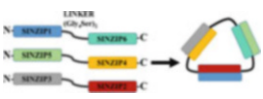
The newly constructed supramolecular architectures can be stabilized by the contribution of noncovalent weak bonds (Hosseinkhani et al. 2013) or by colloidal forces (attractive driving force, repulsive opposition force, directional/functional forces) in balance, ensuring the structural integrity and dynamic flexibility (Lee 2012). Weak interactions are essential in supramolecular architectures providing them with plasticity (Holliday and Mirkin 2001). However, these forces are complex and statistical physics models are used to solve all the possible geometrical structures (Nash 2017; Tramontano 2017; Lensink et al. 2016) and generate more sophisticated biomaterials (Lensink et al. 2016).

The main strategies for protein nanostructure design are based on symmetry design, receptor–ligand recognitions, metal coordination, electrostatic induction, host–guest interactions, precisely designed interface interactions and site-specific covalent ligation (Kuan et al. 2018; Shen et al. 2018; Luo et al. 2016; Li et al. 2017; Sun et al. 2017; Bai et al. 2019). Table 5.1 summarizes some schematic diagrams of nanostructures' formation that have been reported. Supramolecular architectures can be determined through electron microscopy, solution small-angle X-ray scattering (SAXS), ion mobility spectrometry-mass spectrometry (IMS-MS), as well as static and dynamic light scattering (Pieters et al. 2016; Bale et al. 2016; Zhang et al. 2012; Ljubetič et al. 2017).

5.2 Types and Principles of Protein Assemblies

Proteins are powerful molecules that perform a wide range of functions. This significant ability underlies their exploitation as therapeutics, sensors, and biocatalysts. Natural protein portfolio can be extended by constructing innovative proteins through protein engineering; however, the overall shape of the scaffold protein (Chevalier et al. 2017) is achieved by self-assembling. This process is considered complicated since the target is a three-dimensional structure (Kuan et al. 2018). Computational approaches can unveil this complexity (Kuhlman et al. 2003; King et al. 2012; Doyle et al. 2015), but also enable de novo design of protein nanostructures (Bale et al. 2016; Hsia et al. 2016). Databases like CATH, SCOP,

Table 5.1 Schematic diagrams of the main types of protein assembly formation: (a) The electrostatic surface of apoferritin and green fluorescent protein (GFP) with cationic supercharged polypeptides (SUPs) composed of 72 consecutive lysine containing repeating units (K72) forms protein crystals. (b) Metal-directed protein self-assembling of monomeric engineered protein cytochrome cb562 (having two bis-His clamps H59/H63 and H73/H77) and metal Cu^{+2}. (c) Host–guest interactions of sjGST and a maleimide Phe-Gly-Gly tag/CB[8] as a guest moiety. (d) Design of de novo interface interactions between sulerythrin and cysLARFH variant. (e) Coiled-coil interactions of linked-SYNZIP fusion proteins that self-assemble through thermal annealing process

Driving force of the assembly process	Schematic diagram	References
(a) Electrostatic interaction between the building blocks Characteristics: *tunable and reversible*	Apoferritin GFP (+)72 SUPs 	Korpi et al. (2018)
(b) Metal coordination Characteristics: *directional*, *reversible and stable*		Salgado et al. (2007)
(c) Host–guest interactions Characteristics: *morphological diversities (nanorings*, *nanospirals*, *nanowires to superwires)*, *reversible*, *selective*		Li et al. (2017)
(d) Interface interactions characteristics: de novo *design*		Yagi et al. (2016), Yagi et al. (2014)
(e) Coiled-coil Characteristics: *geometrically irregular structures*		Park et al. (2017), Thompson et al. (2012)

Protein images were created using the NGL viewer (Rose et al. 2018) and PyMOL (De Lano 2002)

PFAM—that share information for consensus protein domains and their function—in combination with improved algorithms are useful tools for protein nanostructure design (Lai et al. 2012; Glover and Clark 2016; Bromley et al. 2008).

There are two designing strategies for protein assembly generation based on the flexibility of the new complex structure: stochastic and deterministic. The stochastic approach follows the principles for a higher-order and geometrically irregular assembly, while the deterministic approach aims to specific structures (Lai et al. 2012). Significant advances for both approaches have been attained using chemical

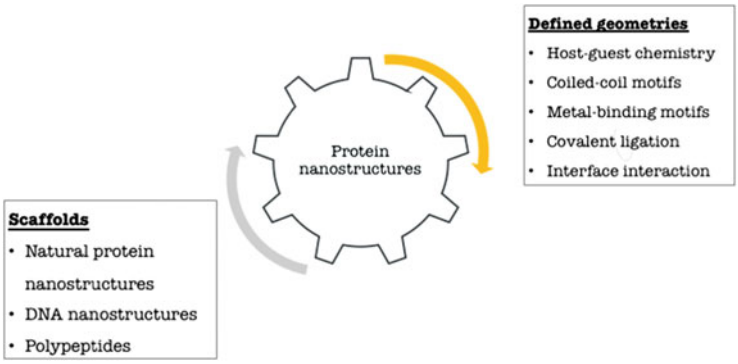


Fig. 5.2 Strategies and scaffolds for protein nanostructures

and biotechnological methods that follow different pipelines according to the type of starting material (e.g., basic units, tectons, self-assembled units) (Bromley et al. 2008; Parmeggiani and Huang 2017). In addition, protein modularity contributes to customized structural design (Parmeggiani and Huang 2017) with different ways of assemblies, like host–guest chemistry, electrostatic and hydrophobic interactions, linker engineering, metal coordination, and covalent bonding.

Host–guest chemistry is considered a powerful method based on hydrophobic interactions, using several synthetic host molecules (e.g., cucurbiturils, cyclodextrins, porphyrins, etc.) (van Dun et al. 2017). Cucurbit[8]uril (CB[8]) is considered versatile supramolecular platform, as it can bind at the N-terminal Phe residues, enabling protein dimerization and enhancing enzyme activity like in the case of caspase-9 (Dang et al. 2013) or of Ca^{+2} stimulated GSTs nanowires that are able to form extended and contracted states (Hou et al. 2013; Si et al. 2016). Bosmans and coworkers developed a method for supramolecular split enzyme complementation allowing for on–off switching of enzymatic activity, using split-luciferase fragment pairs with N-terminal FGG sequences and cucurbit[8]uril Q8 for complementation through host–guest binding (Bosmans et al. 2016). This way of assembling can also lead to reversible modification through redox conditions, as in the case of FGIG protein assemblies, that form nanowires and nanorings, under oxidation and reduction, respectively (Wang et al. 2017) (Fig. 5.2).

Polypeptides scaffolds can serve for the de novo design of polyhedral protein cages. Ljubetič et al. described how protein coiled-coil (CC) dimers can efficiently self-assemble in vitro and in vivo, creating protein cages of different shapes with comparable stability to that of natural proteins (Ljubetič et al. 2017). A rational design approach was reported by Park et al. that exploits interacting coiled coils that generate self-assembled protein nanotriangle (Park et al. 2017). In addition, hybrid nanostructures consisting of coiled-coil heterodimer peptide pairs with DNA handles that link to DNA origami have also been reported (Buchberger et al. 2020). A hybrid protein cage with protein building blocks has also been constructed using a modified homotrimeric protein (KDPG aldolase) with three identical DNA single-strands that

was co-assembled with a triangular DNA structure, resulting in tetrahedral cages (Xu et al. 2019).

Receptor–ligand strategy is based on the unique "lock-and-key" recognition that dominates in cellular biochemical pathways, characterized by diversity, reversibility, and high binding affinity. Utilization of this design strategy has been tested for the construction and modulation in vitro of 1D, 2D, or 3D protein self-assemblies (Bai et al. 2016). Carlson et al. developed highly stable nanorings with dihydrofolate reductase (DHFR) fused with peptide linkers and bivalent methotrexate (bisMTX) ligand as the self-assembling mediator (Carlson et al. 2006). Linker engineering has also been leveraged for protein nanostructures by using protein nanobuilding-blocks of the de novo dimeric structure of WA_{20} and the trimeric fold on domain of fibritin from bacteriophage T4, forming various structural homo-oligomers (Kobayashi et al. 2018, 2015).

Following extensive sequence optimization, new electrostatic and hydrophobic interactions can be generated and yield novel protein–protein interfaces for tailor-made protein architectures (Yang and Song 2019; Sasaki et al. 2017). Therefore, taking into consideration the pH value and ionic strength of the solution, tunable protein assemblies can be designed and produced (Bai et al. 2016). Sasaki et al. based on electrostatic effects managed to expand two- to threefolds the size of lumazine synthase (from 1 MDa to 3 and 6 MDa), by increasing the number of negative charges on luminal surfaces (Sasaki et al. 2017).

Moving on, considering the significant role of metals (Zn^{+2}, Mg^{+2}, Ca^{+2}) on protein structural stability and function (Bailey et al. 2016; Dudev and Lim 2003) as well as taking into account the advantages of metal-binding properties (bonding strength, reversibility, directionality, specificity) (Luo et al. 2016; Salgado et al. 2007; Tezcan 2013; Brodin et al. 2012), protein sequence modification by adding metal coordination motifs on the protein surface was found to promote protein self-assembly under thermodynamic control (Brodin et al. 2012; Kuan et al. 2018; Luo et al. 2016; Garcia-Seisdedos et al. 2017; Yagi et al. 2016; Salgado et al. 2010). Thus, introducing metal-binding sites in proteins constitutes another design strategy that endows proteins with new functions and stability (Regan et al. 2015).

Metal-directed protein self-assembly is a proven method for creating defined supramolecular architectures. The interface bonding takes place through metal coordination and cytochrome c is a useful building block (Salgado et al. 2010; Song and Tezcan 2014; Tavenor et al. 2017). Metal bonds with amino acid side chains show significantly higher binding energies than noncovalent interactions under geometric limitations (Huard et al. 2013; Salgado et al. 2009). The amino acids that most frequently interact with metals are histidine, cysteine, aspartate, and/or glutamate and are referred to as "CHED" (Kuan et al. 2018; Der et al. 2011; Dutta and Bahar 2010). Metal-binding cysteine residues exhibit higher mobility than His, Asp, and Glu (metal-binding HED residues) and notably prefer more buried positions (Dutta and Bahar 2010). Systematic analysis that has been carried out by Dutta and Bahar reveals the tendency of metal sites in proteins (Dutta and Bahar 2010). Late-first-row transition metal ions Ni^{+2}, Cu^{+2}, and Zn^{+2}, which are all reactive and form stable complexes with a bi- or tridentate metal-chelating motif

with most common metal-binding residues, provide different metal coordination geometry on supramolecular assembly (Salgado et al. 2010), ensuring the development of thermodynamically favored structures based on their rapid ligand substitution rate (Sontz et al. 2014). Hydrogen bonds and salt bridges in metal-coordination protein superstructures contribute to the stability, geometry alignment of protein partners, and protein conformation (oligomerization/aggregation) (Salgado et al. 2008). Additionally, disulfide cross-links can enhance the protein scaffold robustness (Bailey et al. 2016). Non-natural metal chelates like phenanthroline have also been used successfully, depicting significant lower metal dissociation constant than those of the *i*/*i*+4 bis-His motifs for each metal (Radford et al. 2010; Radford and Tezcan 2009). In another example, iodoacetamide or maleimide derivatives—attached to α-helix surface-exposed Cys—can develop a tridentate hybrid coordination motif with His (HCM) (Bailey et al. 2016). Moreover, metal-coordination bonding for self-assembled protein structures can be formed through unnatural chelating amino acids, e.g., bidentate bipyridine-alanine. This approach can create tunable structures (1D and 2D assemblies) by altering conditions related to thermodynamics and kinetics of metal coordination (Yang and Song 2019).

Metal ions contribute considerably to the structure and function of proteins in an environmentally dependent manner. Factors like external chelators, pH, and solution redox state can affect the coordination or reactivity of the metal ion (Sontz et al. 2014; Sendai et al. 2013). Therefore, the shape and dimensions of the protein nanostructures can be adjusted by these factors. For example, the diameter of created glutathione S-transferase (GST) nanorings was modulated by adjusting the ionic strength (Bai et al. 2013). The example of tunable arrays of RIDC3 from external stimuli is typical. In this case, fast nucleation/growth conditions (high pH or high [Zn]:[RIDC3] ratio) promoted the formation of helical nanotubes, while under slow nucleation conditions (low pH and low [Zn]:[RIDC3]), the shape was altered in 2D and 3D planar arrays (Brodin et al. 2012). The differences in metal coordination chemistry and sequence preferences of the metal-binding motifs can be exploited for distinct designs of protein self-assembling architectures, while there are also tools available for predicting metal-binding sites, based on already known protein structures (Lin et al. 2005; Passerini et al. 2006; Ebert and Altman 2008). In addition, the stimuli-responsive change of architecture achieved by this strategy, is considered a hallmark of biological assemblies (Brodin et al. 2012).

Two approaches have been developed for the construction of metal-mediated protein assembly: the metal-directed protein self-assembly (MDPSA) and metal-templated interface redesign (MeTIR) (Sontz et al. 2014). In the former approach, the formation of protein assemblies and protein interfaces is directed by designing metal coordination sites in protein scaffolds, while in the latter, protein structure is reengineered around the metal to tune its properties and stabilize the oligomer (Tezcan 2013). MDPSA can be the platform for designing new redesigns, while MeTIR can be considered the evolutionary approach. In MDPSA strategy, multidentate metal coordination motifs are installed following specific geometric requirements: histidine (His), aspartate (Asp), glutamate (Glu), and cysteine (Cys) residues can be engineered in α-helices at i and $i + 4$ or i and $i + 3$ sites (Bailey et al.

2016) and in surface-facing β-strands and reverse β-turns through *i* and *i* + 2 pairings, respectively (Arnold and Haymore 1991; Harding 2004). Late-first-row transition metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) are considered appropriate, because of strong interactions with amino acid residues and fast ligand exchange rate (Bailey et al. 2016). Moreover, these metal ions exhibit different preferences in coordination geometry (Ni^{2+}, octahedral; Cu^{2+}, tetragonal/square planar; Zn^{2+}, tetrahedral) (Rulíšek and Vondrášek 1998). The coordination of Mg^{2+} and Ca^{2+} is limited to carboxylate and hydroxyl groups. Second- and third-row transition metal ions can bind tightly with protein surfaces, but exhibit very slow ligand exchange rates at room temperature (Bailey et al. 2016; Bertini et al. 2007; Lippard and Berg 1994) (Fig. 5.3).

A revision of MeTIR approach (Brodin et al. 2010; Der et al. 2011), was also developed. This modification, termed reverse metal-templated interface redesign (rMeTIR), is based on the direct chemical switch of protein–protein interaction, exploring the metal ions driving force, which are considered as chemical inducers (Huard et al. 2013). The starting material in this case is a self-assembling protein (such as ferritin—an iron storage protein), which is engineered in order to contribute metal-binding sites for obtaining the appropriate rigid self-assembling. In rMeTIR strategy, metal is essential for the assembling, since complementary interactions are eliminated (Huard et al. 2013) (Fig. 5.3).

Using the MeTIR strategy, Churchfield et al. designed an allosteric supramolecular protein assembly ($Zn^{-C38/C81/C96R14}$), in which the dissociation and binding of Zn^{2+} ions are coupled over a distance of15 Å with a hydrolytic breakage (without reductant) and a disulfide bond (Churchfield et al. 2016; Lewis et al. 2018). Finally, allosteric protein complexes, in which the assembly–disassembly process is controlled externally through an engineered fold switch—mimicking the metamorphic protein—are promising for biomedical applications. Protein engineering of chymotrypsin inhibitor 2 (CL2) enables a switch between the native and an alternate fold. The alternative fold allows the self-assembling process that leads to on-demand hexagonal toroidal particles via the competing effects of temperature and a designed short peptide (Campos et al. 2019). Such assemblies can underpin new drug delivery systems (Campos et al. 2019).

Significant results have also been obtained by covalently ligated multi-subunit nanostructures. Split intein chemistry (SI) allows the assembly of proteins by forming peptide bonds between compatible SIs fused to the termini of two protein building blocks. Following this strategy, hyperstable 2D nanostructures of three-helix bundle protein (3HB) were constructed shaping triangles and squares. These assembled protein nanostructures showed exceptional thermal stability (Bai et al. 2019). Furthermore, other biochemical tools like SpyTag/SpyCatcher that are based on isopeptide bonds formed on C-terminus or N-terminus can be harnessed for higher-order and unfamiliar protein assemblies (Reddington and Howarth 2015; Zakeri et al. 2012). This strategy is characterized by strong interactions that enable new approaches for basic research and biotechnology (Liu et al. 2014; Schoene et al. 2014; Veggiani et al. 2015).

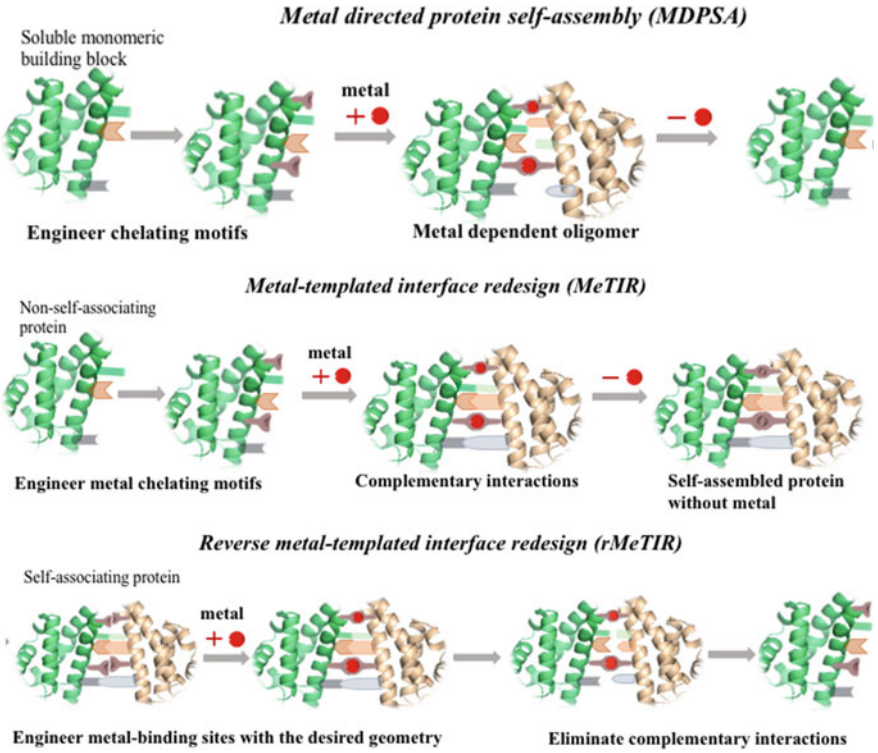


Fig. 5.3 Comparative schemes for MDPSA, MeTIR, and rMeTIR. Red spheres show metal ions. MDPSA strategy follows the incorporation of chelating motifs (e.g., bis-His clamps) into the protein surface, resulting in the development of metal-directed oligomers. MeTIR strategy reinforces metal-mediated oligomers with the installation of complementary interactions, in order to form self-assembling complexes even in the absence of templating metals. rMeTIR strategy follows the incorporation of metal-binding motifs and self-assembled protein upon metal-binding and complementary interactions

5.3 Protein Nanostructures in Biotechnology and Medicine

The purpose of the development of higher-order protein architectures is to: (1) construct biomaterials showing specific catalytic activities and functions, (2) endow these biomaterials with tailored physical and chemical characteristics in order to meet the requirements for applications in drug delivery, tissue engineering, biocatalysis, and biosensing, (3) reveal the mysteries of protein–protein interactions. Molecular biology strategies help to fulfill this purpose, since they allow the sufficient production of recombinant protein scaffolds for higher-order protein architectures with specific characteristics (Luo et al. 2016; Regan et al. 2015; Loo et al. 2015).

5.3.1 Biotechnology

Through exploring the inherent ability of biological molecules to self-assemble into nanostructures of defined shapes and sizes, along with following engineering strategies, new biomaterials can be produced for a plethora of biotechnological applications (Cluskey et al. 2020). For instance, protein self-assemblies can be developed as immobilization matrices or nanocontainers of enzymes (Howorka 2011) and virus-like protein self-assemblies are being used as nanoscaffolds for enzyme selection, enzyme patterning, phage therapy, raw material processing, and single molecule enzyme kinetics studies (Gerrard 2013). New biocatalysts can also be engineered by adding catalytic moieties onto protein surfaces in order to endow proteins with new functions and high catalytic activities (Luo et al. 2016), like the nanostructures of tobacco mosaic virus (TMV) coat protein with a glutathione peroxidase (GPx)-like active site that exhibited high GPx activities (Hou et al. 2012).

In the aquaculture, a novel vaccine strategy against nervous necrosis virus (NNV) infecting soles of Senegal has been developed by Thwaite et al. (2020). The designed nanostructure (VNNV-C^{NP}) was based on the coat virus protein and was able to enhance immunization. Moreover, nanostructured inclusion bodies of TNFα protein were applied to Zebrafish resulting to a boost in their immune responses, showing potential as a mucosal adjuvant (Ji et al. 2019).

Nanostructures that are based on plant proteins, such as whey globular or soy proteins, have been previously described in food industry applications (Ramos et al. 2019; Tang 2019). In these cases, protein nanostructures are formed by factors that promote aggregation, such as heat or enzymatic treatments and pH changes. They can potentially be utilized in nanohydrogel or nanoemulsion forms for encapsulation and more effective delivery of bioactive compounds during food consumption (Ramos et al. 2019; Abaee et al. 2017; Mokhtari et al. 2017).

Hybrid nanostructures (proteins and nucleic acids) are another rapidly developing field which encompasses the advantages of cost-effectiveness (intracellularly self-assembly and cell-free transcription and translation), structural predictability, and functional potential (Cluskey et al. 2020). The precise position of proteins on nucleic acid nanostructures leads to materials endowed with interesting capabilities such as switchable enzyme activity (Cluskey et al. 2020), like in the case of compartmentalization of glucose oxidase and horseradish peroxidase in DNA nanotubes. This approach created a nanoreactor of controllable length with ordered enzyme reactions (Linko et al. 2015). Multienzyme cascades have also been designed with DNA nanostructure as a scaffold, allowing the arrangement of enzymes at nanoscale (Fu et al. 2016, 2012).

5.3.2 Biomedicine

Protein assemblies' biophysical and biochemical properties render them a promising tool with various biotechnological and biomedical applications such as encapsulation, targeted drug delivery and therapy, tissue engineering and design of synthetic

vaccines (Lai et al. 2012; Luo et al. 2016; Jang and Champion 2016; Howorka 2011; Regan et al. 2015).

As it has already been mentioned, the research based on protein nanostructures can offer a deeper understanding of protein–protein interaction (PPIs) and study of the mechanism along with the factors that affect self-assembling in pathological conditions, like Alzheimer's and Parkinson's disease (Hosseinkhani et al. 2013). Protein nanostructures play a significant role in basic research since they can unveil key factors of protein folding, but they can also contribute in applied research. Recent published studies focus on protein cages, which are characterized by stability and structural integrity (Zhang and Ardejani 2015). Protein cages have already attracted scientific attention in pharmaceutical setting, as drug delivery vehicles (Pieters et al. 2016), nanoreactors, or artificial organelles (Sasaki et al. 2017; Kim and Tullman-Ercek 2013). Regarding their application as drug delivery platforms, they are considered highly promising, since protein nanocages exhibit perfectly defined structures, biocompatibility, minimized premature drug degradation, enhanced permeability and low toxicity (Schoonen and van Hest 2014; Bhaskar and Lim 2017; Lee et al. 2016). In addition, an important aspect of protein nanocages that can be exploited is their design flexibility in interior, exterior and intersubunit surfaces for loading therapeutic and diagnostic molecules, target specificity, and finally tuning molecular release (Bhaskar and Lim 2017; Lee et al. 2016). Ferritin is an adequate candidate in drug delivery and has already been used for the encapsulation of several molecules, such as cisplatin and doxorubicin (Yang et al. 2007; Blazkova et al. 2013). Other natural cages like heat shock proteins, vault, virus-like nanocages have been studied as drug carriers (Lee et al. 2016; Bhaskar and Lim 2017). Virus-like icosahedral protein assemblies have also been fabricated intracellularly in *E. coli,* for the encapsulation of RNA genomes that can be effective in drug delivery and vaccine applications (Butterfield et al. 2017).

Another significant perspective in vaccine design is the construction of nanoparticles from self-assembled proteins with suitable size range (diameters of 10–150 nm), for optimal interactions with various cells of the immune system. Specifically, self-assembled protein scaffolds of immunogen can mimic the repetitive surface architecture of a microbe (López-Sagaseta et al. 2016). Therefore, self-assembling proteins show significant potential for the development of innovative vaccines and an effective way to overcome limitations such as lower level of protective immunity and mutagenic effects within the host organism (López-Sagaseta et al. 2016). Kanekiyo et al. (2013) have successfully designed viral hemagglutinin nanoparticles through genetic fusion to ferritin, a protein that naturally forms spherical nanoparticles of 24 identical polypeptides. These generated self-assembling hemagglutinin nanoparticles, exhibited enhanced and broader immunogenicity against influenza (Kanekiyo et al. 2013).

Protein nanostructure principles have also guided the generation of new architectures of protein drugs with modified pharmacokinetic profile. For instance, novel quaternary insulin structure was formed through the artificial ligand 2,2-′-bipyridine and metal-directed self-assembly. Additionally, the use of Fe^{2+} in coordination with the engineered insulin resulted in a homotrimer that can be

followed visually (Munch et al. 2011). Phillips et al. have also demonstrated enhanced pharmacological properties of insulin through rigid protein assembly formation by paired (*i*,*i*+4) His substitutions at an helical surface and novel zinc ions at hexamer–hexamer interfaces (Phillips et al. 2010).

De novo protein assemblies are a challenging process; however, the use of peptides, that can be easily obtained by de novo design, can be efficiently employed due to their structural simplicity, ease of modification and resistance to non-native conditions (Lee et al. 2016). For this purpose, coiled-coil motifs have been selected as scaffolds for the construction of self-assembling structures (Lee et al. 2016). For instance, coiled-coil motifs were used for the generation of self-assembling nanofibers that mimic collagen (Luo and Tong 2011). Three-dimensional architecture of collagen networks also has been studied, through the radial growth of a collagen mimetic peptide H-byp and Fe^{2+}-assisted self-assembling of fibers, with promising potential in tissue regeneration (Przybyla and Chmielewski 2008). Stimuli-responsive biomaterials like hydrogels, biofilms, and fibrils with specific characteristics can also be constructed through self-assembling process (Main et al. 2013). Tunable hydrogels—dependent on sequence, pH, and ionic strength (Grove et al. 2012; Olsen et al. 2010)—can find applications in tissue engineering and drug delivery (Hosseinkhani et al. 2013; Lin et al. 2005; Schloss et al. 2016; Pandya et al. 2000; Ghosh et al. 2012). Variable muscle-mimetic biomaterials have also been developed by artificial elastomeric proteins that mimic titin and can be photochemically crosslinked (Lv et al. 2010).

5.4 Conclusion

Proteins are complex systems encompassing conformational and structural diversity. These features are being explored for developing highly ordered nanostructures with well-defined properties and structures. Over the last years, highly successful approaches for designing highly ordered protein nanostructures have been reported. These approaches allowed scientists to design suitable structural modules, with diverse functional properties, contributing to the construction of proteinaceous molecular machines with sophisticated characteristics.

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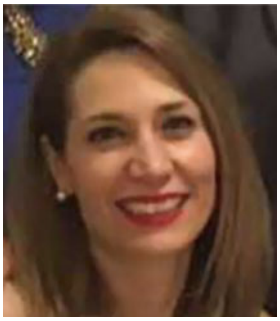

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6 Biosynthetic Pathways in Microalgae Towards Production of Biopigments: Progress and Advances

Dinesh Kumar Saini, Sunil Pabbi, and Pratyoosh Shukla

Abstract

Microalgae gained attention as eco-friendly, sustainable, and potential sources of various bioactive compounds. These have distinctive capability to synthesize and produce a wide range of compounds especially biopigments along with CO_2 fixation capabilities. Biopigments are the essential components of light harvesting complex of microalgae. These accessory pigments (carotenoids and phycobiliproteins) have their primary role in light harvesting but they also have photo-protective role during excess light energy. During recent times, the demands of these biopigments are on the rise due to their great biological potential and beneficial effect on human health. These biopigments show wide range of beneficial applications such as antioxidant, anti-cancerous, anti-inflammatory, immunomodulatory etc. These are also utilized in industries such as food, cosmetic, and pharmaceutical. In this chapter, we discuss about the prospective microalgae as a source of various biopigments and their application in various commercial areas as well as their unique properties. In addition, various challenges and bottleneck in the production of microalgal biopigments are also summarized along with the efforts to explore recent biotechnological approaches applied on microalgae in enhancing biopigments production.

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Keywords

Microalgae · Carotenoids · Phycobiliproteins · Phycocyanin · β-carotene

6.1 Introduction

Microalgae are the microscopic algae, which are present in aquatic as well as terrestrial ecosystem (Christenson and Sims 2011; Jalilian et al. 2020). Around 40,000–60,000 of total known microalgal species have been reported and still hundreds and thousands more need to be discovered around the globe (Lafarga et al. 2020). Moreover, only 1000 species are kept in collection and very few were investigated for bioactive compound and just a handful species are used for commercial purposes (Cadoret et al. 2012). These microalgae are well known for their photosynthetic ability and produce almost 50% of total atmospheric oxygen apart from the fact that only 0.2% of their biomass is available on the land (Parker et al. 2008; Suganya et al. 2016; Mata et al. 2010). They require simple biological components for their growth such as sunlight, carbon, and some inorganic nutrients (N, Mg, P, etc.) and produce various kinds of organic products (Parmar et al. 2011). These products are used as feedstock for food, feed, fuel, cosmetic and pharmaceutical (Dobson et al. 2012). Besides the fast growth rate, microalgae have several advantages like it does not require arable land for its cultivation, thereby do not compete with food crops and moreover these are adaptable to various extreme conditions which give them high survival rate (da Silva Vaz et al. 2016). Microalgae have recently gained attention for the production of products like lipids, polyunsaturated fatty acid, carbohydrates, sulfated polysaccharides, proteins, biopigments, and natural dyes which are used in several industries, viz. cosmetics, biofuel, energy, pharmaceutical and food (Samantaray et al. 2020; Priyadarshani and Rath 2012; Raja et al. 2008; Saini et al. 2020a; Banerjee et al. 2018). Additionally, some species such as *Chlorella*, *Spirulina*, *Schizochytrium*, *Dunaliella,* and *Haematococcus* have the status of GRAS (generally regarded as safe) from US Food and Drug Administration (FDA) (Chacón-Lee and González-Mariño 2010).

Microalgal pigments are extensively used in various industries, including food, nutraceutical, pharmaceutical, aquaculture, and cosmetic (Begum et al. 2016). These pigments also exhibit properties like antioxidants, anti-inflammatory and anti-carcinogenic (Gumbo and Nesamvuni 2017; Gong and Bassi 2016) as mentioned in Table 6.1. The various kinds of pigments produced by microalgae include β-carotene, phycobiliproteins, and astaxanthin which are produced at commercial level from *Dunaliella*, *Spirulina* and *Haematococcus* respectively (Rao et al. 2015; Wu et al. 2016; Sui and Vlaeminck 2020).

This chapter describes biosynthesis of microalgal biopigments and their wide range of applications of some important biopigments, such as β-carotene, astaxanthin, lutein, phycocyanin and phycoerythrin in different industries especially in pharmaceutical industries. We also describe the effects of different abiotic stress on the synthesis of biopigments in microalgae. Moreover, recent molecular

Table 6.1 Commercial value and physiological properties of some important biopigments

S. No.	Pigment	Market price[a] (analytical grade/ 10 mg) in USD[b]	Physiological properties	References
1.	Phycocyanin	US $213.57	• Antioxidant • Anti-inflammation • Anti-cancer • Anti-angiogenic activity	Bannu et al. (2019)
2.	Phycoerythrin	US $11,1497.22	• Antitumor activity • Immune-fluorescent activity	Gargouch et al. (2018)
3.	β-carotene	US $194.71	• Antioxidant activity • Enhances immune function • Source of vitamin A	Marino et al. (2020)
4.	Astaxanthin	US $3136	• Antioxidant activity • Antitumor activity • Prevention of cardiovascular diseases • Anti-diabetic activity • Anti-inflammatory activity	Zhao et al. (2019)
5.	Zeaxanthin	US $7930.14	• Prevention from age-related macular degeneration • Prevention from cataract	Cezare-Gomes et al. (2019)
6.	Lycopene	US $733.54	• Antioxidant activity • Prevention of cardiovascular diseases • Anti-cancerous activity	Viuda-Martos et al. (2014)
7.	Lutein	US $5494.14	• Antioxidant activity • Prevention of cardiovascular diseases	Fernández-Sevilla et al. (2010)

[a]Commercial prices (analytical grade) are listed as per Sigma-Aldrich price list (2018–2019)
[b]1$ = 70 INR

approaches such as genetic and metabolic engineering were also described for the enhancement of biosynthesis microalgal biopigments.

6.2 Carotenoids

Carotenoids are accessory light-harvesting pigments which absorb sunlight at various wavelengths and provide energy in photosynthetic metabolism. These pigments are present in vast variety in plant, algae, and cyanobacteria and play a significant role during stress conditions. Carotenoids are lipophilic in nature and all of them

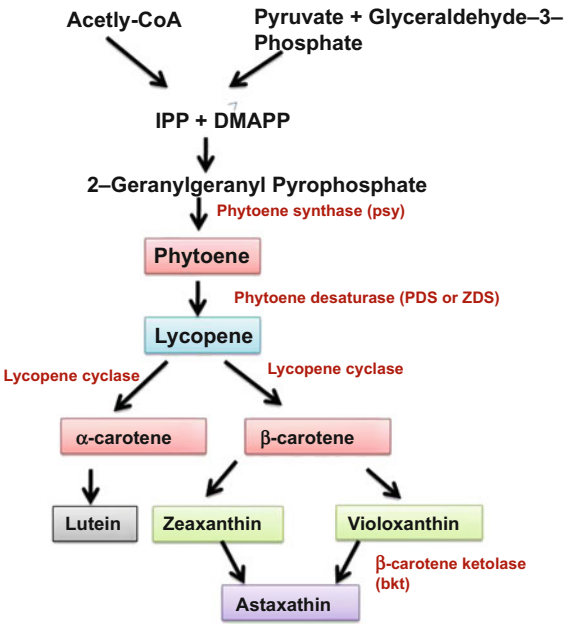

Fig. 6.1 An overview of biosynthetic pathway for carotenoids synthesis

have C_{40} carbon backbone in their structure (Saini and Keum 2018). The biosynthesis of these pigments starts with C_5 bio-molecules isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are derived from acetyl-CoA or pyruvate and glyceraldehydes 3 phosphate. It was considered that IPP and DMAPP in microalgae were derived from 2-*C*-methyl-D-eryhritol 4-phophate (MEP) pathways and known as precursors of carotenoids synthesis (Barredo 2012; Varela et al. 2015). Biosynthesis of carotenoids was started with the condensation of 2 GGPP molecules catalyzed by the enzyme; phytoene synthase (synthesized by *pys* gene) to form the foremost carotenoids called phytoene. Phytoene is colorless carotenoids and acts as precursor for all other carotenoids. The enzyme involved in phytoene synthesis has been present in bacteria, fungi, algae, higher plants as well in human plasma (Meléndez-Martínez et al. 2015). C_{20} geranylgeranyl pyrophosphate was formed by elongation reactions with different cellular enzymes. The two geranylgeranyl pyrophosphate molecules further condense which leads to the formation of the next carotenoid, lycopene. Lycopene is the first color carotenoid and then with two different cyclic reactions forms β-carotene and α-carotene. These two pigments are further separated into different types of carotenoids by undergoing various reactions such as glycosylation, hydroxylation, ketolation, and oxygen cleavage as shown in Fig. 6.1 (Varela et al. 2015).

These carotenoids pigments are structurally divided into two types, viz. (1) non-oxygenated hydrocarbons and (2) oxygenated hydrocarbons known as xanthophylls. The non-oxygenated carotenoids also called carotene include lycopene, α-carotene, and β-carotene, whereas oxygenated carotenoids include lutein, canthaxanthin, astaxanthin, fucoxanthin, etc. (Saini et al. 2018). Commercially

β-carotene and astaxanthin are important carotenoids which are produced at large scale from microalgae such as *Dunaliella salina* and *Haematococcus pluvialis* (Rammuni et al. 2019).

6.2.1 β-carotene

β-carotene is among well-known carotenoids as it acts as pro-vitamin A and helps in the synthesis of retinoid. This pigment also possesses many other beneficial properties like antioxidant, immune modulation, anti-cancerous, etc. (Saini and Keum 2018). It is used as vitamin supplement, additive, and also as colorant in many human and animal food supplements and in cosmetic products. Many oncological investigations have shown that foods rich in β-carotene can reduce the chances of cancer and other degenerative diseases. In recent times, 95–98% demand of β-carotene was covered with synthetic production of this pigment, whereas 2–5% was fulfilled with bio-resources such as plant, algae, and bacteria. The first carotenoid whose production was commercialized via microalgae was β-carotene by *Dunaliella salina*. *D. salina* is a halotolerant strain of microalgae which produces β-carotene up to 10% of its dry weight under nutrition starvation conditions (Rammuni et al. 2019).

6.2.2 Astaxanthin

Astaxanthin is another economically important xanthophyll whose production is commercialized via microalgae. *Haematococcus pluvialis* is a freshwater microalga which accumulates high amount of astaxanthin in specific culture conditions. Astaxanthin has recently gained attention because of its antioxidant potential leading to an increase in its commercial value in pharmaceutical and nutraceutical industries (Rammuni et al. 2019). Astaxanthin has terminal-carbonyl group conjugated with polyene backbone and it provides high free radical scavenging activity which makes it a potent antioxidant carotenoid than β-carotene. Thus, food supplements with astaxanthin can protect damaging cells from free radicals, boost immune system, protect skin cell from UV radiation and from cancer, reduce the occurrence of cardiovascular disease and degenerative diseases (Ambati et al. 2014). It is also used in aquaculture as it imparts decent color to muscle of the fishes and aquatic animals which make them attractive, give fresh appearance, thereby enhancing their market value as well as antioxidant and vitamin content of the fishes. The commercial value of astaxanthin goes up to US $15,000/kg pigment and is commercially produced by several companies in countries like China, Israel and Chile.

6.2.3 Lutein

Lutein (β,ε-carotene-3,3′-diol) is a yellow pigment which is present in plant and some of the microalgae. It is consumed as an additive and coloring agent in food,

feed, pharmaceutical, and cosmetic industries. It is used in poultry farming to brighten feathers colors of poultry animals and deepening the yellow color of the egg yolks. Moreover, it is also used as nutrient supplement by humans to increase the health benefits and immunity against diseases like cancer, age-related macular degeneration and cardiovascular diseases. Commercially, lutein was extracted from marigold flowers where 10–20% of it is used in fodder industries, while rest is processed as health supplemented foods (Gong and Bassi 2016). At present, no commercial production of luteins is reported from microalgae but some organisms have shown promising results for its production.

6.3 Phycobiliproteins

Phycobiliproteins (PBPs) are the light-harvesting protein-pigment complex which is primarily found in all cyanobacteria, some red algae and cryptomonads (Pandey et al. 2013). These protein complexes are water soluble in nature and have open tetrapyrrole structure as a functional group. These phycobiliproteins have high commercial value due to their high application in the field of biotechnology and pharmaceutical industries (Saini et al. 2018). Because of their non-toxic and non-carcinogenic nature, these pigments are gaining importance in food and cosmetic industries over synthetic colors. Apart from used as natural dyes in food and cosmetic products, these are also well known for their bioactive properties such as antioxidants, anti-viral, anti-inflammatory and antitumors (Manirafasha et al. 2016). PBPs are the major light-harvesting pigments in cyanobacteria. The synthesis of PBPs starts with the action of heme oxygenase on the heme bio-molecule which converts them into biliverdin, which further changes into phycobilin molecules by various enzymes actions. These phycobilin molecules covalently attach to specific cysteine residues with the help of enzyme known as bilin lyase. These phycobilin proteins are of three main types: (1) phycocyanobilin (PCB) whose reduction is catalyzed by phycocyanobilin synthase (*Pcy*A), (2) phycoerythrobilin (PEB) formed by reduction of ferredoxin oxidoreductase (*Peb*A and *Peb*B), and (3) phytochromobilin whose formation is catalyzed by ferredoxin oxidoreductase (H2Y) shown in Fig. 6.2. Apart from these three, other types of phycobilin proteins are present such as phycoviolobilin (PVB) and phycourobilin (PUB) (Li et al. 2019; Pagels et al. 2019; Chakdar and Pabbi 2016). PBPs are divided mainly into three types on the basis of absorption spectrum, viz. phycocyanin (610–625 nm), allophycocyanin (650–655 nm), and phycoerythrin (550–565 nm). Among them, phycocyanin has more commercial value followed by phycoerythrin.

6.3.1 Phycocyanin

Phycocyanin (PC) is a blue color phycobiliprotein with absorption maxima at 620 nm, present primarily as light-harvesting pigment in most of the cyanobacteria. It is responsible for the bluish color of the cyanobacteria and may be present up to

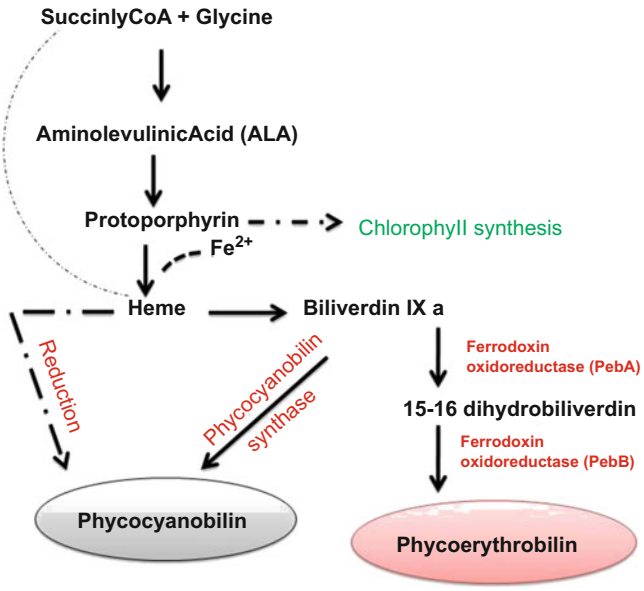

Fig. 6.2 An overview of biosynthetic pathway for phycobilin synthesis

concentrations ranging from 23 to 30% of the total protein. Phycocyanin commonly called C-PC, where C stands for cyanobacteria, is also extracted from red algae called R-PC (Fernández-Rojas et al. 2014). Structurally, phycocyanin is composed of α-chain with single phycocyanobilin attached to cysteine residues at 84 position and β-chain has two phycocyanobilin attached at 84 and 155 cysteine residues. The overall structure and amino acid composition of PC were similar in different organisms except with minor species dependent variations.

Phycocyanin is commercially produced in open ponds or raceways via photoautotrophic cultivation of *Spirulina platensis* in sub-tropical and tropical locations. Other cyanobacterial species of genera such as *Nostoc*, *Anabaena*, *Leptolyngbya*, *Phormidium* and *Lyngbya* have shown potential to produce PC at commercial level (Johnson et al. 2014; Khatoon et al. 2018; Khattar et al. 2015; Saini et al. 2020b). Phycocyanin is commercially useful in food and cosmetic industries as natural coloring agent and marketed in countries like Japan, China, Philippines, etc. but as it imparts blue color to foods limits its consumption in these industries (Kuddus et al. 2013). PC has high fluorescent and antioxidant properties which are utilized in many food, cosmetic, pharmaceutical and biotechnological industries (Nwoba et al. 2020).

6.3.2 Phycoerythrin

Phycoerythrin is another important red-pink color PBP with absorption maxima of 562–565 nm. The phycoerythrin is of three types: R-phycoerythrin, B-phycoerythrin, and C-phycoerythrin where R, B, and C stand for Red algae, Bangiales, and Cyanobacteria, respectively (Munier et al. 2014; Hsieh-Lo et al. 2019). The prosthetic group attached to phycoerythrin is phycoerythrobilins. Similarly, phycoerythrin is also composed of α-β subunits and shows antioxidant, anti-cancerous, and hepato-protective properties (Wang et al. 2015). Generally, phycoerythrin is extracted from red algae but some studies show cyanobacteria like *Anabaena*, *Nostoc*, *Porphyridium* spp. can be the potential sources for their production at large scale (Khattar et al. 2015; Kathiresan et al. 2007).

6.4 Advances in the Production of Biopigments from Microalgae

During the last decade or so, the demand for natural pigments has increased tremendously which is largely because of their non-toxic and health promoting properties. Some of the pharmaceutical application of various biopigments are summarized in Table 6.2 The growing demand further emphasizes on their large scale production from sources which are inexpensive, do not compete with food productivity, and are also eco-friendly (Sánchez-Muñoz et al. 2020). Microalgae fit in this category and show their potential to produce such biopigments in short time intervals (Hu et al. 2018). But, lots of investigations are still required in lowering the cost of production of these pigments. The various ways and means to enhance pigments production in microalgae include process optimizations, effect of various abiotic factors as well as integration of new synthetic and molecular tools to increase the productivity.

6.4.1 Strategies for the Enhancement of Biopigments Production

Light is the primary source of energy for the growth of microalgae and to carry out basic metabolic processes such as photosynthesis. The rate of microalgal growth enhances with the increase in light intensity but after a certain limit, photo-inhibition decreases the growth of microalgae (Singh and Singh 2015). Quality of light has a great impact on these pigments. Many investigations concluded that high phycocyanin content can be achieved under red light in case of cyanobacteria as these lack chlorophyll *b* and unable to absorb longer wavelength. Hence, cyanobacteria synthesize a large amount of phycocyanin to absorb longer wavelengths under red illumination (da Fontoura Prates et al. 2018). In contrast, phycoerythrin synthesis was inhibited under red illumination and increases under green light. The changes on pigment composition due to the light quality in cyanobacteria were well known as complementary chromatic adaptation. Similarly, higher light intensity increases the

Table 6.2 Beneficial effect and pharmaceutical applications of various biopigments

Biopigments	Potential strains	Health benefits/application	References
Phycocyanin	*Spirulina maxima* *Spirulina platensis*	Demonstrate anti-pancreatic cancer activity	Liao et al. (2016)
		Induce apoptotic mechanism and enhance the effect of anti-cancer drug on LNCaP cells	Kaur et al. (2020)
		Prevention of diabetic nephropathy	Zheng et al. (2013)
Phycoerythrin	*Lyngbya* sp. A09DM	Shows antioxidant and anti-aging properties	Sonani et al. (2014)
	Phormidium sp. A27DM and *Halomicronema* sp. A32DM	Shows apoptotic property against A549 human lung carcinoma cells	Madamwar et al. (2015)
	Lyngbya sp. A09DM	Demonstration of therapeutic potential against Alzheimer's disease	Chaubey et al. (2019)
β-carotene	*Dunaliella salina*	Source of vitamin A, antioxidant activity, act as coloring agent in nutraceuticals	de Jesús Bonilla-Ahumada et al. (2018)
Astaxanthin	*Haematococcus pluvialis*	Antioxidant properties, inhibit low density lipoprotein oxidation Anti-aging activity	Fang et al. (2019)
Lutein	*Scenedesmus almeriensis* and *Chlorella fusca*	Improve age-related macular degeneration, cardiovascular diseases, and strong antioxidant potentials	Becerra et al. (2020)
Lycopene	–	Antioxidant, induce cellular communication, act as a pro-vitamin A, and help in the regulation of apoptosis and cell proliferation	Caseiro et al. (2020)

carotenoids content. For example, higher light intensity increases the carotenoids content where increase in astaxanthin and lutein content has been reported in *C. zofingiensis* with the increase in light intensity (Del Campo et al. 2004). Similarly, β-carotene content also increased in *D. salina* (Lamers et al. 2010). Thus it is interesting that carotenoids content enhances with an increase in light intensity, whereas phycobiliproteins production can be increased under low light intensity. Temperature also plays an important role in the accumulation of various biopigments and growth of microalgae. The lutein production increases at higher temperature, whereas carotenoids like β-carotene and astaxanthin have shown contradictory result. Furthermore, phycobiliproteins production varies with the growth of the organisms under different situations.

Nutrient stress was among one of the old used strategies to increase productivity of biopigments especially carotenoids. There are lots of reports, where high

carotenoids content was observed under nitrogen deprivation condition as it stimulates the secondary biosynthetic pathways. *D. salina* accumulates 2.7% high β-carotene content under nitrogen depleted conditions and similar results were also found for astaxanthin and lutein (Lamers et al. 2010). Additionally, under salt stress up-regulated synthesis of carotenoid ketolase (BKT) was observed in *C. zofingiensis* which leads to the enhanced production of astaxanthin and canthaxanthin (Paliwal et al. 2017). However, phycocyanin synthesis increases with high nitrate concentration as it acts as nitrogen source during nitrogen-limiting condition for cyanobacteria (Chen et al. 2010). Similarly, high nutrient content enhances the growth rate which leads to high accumulation of biomass which directly influences the productivity of phycobiliproteins. Phycobiliproteins synthesis was also regulated by the iron, as it regulates the synthesis of key enzymes ferrochelatase and phycocyanobilin/ferredoxin oxidoreductase (PcyA) which are responsible to form heme molecules. These molecules act as substrate for heme oxygenase (HO) which is a rate limiting step for phycocyanobilin synthesis (Mogany et al. 2018). Additionally, Fe also regulates the expression of PC αβ subunit gene (Chakdar and Pabbi 2016). Similarly, Mg^{2+} acts as a cofactor for porphobilinogen (PBG) synthase enzyme which is responsible for protoporphyrin synthesis. It is also a rate limiting step, but excess of Mg^{2+} incorporated protoporphyrin into chlorophyll synthesis, thus decrease in the phycocyanobilin synthesis which reduces phycobiliproteins concentration (Beale 1999; Mogany et al. 2018).

Microalgae are an excellent model to understand the complex cellular mechanism of eukaryotic system as these mimic the plant cell. The high growth rate and ease to handle make microalgae ideal candidates for the metabolic and genetic engineering (Saini et al. 2020b; Zeng et al. 2011). Microalgae are an excellent source for the expression of various genes of enzymes belonging to metabolic pathways to increase the productivity of secondary metabolites. It also acts as an ideal chassis for the expression genes related to cartenogenesis as it has a sufficient supply of precursor for terpenoids synthesis as well as possess enough space for the storage of enhanced carotenoids molecules. But, still there are only a few reports for the manipulation of carotenogenic pathways (Varela et al. 2015).

Several techniques to enhance the productivity of carotenoids by overexpression and inactivation of rate limiting gene/enzymes and endogenous expression gene from different genes have been reported. Even though regulatory mechanism of biosynthetic pathway of carotenoids synthesis is not well studied, but there is plentiful evidence that phytoene synthase acts as a rate limiting enzyme and central point in their synthesis (Nisar et al. 2015; Shang et al. 2018). Moreover, this enzyme was observed upregulated during stress condition along with enhanced production of carotenoids in cases of *D. salina* and *Chlamydomonas reinhardtii* (Couso et al. 2011) or *Haematococcus pluvialis* (Vidhyavathi et al. 2008). Genome study of *C. reinhardtii*, *C. vulgaris,* and V*olvox carteri* shows a single type coding region for *psy* gene, whereas in chlorophytes like *D. salina,* two classes of *psy* gene families are found in their genomes which are regulated during stress conditions. This enzyme was well targeted for the metabolic and genetic engineering approaches for enhancing the carotenoids contents in the organisms. For example, Cordero et al.

isolated *psy* gene from *C. zofingiensis* and expressed in *C. reinhardtii.* This nuclear transformation increased the transcript level of CZpsy gene along two- to threefold violaxanthin and lutein content in the transformed cells as compared to control cells (Cadoret et al. 2012). Similarly, the production of violaxanthin and lutein increases with the expression of *psy* gene (Cordero et al. 2011; Couso et al. 2011). Further to enhanced production of keto-carotenoids, expression of endogenous *BKT* (β-carotenoid ketolase) enzyme in *C. reinhardtii* was targeted (León et al. 2007). Similarly, Lin et al. integrated modified genes β-carotene hydroxylase (*CRTR-B*) and *BKT* via homologous recombination technique in *D. viridis* and enhanced astaxanthin production (Lin et al. 2019).

6.5 Concluding Remark

As described in this chapter, microalgae are getting global attention due to their enormous potential to produce a wide range of metabolites and pigments. Due to ease in cultivation and use of solar energy for growth gives them upper hand over other biological systems for bio-manufacturing of pigments with extensive application in cosmetic, food, feed, and pharmaceutical industries. This chapter also describes the various health promoting properties such as antioxidant, anti-inflammatory, anti-cancerous, etc., of different pigments as well as the potential strains used for their commercial production. We are still at infant stage of understanding metabolic mechanisms of carotenoids and phycobiliproteins synthesis through microalgae. Considering a wide spectrum of possibilities in microalgae, we need to investigate more new species and bridge the path between lab production and large scale production of microalgae. Moreover, combination of metabolic and genetic engineering can be used to improve the productivity of biopigments in existing microalgae along with lowering their production cost.

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7 Revealing the Features of the Oxidative Enzyme Production by White-Rot Basidiomycetes During Fermentation of Plant Raw Materials

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Abstract

White rot basidiomycetes (WRB), belonging to one of the most diverse and important groups of living organisms, have the unique ability to completely degrade lignin through an oxidative process catalyzed by extracellular and non-specific lignin-modifying enzymes (LME) consisting of laccase, manganese peroxidase, lignin peroxidase, and versatile peroxidase with the assistance of several auxiliary enzymes. Because LME are capable of oxidizing a wide variety of natural and synthetic compounds, these enzymes have received tremendous attention for a variety of industrial and biotechnological applications. Consequently, the demand for these enzymes has increased in recent years, leading to the search for cost-effective production systems. To increase LME yields, various approaches and strategies, such as exploitation of cheap plant raw materials as growth substrates, optimization of fermentation media and cultivation conditions, and development of better bioprocess technologies, have been widely exploited. Literature date evidence that many factors influence the synthesis and secretion of LME and their isoenzymes, but the effects of these factors differ among the fungal species and we still have to understand the whole spectrum of mechanisms that modulate LME production. In this chapter, we summarize recent literature reports and our data on the physiological features of LME production by WRB, focusing on the diversity, common characteristics, and unique properties of individual fungi as well as on several approaches and strategies that provide enhanced (or reduced) secretion of laccases and peroxidases.

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Keywords

White-rot basidiomycetes · Lignin-modifying enztmes · Lignocellulose · Fermentation

7.1 Introduction

White-rot basidiomycetes (WRB) belong to one of the most diverse and important groups of living organisms because of their role in ecosystem function and unique ability to degrade and completely metabolize all wood polymers due to their capability to synthesize required hydrolytic and oxidative enzymes. Lignin degradation is an oxidative process catalyzed by extracellular and non-specific lignin-modifying enzymes (LME) composed of laccase (EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (LiP, EC1.11.1.14), and versatile peroxidase (VP, EC1.11.1.16) with the assistance of several auxiliary enzymes. Some WRB secrete several different LME, while others produce only one or two of them, usually as a set of isoenzymes encoded by multiple genes within one fungal species (Lundell et al. 2010; Piscitelli et al. 2011; Janusz et al. 2013; Peng et al. 2018).

Among various industrial enzymes, LME have attracted tremendous attention due to the wide range of industrial and biotechnological applications (Yadav and Yadav 2015; Mäkelä et al. 2016; Chowdhary et al. 2019). However, the wide implementation of LME at the industrial scale is delayed because of too high enzyme cost due to the relatively low yield in the hundreds of explored strains belonging to various taxonomic groups. To solve this problem, the exploitation of molecular biology approaches was widely considered. Various techniques have been utilized to modify enzymes for industrial purposes. Artificial intelligence and computational tools have provided better results for modification and utilization of enzymes (Kumar et al. 2019; Dixit et al. 2019). Nevertheless, although many industrial enzymes (amylases, cellulases, etc.) are efficiently overproduced in heterologous systems, high expression of LME by recombinant organisms has not been yet obtained (Mekmouche et al. 2014). The highest yields of laccase were obtained when the enzyme from *Moniliophthora roreri* and *Pycnoporus cinnabarinus* was expressed in *Pichia pastoris* (281 U/mL) (Bronikowski et al. 2017). At the same time, several wild strains of WRB overproducing individual LME have been discovered (Galhaup et al. 2002; Revankar and Lele 2006; Elisashvili et al. 2014; Coconi-Linares et al. 2014; Kachlishvili et al. 2014; Schneider et al. 2019).

To increase LME yields, various approaches and strategies such as exploitation of cheap plant raw materials as growth substrates, optimization of fermentation media and cultivation conditions, development of better bioprocess technologies have been widely exploited (Elisashvili and Kachlishvili 2009; Piscitelli et al. 2011; Li et al. 2016; Park et al. 2015; Martani et al. 2017). Literature date evidence that the synthesis and secretion of LME and their isoenzymes are affected by many factors, but the effects of these factors vary among the fungal species (Terrón et al. 2004;

Xiao et al. 2006; Piscitelli et al. 2011; Yang et al. 2013; Bertrand et al. 2013, 2014). The results and advances in strain engineering, transcriptional regulation in response to environmental conditions, and biotechnological applications of LME have been comprehensively and excellently reviewed (Yadav and Yadav 2015; Mäkelä et al. 2016; Martinez et al. 2017; Chowdhary et al. 2019), but an exhaustive overview of the basic aspects of modulating enzyme activity through alterations of the cultivation conditions is still lacking in the literature. In this chapter, we summarize recent literature reports and our data on the physiological features of LME production by WRB, focusing on the diversity, common characteristics, and unique properties of individual fungi as well as on several approaches and strategies that provide enhanced (or reduced) secretion of laccases and peroxidases.

7.1.1 Screening of LME Producers

Screening of WRB species is an important initial stage for selecting promising LME producers. It aims to discover novel fungal strains (1) producing high yield and (2) the right set of LME, (3) exhibiting enhanced physical, chemical, and catalytic properties, and (4) suitable for specific industrial applications. Moreover, LME of some basidiomycetes, such as *Phanerochaete chrysosporium* (Kirk and Farrell 1987), are formed during secondary metabolism; therefore, it is important to detect fungi that efficiently produce LME during primary metabolism.

A countless WRB belonging to different taxonomic groups have been screened to identify industrially significant producers of LME, but only a few were able to produce encouraging amounts of target enzymes (Galhaup et al. 2002; Revankar and Lele 2006; Songulashvili et al. 2012; Kachlishvili et al. 2014), MnP and LiP (Kapich et al. 2004; Singh and Chen 2008; Elisashvili and Kachlishvili 2009; Coconi-Linares et al. 2014). It is worth noting that in some cases the direct comparison and correct assessment of WRB biosynthetic potential and enzyme yields are difficult due to the differences in media composition and fermentation conditions as well as the use of various methods and analyses to evaluate the LME activity.

Sergentani et al. (2016) evaluated the enzyme activity of twenty-eight strains in liquid cultures with wheat bran as a suitable growth substrate. Laccase activity (0.12–30.14 U/mL) was detected in the culture supernatants of all strains except *Hydnum repandum*, *Pholiota adiposa*, and *Pycnoporus cinnabarinus*. Neither LiP nor MnP was detected even in *Trametes* spp., although these fungi are known producers of MnP and LiP (Levin et al. 2010; Kachlishvili et al. 2018). Recently, Kinnunen et al. (2017) performed an automated and miniaturized screening and comparison of 53 species of basidiomycetes for LME production in their cultivation in liquid mineral, soy, peptone, and solid-state oat husk media. Among the tested fungi, MnP (96%) and laccase (92%) producers were the most widespread. Strains of *Phlebia radiate* and *Trametes ochracea* showed the highest LiP activity; *P. radiate* appeared to be the best candidate for VP production in soy liquid medium. In this study, the highest enzyme activities were produced on lignocellulose-containing media. By contrast, low laccase activity (0.01–1.97 U/mL) was revealed in

34 basidiomycetes screened in the stationary cultivation using malt extract broth without lignocellulose (Bodke et al. 2012), while only 5 of 21 endophyte fungi were able to produce low laccase activity (0.01–0.04 U/mL) but not peroxidase activity in modified Kirk's liquid medium (Fillat et al. 2016). There is other evidence that not all WRB species readily express LME activity in a defined medium (Schlosser et al. 1997; Kapich et al. 2004; Elisashvili and Kachlishvili 2009).

Therefore, in our screening studies, we simultaneously evaluated the LME activity of fungi during their cultivation in media containing mandarin peels and glycerin, which provide abundant fungal growth and significant enzyme production (Elisashvili and Kachlishvili 2009; Kachlishvili et al. 2014, 2018; Elisashvili et al. 2017). These studies revealed several features that should be considered during screening experiments. Firstly, in concordance with available literature data, our results clearly showed a wide intra- and interspecies diversity in the ability of WRB to produce LME. Thus, in the submerged fermentation of mandarin peelings, the laccase activity of *Ganoderma* spp. varied from 2.0 U/m/L to 75.4 U/mL, while the MnP activity of *Trametes* spp. strains ranged from 0 to 0.9 U/mL. Secondly, the WRB manifested different responses to the used carbon sources. Among them, *Cerrena* spp., *Coriolopsis gallica*, *Pseudotrametes gibbosa*, and *T. versicolor* produced significant laccase activity in synthetic medium, whereas the presence of lignocellulosic material was a prerequisite for the enzyme production by *Ganoderma* spp., *Phlebia radiata*, *Pycnoporus coccineus*, and *Trametes ochracea*. Likewise, 13 fungal strains secreted laccase (0.2–9.4 U/mL) in the 1% glycerol-containing medium, but supplementation of this medium with 20 g/L milled mandarin peels 2 to 22-fold enhanced their laccase activity (to 1.2–38.3 U/mL) (Elisashvili et al. 2017). Moreover, only *Coriolopsis gallica* was capable to express LiP activity in the synthetic medium, whereas in the lignocellulose-based medium all fungi, with the exclusion of *Merulius tremellosus* produced appreciable levels of this enzyme. In another work (Kachlishvili et al. 2018), the substitution of glycerol with mandarin peels two- to tenfold increased the laccase activity of *Trametes* spp., although *T. versicolor* 775 better produced this enzyme in the synthetic medium. Besides, the use of mandarin peels as the fungal growth substrate stimulated or even induced MnP and LiP production. On the whole, an analysis of literature data shows that for the greatest production of individual LME by specific WRB, a certain composition of the nutrient medium is required, and the use of an inappropriate medium may lead to non-detection of promising enzyme producer.

7.1.2 Effect of Lignocellulosic Substrates

Understanding the mechanisms of regulation of the individual LMEs synthesis under specific growth conditions and elucidating the cultivation conditions ensuring their predominant production is a critical necessity. In particular, to ensure abundant fungal growth and efficient production of LME, it is important to select a suitable lignocellulosic substrate rich in readily available carbohydrates, nitrogen, trace elements, and inducers for the synthesis of target enzymes (Winquist et al. 2008;

Elisashvili et al. 2009; Kachlishvili et al. 2018). Thus, a study conducted by Mikiashvili et al. (2006) with *Pleurotus ostreatus* 98 showed that substitution of glucose with mandarin peels 40-fold and 18-fold increased the fungus laccase and MnP activities, respectively. Likewise, the cultivation of *P. radiata* 79 in low-nitrogen defined medium with milled alder as sole carbon source induced tenfold production of LiP and increased MnP activity, in comparison to the glucose-supplemented cultures (Mäkelä et al. 2013). Laccase and MnP activities were the highest in cultures of the *P. radiata* grown on birch wood (Villavicencio et al. 2020). On liquid ME medium, however, the production of laccase by *P. radiata* during 4 weeks of cultivation was very low, although distinct MnP activities were produced by the fungus. In another study, when the basal medium including 20 g/L glucose as a carbon source was supplemented by 10 g/L of orange peel, tea, bagasse, and corn cobs, the laccase production by *P. ostreatus* was improved 9-, 5-, 2-, and 1.1-fold, respectively (Zhao et al. 2017). It is worth noting that the proliferation capacity of the culture with orange peel extract was 0.5-fold higher than that of control. The more orange peel (ranged from 0 to 10 g/L) was added to the culture with glucose, the better was the fungus growth and enzyme production.

Undoubtedly, lignocellulosic biomasses differ greatly in their chemical composition, physical, mechanical, and other properties affecting fungal metabolism and productivity. For example, the tested residues provided equally good growth of *C. unicolor* and *Phellinus robustus* in their submerged fermentation, but the fungal laccase activity varied from 15.7 U/mL to 151.6 U/mL and from 0.9 U/mL to 8.4 U/mL, respectively (Elisashvili and Kachlishvili 2009). Wheat bran and residue after ethanol production appeared to be the best growth substrates for laccase production by *C. unicolor*, whereas mandarin peels and kiwi and walnut pericarp favored enzyme secretion by *P. robustus*. Simultaneously, as compared with wheat bran, the fermentation of kiwi residue stimulated MnP secretion by *P. robustus*, whereas walnut pericarp 12-fold augmented activity of this enzyme in *C. unicolor*.

Coffee husk (CH) and citric pulp pellet (CP) from an orange juice industry used as a carbon source for the submerged cultivation of *Lentinus crinitus* without an additional nitrogen source provided the secretion of 33.4 and 29.1 U/mL laccase activity, respectively(Almeida et al. 2018). Since CH contains caffeine, polyphenols, and tannins, while CP contains polyphenols (mainly flavonoids) soluble in water, the authors assumed that these compounds induced the laccase production by *L. crinitus*. Nevertheless, no MnP or LiP was detected in the fungus cultivation. On the contrary, Conceição et al. (2017) reported the production of MnP but not laccase in the cultivation of *L. crinitus* on a solid medium with barley and cassava residues (1:1). The stimulating effect of water-soluble aromatic compounds obtained from lignocellulosic substrates on the synthesis of LME has been shown by many authors (Crestini et al. 1996; Kapich et al. 2004; Adekunle et al. 2017). The induction of laccase isoforms by aqueous extracts from softwood and hardwood was shown in *T. versicolor* HEMIM-9; specifically, the pine, cedar, and oak extracts addition to the fungal cultures increased 4.6-, 4.5-, and 3.7-fold, respectively, the fungus laccase activity (Bertrand et al. 2014). The authors suggested that not only the concentration of phenolic compounds in aqueous extracts but also the origin or composition of the

extract may influence the induction of laccase. Moreover, native isoelectric focusing of laccases that were isolated from the control and the induced cultures revealed differences in isoform number (from 3 to 6) and pI values. According to the researchers, some isoforms of *T. versicolor* HEMIM-9 are downregulated, while others are upregulated in the presence of the tested inducers.

The available results suggest that kind and composition of lignocellulosic materials determine the set and yield of LME produced by the WRB. Thus, Janusz et al. (2018) compared the transcriptomes of *C. unicolor* FCL139 grown in the solid-state growth conditions on birch, ash, maple sawdust with those of fungus grown on mineral medium. It was found that the expression of MnP XLOC_004360 was upregulated during the fungus growth on maple sawdust, the expression of the MnP XLOC_004631 was specifically induced in the fungus cultivation on ash and maple but it was downregulated during *C. unicolor* growth on the birch medium. Analyses of laccase transcripts (XLOC_008955) revealed their reduced amounts in the fungus cultivation on the birch and ash sawdust-containing media.

It should be noted that the composition of some lignocellulosic substrates may not correspond to the nutritive demands of the fungus; then nutrient medium requires supplementation with an additional carbon source. Thus, the laccase activity of *P. ostreatus* in the fermentation of rice bran and other plant raw materials appeared to be lower as compared with that in the control culture with glucose (2.2 U/mL) (Selvaraj et al. 2014). However, the combinations of rice bran with sugarcane bagasse, corn stalks, or orange peelings showed laccase activity of 3.24 U/mL, 2.96 U/mL, and 2.6 U/mL, respectively, whereas supplementation of these media with 0.2 g glucose further increased laccase activity to 6.47 U/mL, 4.41 U/mL, and 5.71 U/mL, respectively.

Overall, the literature data demonstrate a clear regulatory role of individual lignocellulosic materials in LME activity expression. Undoubtedly, the chemical composition and other characteristics of lignocellulosic biomass play a decisive role in the production of LME; in particular, phenol-containing plant materials, such as coffee husks and by-products of citrus processing, provide the highest production of enzymes. However, the deconstruction of complex lignocellulosic substrates requires the participation of a consortium of various enzymes acting in synergy to provide the microorganism with the necessary nutrients. Therefore, LME producers simultaneously expressing high hydrolytic activity may be more attractive for the successful production of target enzymes during lignocellulose fermentation.

7.1.3 Effect of Carbon Source

Among the medium components, carbon sources and concentrations are the most important factors determining the rate and degree of fungal biomass accumulation, displaying diverse effects on the LME production, depending on the fungal strain (Galhaup et al. 2002; Elisashvili et al. 2002; Stajić et al. 2006; Elisashvili and Kachlishvili 2009; Piscitelli et al. 2011). For example, in the submerged cultivation of *Trametes pubescens* in the presence of glucose, the sugar was rapidly metabolized

and resulted in high laccase activity, but active enzyme secretion by the fungus was observed when the glucose concentration in the growth medium decreased to a certain low concentration (Galhaup et al. 2002). Slowly utilized lactose and cellulose resulted in poor laccase production. Likewise, laccase activity obtained in the cultivation of *Pleurotus sajor-caju* in media containing 0.5 g/L fructose or glucose was 12-fold higher than that obtained with lactose (Bettin et al. 2008). On the contrary, exactly lactose ensured the maximum laccase secretion by *P. gibbosa* (Elisashvili and Kachlishvili 2009). Interestingly, starch appeared to be the best carbon source for the laccase production by WRB*WR-1* fourfold increasing enzyme activity compared with fructose-containing medium (Revankar and Lele 2006). Myasoedova et al. (2015) observed the highest laccase activity of *Lentinus strigosus* 1566 when peptone-yeast extract medium was supplemented with galactose, arabinose, and xylose at a final concentration of 20 g/L. The presence of glucose, sucrose, or maltose in the medium led to a decreased laccase activity. The researchers demonstrated the selectivity of the *L. strigosus* 1566 towards mono- and disaccharides synthesizing different sets of laccase isozymes.

Early observations established that the production of LME in some fungi occurs in response to carbon depletion (Kirk and Farrell 1987). Then it was shown that individual laccase isozymes of basidiomycete I-62 (Mansur et al. 1998), *T. pubescens* (Galhaup et al. 2002), and *Trametes* sp. AH28-2 (Xiao et al. 2006) are differentially regulated by carbon and synthesis of some of them is subjected to catabolite repression by glucose or other easily metabolizable carbon sources. Moreover, many fungi such as *P. coccineus* (Kachlishvili et al. 2016), species of genus *Ganoderma* (Songulashvili et al. 2012; Elisashvili and Kachlishvili 2009) produce very low laccase activity in defined media containing glucose or glycerol. Therefore, in our study, to elucidate if there is a mechanism of catabolite repression of LME synthesis the mandarin squeeze-based medium (control) was supplemented with glucose before inoculation and after 4 days of the submerged fermentation (middle of logarithmic phase of growth). In addition to *P. coccineus* and *Ganoderma lucidum*, *Trametes trogii* and *T. versicolor*, which produce marked laccase activity in the presence of glucose, were studied for comparison. Laccase activity of *P. coccineus* in the control medium gradually increased to 4.2 U/mL peaking on day 5 (Fig. 7.1a). Supplementation of the control medium with 0.5% and 1% glucose delayed laccase secretion and gave lower enzyme activities after 3 days of the fermentation (2.1 and 1.0 U/mL vs. 2.6 U/mL in the control). However, subsequently, laccase activity rapidly increased to 5.6 and 7.5 U/mL, respectively, on day 8 significantly exceeding that in the control medium owing to higher biomass accumulation. When 0.5% glucose was added to the growing fungal culture no increase in enzyme activity was observed during 4 days of cultivation, then the enzyme secretion resumed. In the cultivation of *G. lucidum*, almost no delay in the laccase production was observed due to the supplementation of glucose in the initial medium, but enzyme activity was completely suppressed during 1 day when 0.5% glucose was added to the growing culture (Fig. 7.1b). However, without a transcriptomic analysis, the data obtained are insufficient to confirm the presence of catabolite repression of laccase synthesis in *P. coccineus* and *G. lucidum*, since

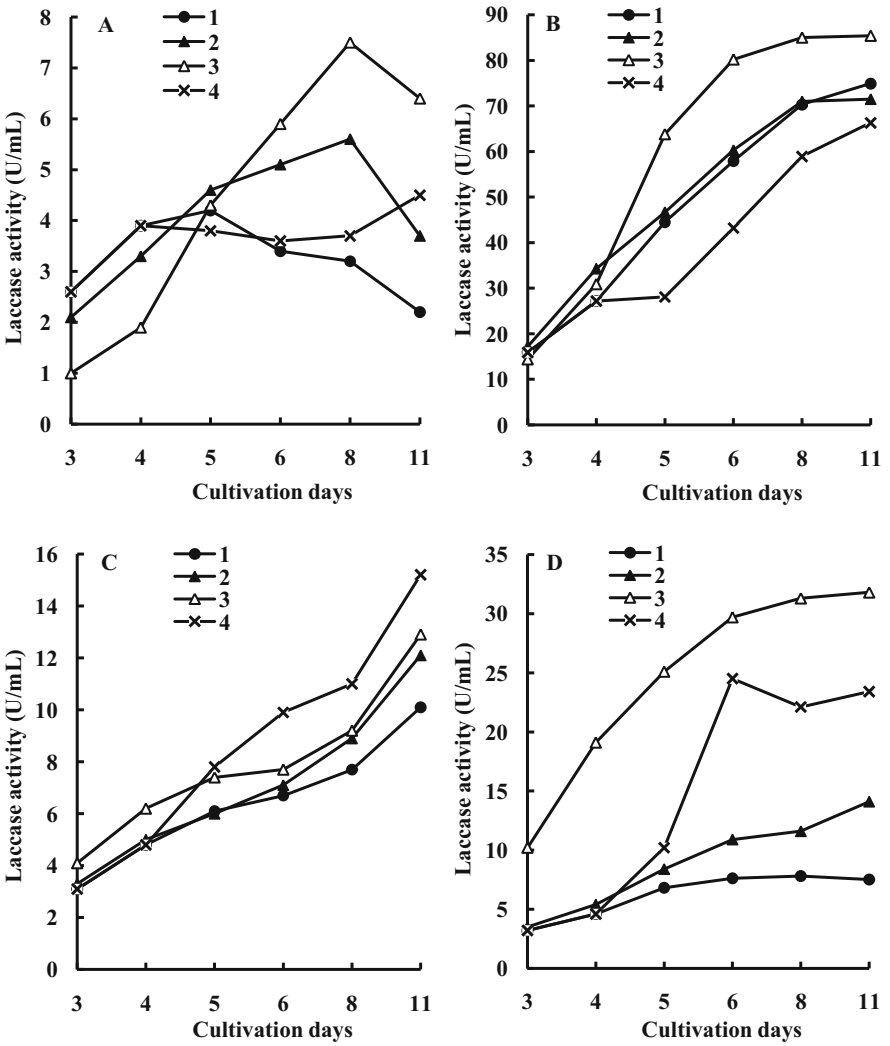

Fig. 7.1 The effect of glucose addition in the production of laccase during the submerged fermentation of mandarin squeeze by four basidiomycetes species. (**a**) *Pycnoporus cinnabarinus* 811; (**b**) *Ganoderma lucidum* 447; (**c**) *Trametes trogii* 146; (**d**) *Trametes versicolor* 159. 1 medium containing 40 g/L mandarin squeeze (control); 2 control + 0.5% glucose; 3 control + 1% glucose; 4 control + 0.5% glucose after 4 days of cultivation

the growth of exactly these fungi in the presence of glucose was accompanied by particularly strong acidification of the medium, which of course negatively affected the synthesis and activity of laccase. Finally, unlike these fungi, *T. trogii* (Fig. 7.1c) and *T. versicolor* (Fig. 7.1d) responded to the addition of glucose to the control medium by an accelerated secretion of laccase activity. This means the absence of

catabolite repression in the tested *Trametes* species and using an additional carbon source is the right strategy to increase the yield of target enzyme.

It is interesting that *Cerrena* sp. HYB07 showed especially distinctive features since very high carbon concentration (malt dextrin, 60 g/L) in the fermentation medium with a high concentration of nitrogen was beneficial for laccase production (280 U/mL) (Yang et al. 2016). Substitution of malt dextrin by glucose slightly increased laccase yield, whereas fructose decreased laccase production by 60%. Moreover, disaccharide maltose did not affect laccase yields, sucrose lowered laccase yield by 15.5%, whereas glycerol threefold decreased the fungus enzyme activity. At the same time, *Coriolopsis gallica* T906 expressed the highest laccase activity in medium with glycerol and ammonium dihydrogen phosphate as carbon and nitrogen sources, respectively (Xu et al. 2016).

Recently, Schneider et al. (2018) investigated LME activity of *Marasmiellus palmivorus* VE111 in media containing glucose, sucrose, or glycerol and showed that the medium prepared with glucose ensured the highest laccase and MnP activities. It is interesting that the effect of the carbon source on laccase secretion significantly depended on nitrogen source (casein was better than peptone). Usually, media that provided higher fungal biomass resulted in higher enzymatic activity, medium prepared with sucrose and casein ensured the best growth of the mushroom, but not the highest laccase activity. In our study with *Trametes multicolor*, the lowest laccase activity was detected in the fungus cultivation in the sodium gluconate-based medium (Kachlishvili et al. 2018). It turned out that mannitol, as well as cellobiose and xylose, were the best carbon sources for laccase production by *T. multicolor* but increased enzyme activities were not due to higher biomasses accumulated in the presence of these carbohydrates. In particular, calculation of the specific laccase activity showed that mannitol, cellobiose, and xylose favored enzyme production by the fungus, increasing more than two- to threefold the specific laccase activity compared with the control medium. Likewise, mannitol more than fourfold increased the *T. multicolor* MnP activity compared to the control medium. However, unlike laccase, it did not promote the secretion of MnP, since the specific activity of MnP in the medium with mannitol decreased 1.5-fold compared with the control medium.

These and many other observations indicate that some carbohydrates can modulate LME expression in WRB; therefore, to maximize the synthesis of LME, it is necessary to find out the carbon source optimal for the fungal growth and the target enzyme production. Yet, to correctly assess fungal enzymatic potential, it is necessary to take into account the amount of biomass accumulated in the presence of a specific carbon source. Moreover, in fungal cultures, the metabolism of various carbohydrates is accompanied by varying degrees of acidification of the nutrient medium. In turn, media with different pH differently affect both the synthesis of a target enzyme and the stability of the already synthesized enzyme.

It is clear that a carbon source, especially, in the form of lignocellulosic biomass, plays a principal role in modulation of basidiomycetes LME activity, but for each enzyme producer, fungus-specific carbohydrate ensuring the highest enzyme activity must be established. Some published data on the laccase activity of WRB, given in

Table 7.1 WRB laccase activity (ABTS) under submerged cultivation conditions

Enzyme producers	Media main components	Laccase (U/mL)	References
Agaricus blazei	10 g/L glucose, 2.8 g/L urea, 0.15 mM $CuSO_4$	45.7	Valle et al. (2015)
Arthrospira maxima	10 mM sucrose, 1 mM guaiacol	56.9	Afreen et al. (2018)
Cerrena unicolor	50 g/L ethanol production residue, 1 mM xylidine, 1 mM $CuSO_4$	507.0	Kachlishvili et al. (2014)
Cerrena sp. HYB07	60 g/L maltodextrin, 10 g/L peptone, 0.25 mM $CuSO_4$	280.0	Yang et al. (2016)
Ganoderma lucidum	50 g/L wheat bran	110.8	Songulashvili et al. (2012)
Ganoderma sp.	40 g/L glycerol, 0.85 mM veratryl alcohol	240.0	Teerapatsakul et al. (2007)
Lentinus crinitus	50 g/L coffee husk, 0.7 g/L urea, microelements	41.2	Almeida et al. (2018)
Lentinus strigosus	20 g/L glucose, 2 mM $CuSO_4$	186.0	Myasoedova et al. (2008)
Pleurotus ostreatus	Rice bran, sugarcane bagasse, 1 mM xylidine, 1 mM $CuSO_4$	37.5	Selvaraj et al. (2014)
Pleurotus pulmonarius	MEB, 0.1% xylidine	349.5	Lallawmsanga et al. (2019)
Pycnoporus cinnabarinus	20 g/L maltose, 35 g/L ethanol	266.0	Lomascolo et al. (2003)
Trametes pubescens	40 g/L glucose, 10 g/L peptone, 2 mM $CuSO_4$	743.0	Galhaup et al. (2002)
Trametes versicolor	2 g/L glucose, 1 mM 2,5-xylidine, 0.5 mM $CuSO_4$	33.6	Birhanlı and Yeşilada (2017)
WR-1	20 g/L starch, 1 mM $CuSO_4$, 0.8 mM xylidine	692.0	Revankar and Lele (2006)

Table 7.1, show their diversity in their requirements for a carbon source to achieve maximum laccase activity.

7.1.4 Effect of Nitrogen Source

Many studies verified the effect of organic and inorganic nitrogen sources in the fermentation medium on the WRB enzyme activity to create the best nutritional conditions for the maximum production of LME. Thus, the assessment of the effect of different nitrogen sources (20 mM as nitrogen) on the LME production by *T. multicolor* was assessed in the submerged fermentation of mandarin peels (Kachlishvili et al. 2018). The lowest activities of laccase, MnP, and LiP were revealed in the medium with potassium nitrate. This nitrogen source and yeast extract provided even lower specific laccase activity (2.4 and 2.9 U/mg, respectively) compared with that in the control medium. On the contrary, casein hydrolysate

favored the laccase production with the highest productivity (12.3 U/mg), obviously, due to available aromatic amino acids. Likewise, the casein hydrolysate ensured the highest specific activity of MnP activity increasing almost twofold specific activity of this enzyme compared with that in the control medium.

It is considered that compared with organic nitrogen sources inorganic ones provide lower enzyme yields despite sufficient biomass accumulation (Piscitelli et al. 2011). Indeed, laccase activity was not observed with any inorganic nitrogen source in the cultivation of *Grammothele fuligo* in glucose-based medium although it was detected in the control medium containing ammonium chloride as a nitrogen source in experiments with various trace elements (Chauhan 2019). It is useful to note that in this study, the enzyme activity was measured only after 12 days of fungus cultivation. At the same time, the fungus showed maximum LiP activity with ammonium chloride and MnP activity with ammonium acetate. In experiments with *T. multicolor*, ammonium sulfate and ammonium nitrate were suitable sources of nitrogen for the production of laccase and MnP, respectively (Kachlishvili et al. 2018). Besides, ammonium sulfate provided two times higher LiP activity compared with that in the control medium, although the calculations showed it was achieved due to the higher fungal biomass. Moreover, the highest values of laccase activity were observed for *P. eryngii* 616 and *P. ostreatus* 493 when $(NH_4)_2SO_4$ and NH_4Cl, respectively, were supplemented to the medium (Stajić et al. 2006). Likewise, inorganic nitrogen supported high laccase activity in the cultures of *Pleurotus dryinus*, *Pleurotus tuberregium*, and *Pleurotus pulmonarius* (Kachlishvili et al. 2006; Stajić et al. 2006), but organic nitrogen was favorable for high production of MnP in the culture of *P. ostreatus* 108 (Mikiashvili et al. 2006). These and many other data indicate the diversity of the nutritional needs of fungi for nitrogen sources necessary to express their enzymatic potential and also evidence that the nitrogen source optimal for one type of enzyme does not necessarily contribute to the production of another.

The highest value of *Coriolopsis gallica* T906 laccase activity was obtained with ammonium dihydrogen phosphate as nitrogen sources (Xu et al. 2016). Ammonium sulfate and casein were also good sources of nitrogen. Under the solid-state fermentation (SSF) of peach waste by *Pleurotus eryngii* supplementation of medium with 4.0 g/L ammonium nitrate resulted in 12.8 times higher laccase activity than in control culture (Akpinar and Urek 2017). Among nitrogen sources tested in the submerged fermentation of coffee husk (CH) and citric pulp pellet (CP) by *Lentinus crinitus*, only urea favored laccase production in both media, while the addition of yeast extract in CH medium and sodium nitrate in CP medium decreased enzyme activity as compared with control medium (Almeida et al. 2018). Interestingly, the urea concentration of 0.7 g/L was sufficient for maximum laccase production in CH medium and the enzyme production was completely inhibited at the urea concentration of 11.2 g/L whereas exactly this concentration of urea provided the highest laccase activity in the CP medium. The authors suggested that the effect of nitrogen on the laccase production by *L. crinitus* depends on the carbon substrate in the culture medium.

The production of fungal LME depends not only on the chemical nature but also on the concentration of nitrogen. In *P. chrysosporium* synthesis of MnP and LiP is suppressed by high-nitrogen concentrations in the medium and it is induced only during secondary metabolism in response to nitrogen limitation (Kirk and Farrell 1987). However, in the fermentation of lignocellulosic substrates, even a high concentration of organic nitrogen did not inhibit enzyme production in *P. chrysosporium* ME-446, but instead stimulated it (Kapich et al. 2004). Laccase, MnP, and LiP activities of *Coriolopsis polyzona* reached significantly higher values under low-nitrogen (2.2 mM) compared with high-nitrogen (20 mM) conditions (Jaouani et al. 2006). On the contrary, a tenfold increase in laccase production of *Ganoderma lucidum* was observed when nitrogen was increased from 2.4 to 24 mM (D'Souza et al. 1999). Interestingly, in the cultivation of *P. dryinus* only laccase activity increased with nitrogen concentration, whereas the MnP production was not affected (Kachlishvili et al. 2006). Especially distinctive features showed *Cerrena* sp. HYB07 since high carbon (malt dextrin, 60 g/L) and nitrogen (peptone, 10 g/L and ammonium tartrate, 1.6 g/L) concentrations in the fermentation medium provided the highest laccase production (280 U/mL), whereas low-nitrogen concentrations reduced laccase yields to 5.3 U/mL (Yang et al. 2016).

Finally, some above-mentioned and other studies indicate that the ratio of carbon to nitrogen (C/N) is important for the maximum production of the LME. Thus, a high carbon–nitrogen ratio favored laccase productions by *Trametes gallica* (Dong et al. 2005); in contrast, high laccase activity of *P. ostreatus* was observed with low carbon–nitrogen ratio (Hou et al. 2004). These data show a wide variety in the nitrogen requirements of WRB for the production of LME as well as some uncertainty regarding the selection of the optimal nitrogen concentration for enzyme production.

7.2 Aromatic Compounds

The effect of a wide variety of aromatic or phenolic compounds, especially of structurally related to lignin compounds, in the production of LME by WRB has been extensively studied (Eggert et al. 1996; Elisashvili and Kachlishvili 2009; Cambria et al. 2011; Bertrand et al. 2017; Daly et al. 2020). 2,5-Xylidine was the most widely used as a potent inducer of laccase synthesis (Elisashvili et al. 2002; Revankar and Lele 2006; Birhanlı and Yeşilada 2017; Lallawmsanga et al. 2019). However, the laccase production by *T. versicolor* increased twofold when veratryl alcohol or guaiacol was used instead of 2,5-xylidine (Lee et al. 1999). At the same time, in *Lentinus strigosus* veratryl alcohol insignificantly increased the laccase synthesis, whereas 2,6-dimethylphenol promoted eightfold compared to the control (Myasoedova et al. 2008). In the submerged fermentation of mandarin squeeze by *T. multicolor* 511, veratryl alcohol and guaiacol twofold increased the specific laccase activity compared with the control medium, guaiacol fourfold increased the fungus MnP activity, while veratryl alcohol favored LiP secretion (Kachlishvili et al. 2018). Kumari et al. (2019) observed a differential effect of inducers on the

secretion of LME by *Stereum ostrea* in a defined medium containing chlorpyrifos. Gallic acid induced maximum secretion of laccase and MnP; however, the highest production of LiP was recorded in the presence of veratryl alcohol. Interestingly, induction with 0.5 mM resveratrol showed the best laccase activity of *C. gallica*, followed by tannic acid and guaiacol, while ABTS and gallic acid did not affect laccase production (Xu et al. 2016). On the contrary, ABTS and guaiacol increased the *Cerrena* sp. HYB07 laccase production by 40.1 and 26.7%, respectively, whereas other aromatic compounds did not significantly affect enzyme production (Yang et al. 2016). It is interesting that in another strain of *C. unicolor* VKMF-3196 laccase production needed Cu^{2+} ions, but not aromatic compounds (Lisova et al. 2010). These results suggest that the effect of inducer for laccase production depends on the species and/or strains of the tested fungus.

In the early work, Soden and Dobson (2001) showed that 300 μM xylidine, 100 μM 1-hydroxybenzotriazole, 100 μM ferulic acid, and 500 μM veratric acid resulted in 21-, 16-, 10-, and 5-fold increases of laccase activity of *P. sajor-caju,* respectively, compared to the basal culture with no additions. Since one of the functions of fungal laccases is the polymerization of aromatic compounds formed during the degradation of lignin these authors, similarly to Eggert et al. (1996), proposed that laccase induction is a protective reaction of fungal culture to avoid the toxic effects of these compounds. Moreover, it turned out that in some WRB different laccase isoenzyme genes responded differently to the same aromatic inducer. Thus, in *Trametes* sp. AH28-2 o-toluidine induced expression of the laccase A gene, while 3,5-dihydroxytoluene favored laccase B production (Xiao et al. 2006). Guaiacol and p-coumaric acid selectively induced lcc1 and lcc2 expression in *Trametes* sp. I-62 but ferulic acid induced lcc3 expression (Terrón et al. 2004). In the cultivation of *P. ostreatus* HAUCC 162 ferulic, vanillic, coumaric, and cinnamic acids increased the expression of lacc8 and lacc11 genes, but not lacc1 and lacc2 (Zhuo et al. 2017).

Besides fungal species peculiarities, the effect of aromatic compounds on LME production depends on inducer concentration and adjusting the concentration of phenolic/aromatic compounds necessary for individual fungi is essential. In particular, a gradual increase in the concentration of ABTS from 0 to 0.05 mM and 2,5-xylidine from 0 to 1 mM in the glucose-yeast extract basal medium (SBM) increased *T. versicolor* ATCC 200801 laccase activity from 0.6 U/mL to 4.76 U/mL and 2.87 U/mL, respectively (Birhanlı and Yeşilada 2017). Higher concentrations of both compounds significantly decreased the enzyme yields. It is worth noting that under the same cultivation conditions, no induction of laccase production was observed in SBM with 0.025–0.5 mM syringaldazine.

Finally, Lallawmsanga et al. (2019) showed that the effect of aromatic compounds may depend on nutrient medium composition. Thus, the addition of xylidine in MEB medium (malt extract broth) resulted in 2.8-fold induction of laccase activity, while a 1.9-fold increase was observed when xylidine was added to potato dextrose broth medium (PDB). In contrast, the use of $CuSO_4$ as an inducer resulted in higher laccase activity in PDB than in MEB.

7.3 Effect of Microelements

The regulating effect of metal ions on LME activity and gene expression in WRB is well established (Piscitelli et al. 2011; Janusz et al. 2013). Copper is the most important and commonly used metal ion for laccase production. However, the effect of metal ions may vary depending on their concentration and fungal species/strain physiological peculiarities. Thus, in the cultivation of *P. coccineus*, the addition of 0.2 mM $CuSO_4$ to potato dextrose broth significantly upregulated the extracellular laccase activity in five strains of *P. coccineus*, whereas two strains did not respond to Cu^{2+} (Park et al. 2015). Supplementation of glucose-yeast extract medium with 0.5 mM Cu^{2+} increased laccase activity of *T. versicolor* ATCC 200801 from 0.60 to 10.25 U/mL (Birhanlı and Yeşilada 2017). Higher concentrations of copper decreased the enzyme yield with complete inhibition of laccase secretion at 5 mM Cu^{2+}. Supplementation of glucose-peptone-yeast extract medium with as high as 0.5–4 mM $CuSO_4$ led to a 13- to 22-fold increase in laccase activity of *L. strigosus* 1566 when compared with the control (Myasoedova et al. 2015). At the same time, low concentrations of copper ions (10–50 μM) were sufficient to 1.1 to 2.6-fold increase in the laccase activity of *G. lucidum* in comparison with control medium (Liu et al. 2020). When the concentration of added copper ions was increased to 60–80 μM, the laccase activity decreased. The optimal copper dose for the maximum laccase activity for *C. unicolor* C-139 in the glucose and L-asparagine containing culture was found to be only 10 μM (Janusz et al. 2007). Copper concentrations increase from 25 μM to 300 μM Cu^{2+} repressed laccase production (up to 90%) with no effect on fungal growth. The authors showed that the copper supplementation mode was also important. Adding 10 μM Cu^{2+} to the culture medium after 3 days and repeating on the 6th and 9th day of the fungus cultivation led to the greatest activity of laccase. Likewise, the time-dependent effect of copper was observed in *P. ostreatus* ACCC 52857 (Zhu et al. 2016). The laccase activity dramatically increased (more than 80-fold) upon the addition of copper to the medium from the 6th to the 9th day of cultivation. It is interesting that unlike *C. unicolor* C-139, the stimulating effect of even 100 times higher concentration of copper on laccase secretion was revealed in the cultivation of *C. unicolor* CBS 117347 (Kachlishvili et al. 2014).

Many studies proved that metal ions may control the expression of individual isozymes through activation of metal responsive elements (Janusz et al. 2013). Thus, the addition of 0.15 mM copper sulfate to the potato dextrose medium increased production (up to 500-fold) of *P. ostreatus* POXA1b laccase isoform, while POXA1w isoform was not affected (Palmieri et al. 2000). Klonowska et al. (2001) reported that *Marasmius quercophilus* produced only one laccase (LacI) in liquid medium with malt extract. Supplementation of the same medium with $CuSO_4$ induced three other isoforms, increasing the total activity 10 times. Concentrations of 0.05–1 mM Cu^{2+} induced Lac1 and Lac2 of *Coprinus comatus*, while Lac3 was strongly induced by the addition of 3 mM Cu^{2+} (Lu and Ding 2010).

The effect of metal ions other than Cu^{2+} on the LME activity was also widely studied. The addition of Mn^{2+} to the cultures of *C. subvermispora* (Manubens et al.

2007), *P. chrysosporium* (Brown et al. 1991), *T. versicolor* (Johansson et al. 2002), and several other WRB is required for the stimulation of MnP production. According to Brown et al. (1991), manganese is directly involved in the regulation of the mnp gene transcription through a mechanism specific for the growth stage and depending on the concentration. Manganese was a highly potent laccase inducer in *C. comatus* (Lu and Ding 2010). Supplemented to the cultures in the range of 0.05–0.8 mM, Mn^{2+} markedly increased the overall laccase activity and caused the synthesis of additional Lac2 and Lac3 isoforms compared with un-supplemented cultures. Increase of $CuSO_4$ and $MnSO_4$ concentrations from 1 to 3 mM as inducers for LME production by *Marasmiellus palmivorus* VE111 in the medium composed of 0.5% glucose, 0.18% casein, and potato broth led to increased production of the enzymes relative to the control (Schneider et al. 2019). Moreover, the presence of a third isoform in the zymogram of laccase was revealed after the fungus cultivation with inducers. For MnP, only 3 mM $MnSO_4$ significantly induced enzyme production over the control, 2 mM $CuSO_4$ also provided greater enzymatic activity as compared to the control medium, while veratryl alcohol rather decreased the fungus MnP activity.

Testing of the effects of eight microelements on *Grammothele fuligo* growth and enzyme production revealed that the fungus did not show any laccase activity with Ca^{2+}, Mn^{2+}, and Zn^{2+} and no MnP activity with Co^{2+} and Fe^{2+}, but it showed three LME activity at different concentration of B^{3+}, Cu^{2+}, and Mo^{6+} (Chauhan 2019). No effect on laccase production by *P. ostreatus* ACCC 52857was observed after the addition to the medium of K^+, Na^+, Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+}, whereas Cd^{2+}, Pb^{2+}, and Cu^{2+} caused a three- to sixfold increase in laccase activity (Zhu et al. 2016). Finally, it should be noted that the same metal ion may have the opposite effect depending on species or enzyme type. For example, Mn^{2+} induced laccase gene transcription in *P. sajor-caju* (Soden and Dobson 2001), but inhibited gene transcription in *C. subvermispora* (Manubens et al. 2007). Fe^{2+} significantly increased laccase activity in *P. eryngii* (Stajić and Vukojević 2011), while it inhibited laccase production in *G. lucidum* (Murugesan et al. 2009). Moreover, Mn^{2+} stimulated lacc3 and lacc12 genes transcription in *P. ostreatus* HAUCC, but decreased transcription of lacc2 and lacc4 (Zhuo et al. 2017). In the submerged fermentation of mandarin squeezes, an augmentation in copper concentration from 0 to 0.5 mM increased 18-fold the activity of *T. multicolor* laccase but did not significantly affect the activity of MnP and LiP, which indicated that the inductive effect of copper was specific for laccase (Kachlishvili et al. 2018). It is interesting that iron ions at the concentration of 0.1 mM increased almost eightfold both volumetric and specific activity of laccase compared with the control medium. In the same culture, increase in the concentration of manganese from 0 to 0.5 mM only slightly increased the activity of *T. multicolor* laccase, decreased the activity of the fungus LiP, but this metal 13–14 times specifically increased the volume and specific activity of MnP when it was added to the control medium at a concentration of 0.5 mm.

Several studies revealed the synergistic effect of different inducing agents on LME production. Manubens et al. (2007) found a synergistic effect between Mn^{2+} and syringic acid on laccase production in *C. subvermispora*. Laccase activity of

P. ostreatus HAUCC 162 increased from 205.5 U/L in the presence of Fe^{2+} alone to 250 and 363 U/L due to the addition of 0.5 mM vanillic or ferulic acid, respectively (Zhuo et al. 2017). In our study with *C. unicolor* CBS 117347, copper and xylidine added separately accelerated laccase secretion and 1.8-fold improved laccase yield. An additive effect and fourfold increase of laccase activity were observed upon supplementation of copper and xylidine to the medium simultaneously before inoculation. Likewise, supplementation of glucose-yeast extract basal medium (SBM) with 0.5 mM Cu^{2+} increased laccase activity of *T. versicolor* ATCC 200801 from 0.60 to 10.25 U/mL (Birhanlı and Yeşilada 2017). Enzyme production was enhanced considerably (to 33.61 U/mL) due to the synergistic effect of 1 mM 2,5-xylidine and 0.5 mM Cu. Interestingly, no induction of laccase production was observed in SBM with 0.025–0.5 mM syringaldazine alone, but laccase activity reached 22.23 U/mL in medium containing simultaneously 0.5 mM Cu and 0.1 mM syringaldazine. However, conflicting results were obtained when optimal concentrations of caffeic acid and Mn^{2+} were added to *C. comatus* culture, resulting in a decrease in total extracellular laccase activity compared to a culture supplemented with caffeic acid only (Lu and Ding 2010). Nevertheless, the supplementation of nutrient medium with different microelements is a suitable and effective tool to regulate the production of individual LME.

7.4 Effect of Co-cultivation

Co-cultivation of WRB may be a promising strategy to significantly improve LME production. Previous studies have shown that laccase activity of *L. edodes* (Savoie et al. 1998), *C. unicolor* (Elisashvili et al. 2014), *P. ostreatus*, *T. versicolor*, and several other basidiomycete species (Baldrian 2004) significantly increased in their co-cultivation with *Trichoderma* sp. The mixed cultivation of *Gongronella* sp. W5 with *Panus rudis* (Wei et al. 2010) and *Shiraia bambusicola* and *Phoma* sp. BZJ6 (Du et al. 2017) caused a 25-fold and 9.2-fold increase, respectively, of dual cultures laccase activity as compared with those in their monocultures.

Few studies were performed in the co-cultivation of basidiomycetes. Among four fungi tested, high stimulation of laccase production was observed only in the co-cultivation of *P. ostreatus* with *C. subvermispora*, while MnP activity was stimulated in the co-culture with both *C. subvermispora* and *Physisporinus rivulosus* and Western blotting revealed proteins from both fungi (Chi et al. 2007). However, in the co-cultivation of *C. subvermispora* with *P. rivulosus* low MnP activity was detected and the protein pattern resembled that of *P. rivulosus*, indicating that this fungus suppressed *C. subvermispora* growth. Similarly, among six WRB cultured in pairs for LME production, *Hypoxylon fragiforme* inhibited the expression of LME during co-cultivation with *P. ostreatus*, *Dichomitus squalens* inhibited MnP expression by *P. ostreatus* or *Phlebia radiata* (Qi-he et al. 2011). At the same time, the co-culture of *P. radiata* with *D. squalens* showed the maximum specific laccase activity but the co-culture of *P. radiata* with *P. ostreatus* was beneficial for MnP and LiP expression.

Table 7.2 Enzymatic activity of mono- and dual cultures of WRB in fermentation of mandarin squeeze on agar plates and in submerged cultures

	Laccase (U/mL)			MnP (U/mL)
	Solid medium		Liquid medium	Liquid medium
	Distance between colonies			
Fungi	1 cm	4 cm		
C. unicolor 305	4.9 ± 0.3	4.5 ± 0.2	140.3 ± 19.3	1.28 ± 0.20
L. betulina 141	4.3 ± 0.2	3.9 ± 0.2	16.1 ± 2.0	0.05 ± 0.01
P. lecometei 903	2.2 ± 0.1	2.6 ± 0.2	6.4 ± 1.0	0
P. coccineus 310	1.8 ± 0.1	1.5 ± 0.1	4.3 ± 0.6	0
T. hirsuta 82	2.2 ± 0.1	2.3 ± 0.2	27.2 ± 3.0	0.21 ± 0.02
T. versicolor 159	5.2 ± 0.2	5.3 ± 0.2	65.8 ± 10.4	0.58 ± 0.09
C. unicolor 305 + *L. betulina* 141	7.3 ± 0.5	6.0 ± 0.3	254.8 ± 32.9	0.35 ± 0.05
C. unicolor 305 + *P. lecometei* 903	2.3 ± 0.2	2.5 ± 0.3	224.4 ± 37.0	0.25 ± 0.05
C. unicolor 305 + *P. coccineus* 310	5.7 ± 0.4	5.1 ± 0.5	21.3 ± 3.8	0.44 ± 0.05
C. unicolor 305 + *T. hirsuta* 82	4.8 ± 0.2	5.6 ± 0.7	16.6 ± 2.1	0.10 ± 0.01
C. unicolor 305 + *T. versicolor* 159	6.5 ± 0.5	9.2 ± 0.5	318.0 ± 43.5	0.81 ± 0.15
P. coccineus 310 + *T. hirsuta* 82	1.5 ± 0.1	2.6 ± 0.2	51.8 ± 5.5	0.03 ± 0.01
P. coccineus 310 + *T. versicolor* 159	3.8 ± 0.2	7.7 ± 0.6	5.4 ± 0.6	0

In our work, we attempted to improve LME production through interspecific interaction of the best enzyme producer *C. unicolor* 305 with 5 different WRB species in the submerged fermentation of mandarin peels. Moreover, their growth and interactions were simultaneously verified on agar plates using the same medium composition. On the solid medium, a distance between inoculated fungal colonies was 1 and 4 cm. None of the dual cultures demonstrated invasion or replacement over 10 days after the creation of confrontation zones. All pairs inoculated at a distance of 4 cm formatted delineated barrages in mycelial contact zones although without brown pigmentation. Among closely inoculated fungi, only *C. unicolor* 305 and *L. betulina* 141 formed a faintly visible barrage.

The results presented in Table 7.2 show several distinctive features of the tested cultures. Firstly, like the above-mentioned data, the enzymatic activity of the dual cultures depended on the fungal species combination. Co-cultivation of *C. unicolor* 305 with *L. betulina* 141 and *T. versicolor* 159 on both solid and liquid media was the most beneficial for the laccase production and their synergistic interaction led to a much higher enzyme activity as compared with those produced by either monoculture. Secondly, it turned out that the cultivation method strongly affected the laccase activity of individual fungal pairs. Thus, pairs of *C. unicolor* 305 + *P. lecometei* 903 and *P. coccineus* 310 + *T. hirsuta* 82 demonstrated an upregulation of laccase activity when replacing SSF with submerged liquid fermentation. On the contrary,

interspecies interaction of *C. unicolor* 305 + *P. coccineus* 310 and *P. coccineus* 310 + *T. versicolor* 159 on the solid medium promoted laccase production, whereas in the submerged fermentation their co-cultivation inhibited laccase activity. Also, it should be noted that in monocultures grown on a solid medium, the laccase activity of the tested fungi differed less than three times, while in submerged cultures the enzyme activity varied from 4.3 to 140.3 U/mL. Thirdly, although no evident regularities were found between a distance of inoculation and enzyme activity values our findings indicate that in some pairs (for example, *P. coccineus*310 + *T. versicolor* 159) the distance between inoculated colonies can determine the outcomes of the interaction. Fourthly, no stimulation of MnP activity was observed in this study; on the contrary, a combination of *C. unicolor* 305 with other fungi 3 to 12-fold downregulated MnP activity of this fungus. Finally, the interaction of only live fungi is required to observe an increased outcome since the cultivation of *C. unicolor* 305 with a thermally inactivated mycelium of *T. versicolor* 159 did not increase laccase activity. Overall, it is clear that the process of interspecies interaction is complex and its result may depend not only on the particular microbe's combination, but also on physiological peculiarities and enzymatic systems of individual competitors, nutritional conditions in dual culture, and space they occupy. Compared with the SSF, in the submerged fermentation, mycelium of the dual culture has permanent access to the available nutrients, but the degree of fungal competition is closely related to the initial concentration of the nutrients. Undoubtedly, individual competitors' inoculum size, ratio, time, and sequence of their inoculation should be considered in the co-cultivation study since these parameters can significantly affect enzyme activities. An understanding of the peculiarities and mechanisms of interspecies interaction of WRB is essential for the development of improved technologies of LME production.

7.5 Conclusion

LME constitute one of the most important groups of versatile enzymes for industrial applications. Owing to the progress in recent research, screening of potent enzyme producers, genetic engineering, omics-based data, understanding of physiological and transcriptional regulation of individual LME synthesis, the production of these enzymes was maximized. Nevertheless, there are still many limitations that need to be overcome, and further research is needed, in particular, using various genetic methods and the recombinant technologies to improve the production of LME and to obtain these biocatalysts in quantity and quality, satisfying various fields of application. The use of cheap lignocellulosic materials, metals, and aromatic inducers, clarification of the molecular mechanisms triggering and regulating LME expression in a response to different stimuli may boost additional production of LME at low cost. Besides, elucidation of the factors that impede the production of enzymes is also one of the most challenging tasks. Mixed cultivation, as well as the development of process design in the lignocellulose SSF, should be considered as encouraging and cost-efficient strategies.

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Endophytic Bacterial Applications in Phytoremediation of Organic Pollutants and Toxic Metals

8

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Abstract

The rising concentration of organic pollutants in soil and water matrices has always posed a global concern of health hazards, including humans. In the process of bioremediation, phytoremediation is supposed to be the best eco-friendly process. With the endophytes, phytoremediation promises efficient removal of organic pollutants and heavy metals. The mutual interaction of endophytes and plants provides shelter to microbes and growth promotion of plants with many more benefits. Their interaction leads to accumulation and removal of pollutants with minimum external chemical entities leaving wastewater. Although phytoremediation is a slow and time-consuming process, many plants showed excellent results in removing the organic pollutants and heavy metals combined with their endophytic communities. The enzymes of endophytes can change the chemical moieties of contaminants and make them less toxic to the environment, and hence they may be explored more with the help of 'Omics.' The Omics and systems biology approach may be useful in future applications on the contaminated sites. This chapter provides collective information about the endophytic bacteria assisted phytoremediation and updates us to create an eco-friendly environment.

Keywords

Endophytes · Phytoremediation · Organic pollutants · Metals · Omics

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8.1 Introduction

Persistence of organic pollutants and heavy metals over a longer period has resulted in pollution of soil and water bodies along with the disruption in the metabolic activities of the related plants, animals, and microbial community (Sun et al. 2013; Havelcová et al. 2014). The decontamination of the pollutants is an emerging global problem (Wei et al. 2014). Endophytic bacteria, along with their associated plants, can play a crucial role in minimizing the load of organic pollutants from the environment. Bacteria and plants have a symbiotic relation, where bacteria help in the metabolic reactions of the plants (Tervet and Hollis 1948; Hollis 1949). Phytoremediation emerged as an environment-friendly, non-hazardous, and economically beneficial technology for the removal of the organic contaminants while the traditional methods are inappropriate and non-eco-friendly (Pilon-Smits 2005; Xia 2004; Schwitzguébel et al. 2009; Pandey et al. 2009). The organic pollutants inhibit metabolic activities of soil and water-related microbes because of their hydrophobic nature, toxic effect, and long persistence (Karickhoff et al. 1979) and even disturb the food chain of surrounding organisms (Kelly et al. 2007), so for eco-friendly removal of these organic pollutants from contaminated sites, plants which show phytoremediation activities are used for the clean up of natural bodies. In soil, there is a reduction in the efficiency of phytoremediation of the organic pollutant hence the plants have to be adapted by the contaminated sites for their complete action (Escalante-Espinosa et al. 2005). Plant and their associated microorganisms might play a role in pollutant degradation or plant growth promotion (Weyens et al. 2009a; Glick 2010; Ma et al. 2011).

In the symbiotic relationship, plant provides shelter and nutrients while bacteria help in plant growth promotion (Fig. 8.1), stress tolerance, provide metabolites, and make clean and detoxified environment for the plants (Hallmann et al. 2001). Endophytic bacteria are non-pathogenic to plants and reside and colonize inside them. Rhizobacteria are found in the close proximities of plant roots, and their phytoremediation capacities have been reported in the scientific studies, but in comparison to endophytic bacteria, they colonize inside the plants so interact more closely and efficiently with their host plant (Tan and Zou 2001; Rosenblueth and Martínez-Romero 2006; Ma et al. 2013; Kumar et al. 2016). Recent studies show the enhancement in the phytoremediation efficiency of plants in association with endophytic bacteria (Barac et al. 2004; Doty 2008; Andreolli et al. 2013; Oliveira et al. 2014; Phillips et al. 2008). Bacteria can help in the degradation of the organic pollutants (Kumar et al. 2019a, b) and show enhanced metabolic activities when associated with plants (Khan et al. 2013a; Weyens et al. 2009c). According to the reports of Afzal et al. 2011, endophytic bacteria have certain genes that have the potential to mineralize organic contaminants. Endophytic bacteria can also help in the accumulation of heavy metal from soil (Ma et al. 2011; Rajkumar et al. 2009). Thus phytoremediation promises a sustainable future with minimal residues post-treatment.

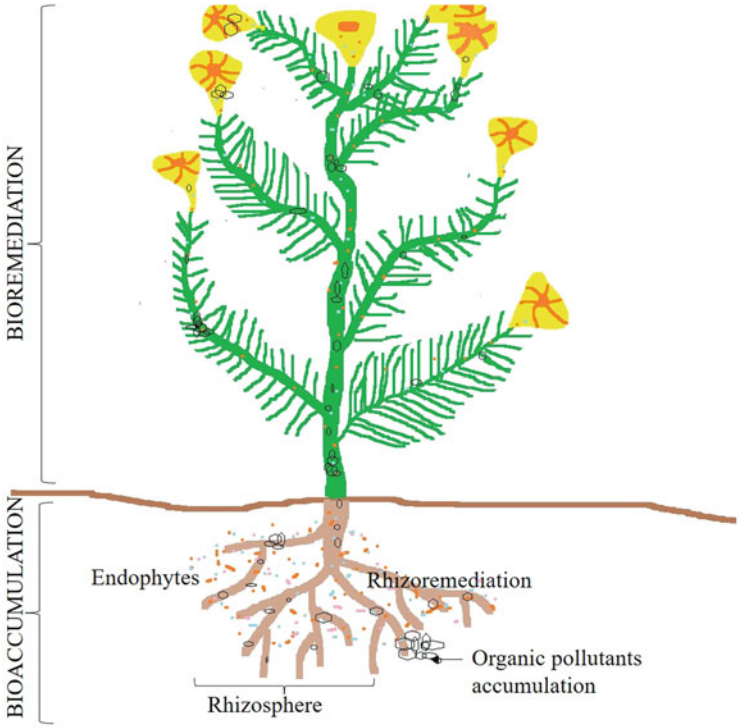

Fig. 8.1 Plant showing diverse microbial communities colonizing the root, stems, leaf, and flower along with the organic contaminants

8.2 Endophytic Bacteria

Endophytic bacteria reside inside the plant in a mutual benefit relation without causing any harm to plants (Sturz et al. 2000; Weyens et al. 2009a, b, c; Hamilton et al. 2012). Unlike Rhizobacteria which reside near roots, endophytes can live in any part of the plant (Figs. 8.1 and 8.2) including roots as the central entry area (Ryan et al. 2008). The majority of them resided and flourished in the soil environment (Compant et al. 2010).

Before entering the plant, they are first attracted by the phytochemo-attractant and then attach to plant root or tissue wounds via flagella and pili (Sturz et al. 2000; Zeidler et al. 2004). These bacteria secrete some cell wall degrading enzymes for making their path easy for penetration (Quadt-Hallmann et al. 1997; Reinhold-Hurek et al. 2006). After entering the plants either they reside at the entry point or spread to other parts with the help of flagella (Hallmann et al. 1997; Hurek et al. 1994). These endophytes produce degradation enzymes and show significant metabolizing activities against them (Hurek et al. 1994; Rosenblueth and Martínez-Romero

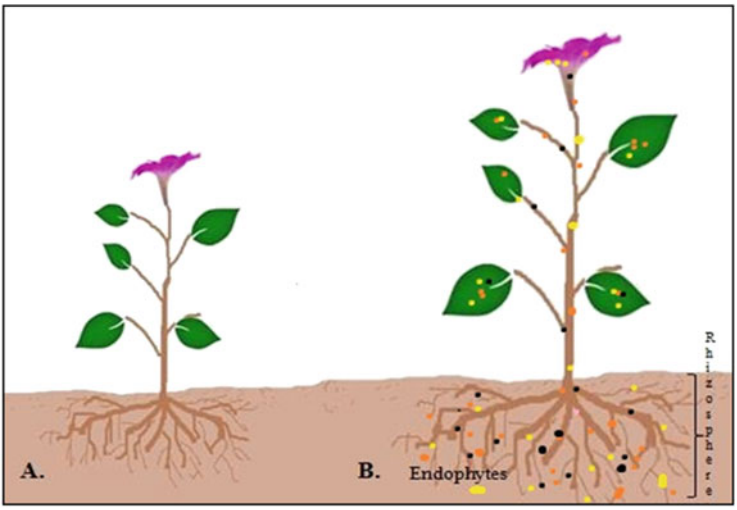

Fig. 8.2 The plant (**a**) without endophytic bacteria and plant (**b**) with endophytic bacteria showing growth promotion

2006). Rhizosphere is the primary nutrient-rich niche for microbes, but due to competition, some microbes have penetrated inside the plant tissues and are called as endophytic bacteria (Hardoim et al. 2008).

However, the study of the whole microbiome, inter-microbial interaction, and relation between genetic content, their expression, and its effect on transcriptional and translational mechanism will collectively provide us information about the molecular behavior and the switching of genes in the microbes (Bell et al. 2014a, b; Imam et al. 2016, 2017; Dangi et al. 2019). Different metabolites (e.g., ACC deaminase, siderophores, Indole-3-acetic acid, and organic acids.) produced by plant-associated bacteria (plant growth-promoting bacteria) are known to be involved in many processes operating in plants rhizosphere (Rajkumar et al. 2012). These bacteria move from a root-soil environment to the other tissues and organs of the plant and reside successfully because of natural adaptation mechanisms (Glick 2010; Barret et al. 2011; Loaces et al. 2011). These endophytes are of different origin and have an independent life at the molecular level. These bacteria, in comparison to the rhizobacteria, have the advantage of residing inside the plant and protect themselves from the external biotic and abiotic factors (Seghers et al. 2004; Reinhold-Hurek and Hurek 2011). Endophytes not even degrade the organic pollutants but also make a cleaner environment for plant growth (Hurek et al. 1994; Khan et al. 2013b). According to Weyens et al. (2010a, b), endophytes are good colonizer and active metabolizer in endosphere and rhizosphere, but some endophytes show more efficient colonizing and metabolic activities in a specific plant species due to adaptation advantage, and both these characteristics differ in different endophytic bacteria and plants (Andreote et al. 2010; Phillips et al. 2008).

8.3 Ecological Significance of Endophytic Bacteria

In the contaminated site, most of the plants fail to grow, but few of them harbors bacteria, which not only grow but also degrades the organic contaminant efficiently (Phillips et al. 2008). Interestingly some endophytes have not been previously exposed to pollutants but show degradation activities (Sessitsch et al. 2012). The diversity of endophytes depends on the concentration of organic and inorganic contaminants in water and soil (Peng et al. 2013; Glick 2010). These endophytes get simple sugars, amino acids, and other nutrients in plants, intercellular spaces, and proliferate successfully (Bacon and Hinton 2007). Many endophytic bacteria are listed in Table 8.1 in association with their respective plants. Endophytes have been reported to successfully degrade the hydrocarbon in contamination sites; and predominately present in root interior (Siciliano et al. 2001). Different plant species influence different endophyte diversities (van der Lelie et al. 2009; Yousaf et al. 2010). Endophytic bacteria isolated from poplar trees are reported to be a good herbicide degrader and can degrade (2,4-D), xylene, toluene, benzene, and ethylbenzene (Germaine et al. 2006; Taghavi et al. 2011; Weyens et al. 2011). Volatile organic pollutant e.g., trichloroethylene (TCE) is degraded by endophytes and reported in English oak (*Quercus robur*), Common ash (*Fraxinus excelsior*), and poplar trees, vegetated in TCE contaminated soil (Weyens et al. 2010a; Moore et al. 2006). Endophytes are not only able to degrade the organic pollutant but also have resistance against some toxic metals and show enhanced phytoremediation. Some endophytic bacteria utilize persistent organic compounds (present in contaminated soil and water) as a sole carbon source (Wrenn and Venosa 1996; Haritash and Kaushik 2009). These endophytes mainly belong to the family *Alphaproteobacteria*, *Actinobacteria*, *Gammaproteobacteria*, *Flavobacteria*, and *Bacillaceae*. Previous studies depicted that endophytes application can cause degradation of alkanes, hydrocarbons, and pesticides along with that aid in plant growth promotion such as nitrogen fixation, auxin, and siderophore production (Elbeltagy et al. 2001; Rajkumar et al. 2010). The diversity and specificity of pollutant-degrading endophytes vary according to their environment, the quantity and quality of pollutants to which they are exposed, and on the plant genotype as well (Barac et al. 2004, 2009; Ulrich et al. 2008). The endophytic bacteria from rice plants can degrade aliphatic and aromatic hydrocarbons (Sessitsch et al. 2012). The genomic study of *Burkholderia phytofirmans* PsJN, a plant growth-promoting endophyte, suggests that they possess hydrocarbon-degrading genes alkB and CYP450a, although the strains are not exposed to pollutants previously (Afzal et al. 2013; Mitter et al. 2013). The pollutant degrading endophytes selectively colonize the plant parts, e.g., alkane degrader root endophytes, colonize better in plant roots while shoot-endophytes prefer to shoot for better colonization (Yousaf et al. 2010). The other parts of plants are not that helpful in aiding degradation such as stem and leaves. In the early stage of plant growth, lower densities of endophytic bacteria are found which indicates the establishment stage of endophytes and later on these bacteria along with growth show the degradation of organic pollutants (Afzal et al.

Table 8.1 Many endophytic bacteria have been identified and studied for the bioremediation techniques

S. No.	Plant	Endophytic bacteria	Reference
1.	Potato (*Solanum tuberosum*)	*Bacillus* sp.	Hollis (1949)
2.	Tomato	*Pseudomonadaceae*	Samish et al. (1963)
3.	Potato (*Solanum tuberosum*)	*Micrococcus* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Bacillus* sp., *Xanthomonas* sp., *Agrobacterium* sp.	Boer and Copeman (1974)
4.	Rough lemon (*Citrus jambhiri*)	*Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Corynebacterium* sp., *Lactobacillus* sp., *Serratia* sp.	Gardner et al. (1982)
5.	Sugar beet (*Beta vulgaris L.*)	*Bacillus* sp., *Erwinia* sp., *Pseudomonas* sp., *Corynebacterium* sp., *Lactobacillus* sp., *Xanthomonas* sp.	Jacobs et al. (1985)
6.	Alfalfa (*Medicago sativa L.*)	*Pseudomonas* sp., *Erwinia* sp.	Gagné et al. (1987)
7.	Corn (*Zea mays*)	*Pseudomonas* sp., *Bacillus* sp., *Corynebacterium* sp.	Lalande et al. (1989)
8.	Cotton (*Gossypium hirsutum L.*)	*Erwinia* sp., *Bacillus pumilus.*, *B. brevis.*, *Clavibacter* sp., *Xanthomonas* sp.	Misaghi and Donndelinger (1990)
9.	Corn (*Zea mays*)	*Enterobacter* sp., *Pseudomonas* sp., *Klebsiella* sp., *Vibrio* sp.	Fisher et al. (1992)
10.	Sugarcane (*Saccharum officinarum) CV NIF-8*	*Herbaspirillum seropedicae*, *H. rubribalbicans*, *Acetobacter diazotrophicus*	Dong et al. (1994)
11.	Grapevine	*Pseudomonas* sp., *Enterobacter* sp., *Pantoea* sp., *Klebsiella* sp., *Staphylococcus* sp., *Clavibacter* sp., *Bacillus* sp., *Curtobacterium* sp., *Xanthomonas* sp., *Rhodococcus* sp.	Bell et al. (1995)
12.	Cotton (*Gossypium hirsutum L.*)	*Enterobacter* sp.	Musson et al. (1995)
13.	Corn (*Zea mays*)	*Burkholderia* sp., *Enterobacter* sp., *Methylobacterium* sp., *Bacillus* sp.	Mcinroy and Kloepper (1995)
14.	Cotton (*Gossypium hirsutum L.*)	*Agrobacterium* sp., *Serratia* sp., *Burkholderia* sp. *Bacillus* sp., *Staphylococcus* sp., *Rhizobium* sp., *Variovorax* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Arthrobacter* sp., *Enterobacter* sp.	Mcinroy and Kloepper (1995)

(continued)

Table 8.1 (continued)

S. No.	Plant	Endophytic bacteria	Reference
15.	Cucumber (*Cucumis sativus*)	*Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Agrobacterium* sp., *Burkholderia* sp., *Arthrobacter* sp., *Stenotrophomonas* sp., *Chryseobacterium* sp.	Mahaffee and Kloepper (1997)
16.	Rice (*Oryza sativa L.*)	*Azorhizobium* sp.	Reddy et al. (1997)
17.	Wheat (*Triticum aestivum*)	*Azorhizobium* sp.	Webster et al. (1997)
18.	Rice (*Oryza sativa L.*)	*Azoarcus* sp., *Herbaspirillum* sp.	Reinhold-Hurek and Hurek (1997, 1998)
19.	Strawberry	*Pseudomonas fluorescens (type A, F, G)*, *Pseudomonas corrugata*, *Pseudomonas tolaasii*, *Pseudomonas paucimobilis*, *Enterobacter cloacae*, *Xanthomonas* sp.	Tanprasert and Reed (1997)
20.	Red clover (*Trifolium pratense L.*)	*Serratia* sp., *Agrobacterium rhizogenes A.*, *Rhizobium loti B.*, *Acidovorax* sp., *Arthrobacter* sp., *Bacillus* sp., *Curtobacterium* sp., *Enterobacter* sp., *Pseudomonas* sp., *Klebsiella* sp., *Methylobacterium* sp., *Micrococcus* sp., *Pantoea* sp., *Variovorax* sp., *Xanthomonas* sp.	Sturz et al. (1998)
21.	Wheat (*Triticum aestivum*) and Canola (*Brassica napus L.*)	*Flavobacterium* sp., *Micrococcus* sp., *Bacillus* sp., *Rathayibacter* sp.	Germida et al. (1998)
22.	Rice (*Oryza sativa L.*)	*Methylobacterium* sp., *Azospirillum* sp., *Klebsiella* sp., *Sphingomonas* sp., *Flavobacterium* sp., *Cytophagales* sp., *pantoea* sp., *Rhodopseudomonas* sp., *Herbaspirillum* sp.	Elbeltagy et al. (2000)
23.	Citrus plants	*Alcaligenes* sp., *Bacillus lentus*, *B. cereus*, *B. pumilus*, *B. megaterium*, *Burkholderia cepacia*, *B. subtilis*, *Enterobacter cloacae*, *Curtobacterium flaccumfaciens*, *Pantoea agglomerans,* and *Methylobacterium extorquens*	Araújo et al. (2001)

(continued)

Table 8.1 (continued)

S. No.	Plant	Endophytic bacteria	Reference
24.	Yellow lupine	*Bacillus cepacia*	Barac et al. (2004)
25.	Poplar	*Burkholderia cepacia VM 1468*	Taghavi et al. (2005)
26.	Pea	*P. putida* VM1450	Germaine et al. (2006)
27.	Wheat (*Triticum* sp.) and maize	*Enterobacter* sp.	Sheng et al. (2008)
28.	Poplar	*P. putida* W619-TCE	Weyens et al. (2009c)
29.	Poplar	*Enterobacter* sp.	Taghavi et al. (2009)
30.	*Ipomoea aquatica*, *Phragmites australis*, and Vetiveria zizanioides	*Achromobacter xylosoxidans*	Ho et al. (2009)
31.	Italian ryegrass (*L. multiflorum var. Taurus*)	*Pseudomonas* sp.	Reichenauer and Germida (2008)
32.	Pea	*Pseudomonas putida*	Germaine et al. (2009)
33.	Poplar	Indigenous sp.	Barac et al. (2009)
34.	Birdsfoot trefoil (L. corniculatus var. Leo)	*P.* sp. strain ITRI15	Yousaf et al. (2010)
35.	Corn and wheat	*Burkholderia cepacia* strain FX2	Yang et al. (2011)
36.	Poplar	*Pseudomonas putida* W 619-TCE	Weyens et al. (2010a)
37.	Yellow lupin	*Bacillus cepacia* VM 1468	Weyens et al. (2010b)
38.	Poplar	*Burkholderia cepacia* VM 1468	Taghavi et al. (2011)
39.	Poplar	*P. putida* strain	Weyens et al. (2013)
40.	Alfalfa (*M. sativa var. Harpe*) and Birdsfoot trefoil (*L. corniculatus var. Leo*), Italian ryegrass (*L. multiflorum var. Taurus*)	*Enterobacter ludwigii* strains	Pau-Roblot et al. (2013)
41.	Italian ryegrass (*L. multiflorum var. Taurus*)	*Pseudomonas* sp. strain ITRI53, *Pseudomonas* sp. strain MixRI75	Afzal et al. (2011, 2012)
42.	Popla	*Enterobacter* sp. strain PDN3	Kang et al. (2012)
43.	Willow	*Pseudomonas* sp. HU002, *Burkholderia* sp. HU001	Weyens et al. (2013)

2011). In addition to plant growth promotion, endophytic bacteria help in nitrogen fixation (Ali et al. 2012).

8.4 Uptake of Organic Pollutants by Plants

Plants absorb the organic contaminants from the soil through the passage system, and it gets transported to the whole plant via their transportation system. Many plants have been reported to degrade the organic contaminants which include ryegrass (Ryan et al. 2008), rice (Lodewyckx et al. 2002), zucchini (Afzal et al. 2013; Zhu et al. 2014a, b), maize (Ryan et al. 2008), cauliflower (Arshad et al. 2007), poplar (Barac et al. 2004), radish (Shaw and Burns 2003), pumpkin (Arslan et al. 2015), tobacco (Doty 2008), and the nightshade plants (Zhu et al. 2014a, b). These plants have the capability to uptake and accumulate organic pollutants in different organs. Cattle feeding on these plants increase the rate of organic pollutant gets exposed to it and therefore increase the chance of exposure to humans (Cang 2004). Common organic pollutants commonly found in soil and wastewater are listed in Table 8.2.

These absorbed pollutants have different fates. Methyl *tert*-butyl ether (MTBE) is toxic to the environment, absorbed by plants (Yu and Gu 2006) and phytovolatilization is supposed to be only a relevant removal mechanism of it. MTBE is sensitive to photo-oxidation in the presence of plant enzymes. Hence, phytoremediation is an excellent and effective technology for remediation of organic and inorganic pollutants (Zhang et al. 2013). The plant shows a variety of processes by which remediation of contaminants takes place in different kinds of environments. According to Tangahu et al. 2011, rhizodegradation is vital for the degradation of organic xenobiotics, phytovolatilization in which pollutant are converted to volatile forms (Marques et al. 2009), phytoextraction in which pollutant are accumulated in plant biomass (Bhargava et al. 2012), phytotransformation in which highly toxic contaminants convert to less toxic forms (Marques et al. 2009), phytodegradation in which organic xenobiotics degradation occurs within plant tissues (Khan 2005), and phytofiltration in which sequestering of contaminants is done by plants from polluted water (Ghosh and Singh 2005). Table 8.2 gives us an idea about the variety of organic contaminants that pollutes our freshwater. Once uptaken by plants, the plants degrade these pollutants and release volatile pollutants in the environment via evaporation. Therefore, endophytes seem to play an essential role in the degradation of these compounds and to minimize their load from the environmental (Newman and Reynolds 2005).

Organic pollutant uptake by plants is impacted by both abiotic and biotic processes (Wania and Mackay 1999; Singh and Jain 2003). Abiotic processes involve the physicochemical nature of the soil, type of land, contaminants concentration, and their K_{ow} value, the moisture content of soil (Pandey et al. 2009). The biotic processes involved the symplastic and apoplastic mode of transportation (Su and Zhu 2007). The dinitrotoluene and dinitrobenzene follow the symplastic pathway, whereas phenanthrene and pyrene follow the apoplastic pathway. Plants can absorb the organic pollutant from the environment, and it gets transported in the interior of

Table 8.2 Frequently found organic pollutants in the environment

Aniline	Acetanilide
Acetophenone	Allyl phenyl ether
Atrazine	Anisole
Benzophenone	Bromobenzene
Benzyl benzoate	Biphenyl
Benzyl alcohol	Benzonitrile
benzene	chlorobenzene
Cinnamic alcohol	Diphenylamine
Dibenzyl	DDT
Ethyl benzoate	Fluoranthene
Dodecanoic acid	Diphenyl ether
Phenanthrene	n-butyl benzene
Triphenyl amine	Naphthalene
Phenyl benzoate	Isopropyl benzene
Thymol	Ethylbenzene
Toluene	Nitrobenzene
Benzoic acid	p-cresol
Trans-cinnamic acid	Methyl benzoate
Phenyl acetonitrile	Phenol
Phenoxy acetic acid	Trichloroethene
2-butanone	4-acetyl pyridine
4-methoxy phenol	2,4-dinitrophenol
4-methyl benzyl alcohol	4-chloraniline
3-nitrobenzoic acid	2-nitrophenol
3-chlorbenzoic acid	1-Napthol
2,3-dichloro aniline	4-phenyl phenol
1,4-dichlorobenzene	2,4,6-trichlorophenol
2,4-dinitro-6-sec-butyl phenol	1,2,4-trichlorobenzene
2,6-diphenyl pyridine	2,6-dichlorobenzonitrile
Cis-cinnamic acid	

the plant cell and makes conjugate with the endogenous glutathione and tripeptides (Reichenauer and Germida 2008). Some pollutants accumulate in the plant's cell vacuoles from where they are supposed to evaporate in the atmosphere. The fate of organic pollutants may be changed during plant passage because they may be either transformed into less toxic, more toxic, or remain unchanged by the plant's redox enzymes system and associated endophytes (Zhu et al. 2014a, b).

8.5 Oxidation and Reduction of Metals by the Endophytes

Endophyte along with the degradation of organic pollutants also helps in altering the mobility of heavy metals, e.g., copper mobility is enhanced by sulfur-oxidizing rhizospheric bacteria by alkaline reactions (Shi et al. 2011). Iron-sulfur oxidizing

Table 8.3 Some metal uptake plants in association with endophytic bacteria

S. No.	Plant Used	Metal	Bacteria	Reference
1.	*Alnus firma*	As, Cu, Cd, Ni, Pb, and Zn	*Bacillus thuringiensis* GDB-1	Babu et al. (2013)
2.	*Polygonum pubescens*	Cd, Pb, and Zn	*Rahnella* sp. *JN6*	He et al. (2013)
3.	*Sedum alfredii Hance*	Cd and Zn	*Burkholderia* sp. SaZR4, *Burkholderia* sp. SaMR10, *Sphingomonas* sp. SaMR12, and *Variovorax* sp. SaNR1	Zhang et al. (2013)
4.	*Pteris vittata* and *P. multifida*	As	Endophytes belonged to *Firmicutes*, *Proteobacteria*, and *Actinobacteria*	Zhu et al. (2014a, b)
5.	*Pelargonium graveolens*	Cr	*P. monteilii* PsF84, *P. plecoglossicida* PsF610	Dharni et al. (2014)
6.	*Solanum nigrum*	Cd, Zn, and Cu	*Pseudomonas* sp. Lk9	Chen et al. (2014)
7.	*Amaranthus hypochondriacus* and *A. mangostanus*	Cd	*Rahnella* sp. JN27	Yuan et al. (2014)
8.	*Miscanthus sinensis*	As, Cd, Cu, Pb, and Zn	*Pseudomonas koreensis* AGB-1	Babu et al. (2015)

bacteria enhance metal bioavailability in the soil through acidification reaction (Shi et al. 2011; Chen and Lin 2001). Chromium resistant endophyte *Cellulosimicrobium cellulans* reduce Cr uptake in the shoot by 37% and in roots by 56% (Chatterjee et al. 2009). Similarly, selenium uptake reduction has also been reported (Di Gregorio et al. 2005). These oxidizing and reducing endophytes also show synergistic actions (Beolchini et al. 2009). The bacteria possess MerA (mercuric reductase) enzyme, suitable to reduce the toxic ionic mercury into its detoxified form by using NADPH. This showed the potential of plants and their harbored bacteria in the field of bioremediation.

The toxic metals have the potential to inhibit microbial fauna, along with the degradation of toxic waste (Sandrin and Maier 2003). Table 8.3 provides us brief and up-to-date information about the symbiosis of plant and endophytes and their remediation of target metals. Many plants showed good results for the accumulation of cadmium, which is a good sign for phytoremediation as a technique that has a good future prospect in terms of decontamination of pollutants from the environment. Some metals and their related plants, which have the capacity for phytoremediation are given in Table 8.3. Two willows species *Salix babylonica L.* and *Salix matsudana Koidz* were investigated for chromium bioremediation using roots and leaves. Cr (III) removal was observed faster than Cr (VI) in both

the species (Zhang et al. 2013). Although both the species were able to bioremediate the chromium, the hybrid willow (*Salix matsudana Koidz* × *alba* L.) checked for the bioaccumulation of Cr (III) where root and stem were found to be accumulating a significantly higher amount of chromium whereas translocation through leaves was limited (Yu and Gu 2007a, b, c).

The bioaccumulation of Cr (III) and Cr (VI) in plants is affected by the nutrient cycles and seen as a difference in their translocation (Yu and Gu 2008b). Chromium bioaccumulation does not affect the willow plant's physiology and functions, while they are ecologically safe for metal bioremediation (Zhang et al. 2013). Uptake of Cr^{6+} and Cr^{3+} was minorly affected in the presence of EDTA while translocations of both the ionic forms are positively affected (Yu and Gu 2008d; Zhang et al. 2013). Weeping willow (*Salix babylonica*) was also checked for their biotransformation and metabolic capabilities to cyanide (Yu et al. 2007). Most bioaccumulation was seen in roots of the plant while another positive side of this phytoaccumulation was observed as it helps in plant growth-promotion, enhances the transpiration rates, and affects the important metabolic enzymes, e.g., superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). Weeping willows did not show any toxic effect on their growth and other metabolic activities and showed >97% metabolism of the applied cyanide in the experiment, and all this may be due to the well-established detoxification system in willows (Zhang et al. 2013). Maize was also studied for their metabolic removal of cyanide and suggested that high biomass, fast-growing plants should be preferred over the high metabolic rated slow growth plants (Zhang et al. 2013). Maize (Zea mays L. var. ZN 304) and weeping willow (*Salix babylonica*) plants were checked for the phytoaccumulation of ferrocyanide and ferricyanide and it is observed that maize was able to remove them from the provided source, which indicates the potential of maize in phytoremediation technology (Yu et al. 2008; Yu and Gu 2008c, 2009; Zhang et al. 2013). In the year 2008a, Yu and Gu also studied the uptake and translocation of selenate and selenite by wild and hybrid willow plants and significant removal of both observed clearly. An environmental load of heavy metals contamination can influence the activities of the plant. The plant growth promotion was observed at a low concentration of selenium, while at higher concentrations, the transpiration rate decreased (Yu and Gu 2007c; Zhang et al. 2013). Cadmium can slow the growth of plants and can reduce the activity of nitrate reductase but increase the proline content and antioxidant enzymes. Enhanced cadmium and zinc bioaccumulation were seen in transgenic plants (Irfan et al. 2014). *Salix caprea* plants show (Zn) and cadmium (Cd) tolerance, accumulation abilities, and tolerance (Konlechner et al. 2013). The ATP dependent ABC transporter pumps the heavy metal into vacuole with the help of MPR and PDR proteins. In nature, lead is found in ionic form, hence it is highly reactive and makes complexes with their surrounding components immediately in the soil and absorbed by the plants through roots to cause food poisoning through the food chains (Kumar et al. 2017). The metal tolerance protein (MTP), of CDF transporter family, is involved in transporting the cationic metal ions Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, or Mn^{2+} from cytosol to vacuole (Singh et al. 2016).

8.6 Plant–Endophytic Association for the Remediation of Pollutants

The autotrophic nature of plants has provided an opportunity to endophytes for their nutrition and living (Calderón et al. 2012; Chaudhry et al. 2005). As the residence time of volatile and organic pollutants is less than from few hours to 2–3 days, these endophytes are more suitable microorganisms for the remediation of both toxic and non-toxic contaminants (Weyens et al. 2009b). Enzymes produced by the endophytes decrease phytotoxicity as well as evapotranspiration of volatile pollutants. In addition to the degradation of organic pollutants, endophytic bacteria also assist in plant growth and environmental adaptation (McGuinness and Dowling 2009; Wang and Dai 2011; Sharma et al. 2018). Many examples of genetically modified bacteria are reported which helps in plant growth promotion activities such as *Pseudomonas putida* KT2410, *P. putida* 06909, *Mesorhizobium huakuii* sub sp., Angie strain B3, *E. cloacae* CAL2 and *B. cepacia* VM1330, etc. (Yong et al. 2014; Wu et al. 2006; Sriprang et al. 2002; Nie et al. 2002; Newman and Reynolds 2005). Plant–endophyte association is a topic of interest for knowing the phytoremediation competence of plants, cultivated in contaminated soil.

The first endophytic bacteria assisted application is applied in 2,4-dichlorophenoxyacetic acid contaminated soil (Germaine et al. 2006) and the result shows a reduction in 2,4-D accumulation in plant cells, cell's surrounding area, and in the soil also. Later on, the application was also used for pollutant mineralization and plant growth (Yousaf et al. 2011). Figure 8.3 represents the different levels of bioremediation through the plant, which may involve Phytoaccumulation, Phytotransformation, Phytovolatilization, Phytoevaporation, or Rhizoremediation. Endophytes relationship with plants is like "give and take," in which endophytes help in plant growth promotion, pollutant degradation, and lower the pollutant toxicity. On the other hand, the plants provide shelter and nutrition for growth and colonization of endophytic bacteria. Their partnership helps in the bioaccumulation of pollutants (mainly heavy metals). An increase in pollutant-degrading bacteria shows the correlation with the enhanced degradation of organic contamination. Specific plant endophyte, enzymes, and their target in chemical structures are given in Table 8.4. A bacterium in the microbial community transfers its degrading genes to other neighbor bacteria by the process of horizontal gene transfer (Weyens et al. 2010b).

Adaptation in microbes according to their environment results in horizontal gene transfer (van der Lelie et al. 2005). Studies show that different plants when inoculated with endophytic bacteria show enhanced degradation of aromatic compounds (Ho et al. 2013).

The plant endophytes also show biosorption through which they accumulate the contaminants (Ma et al. 2011). These mechanisms are also accounted for a reduction of metal uptake by plants as in tomato roots and shoots (Vivas et al. 2006; Madhaiyan et al. 2007). Mycorrhizal fungi are also reported to reduce metal translocation (Krupa and Kozdrój 2007). Extracellular and intracellular components of microbes help in adsorption, immobilization, and accumulation of contaminants

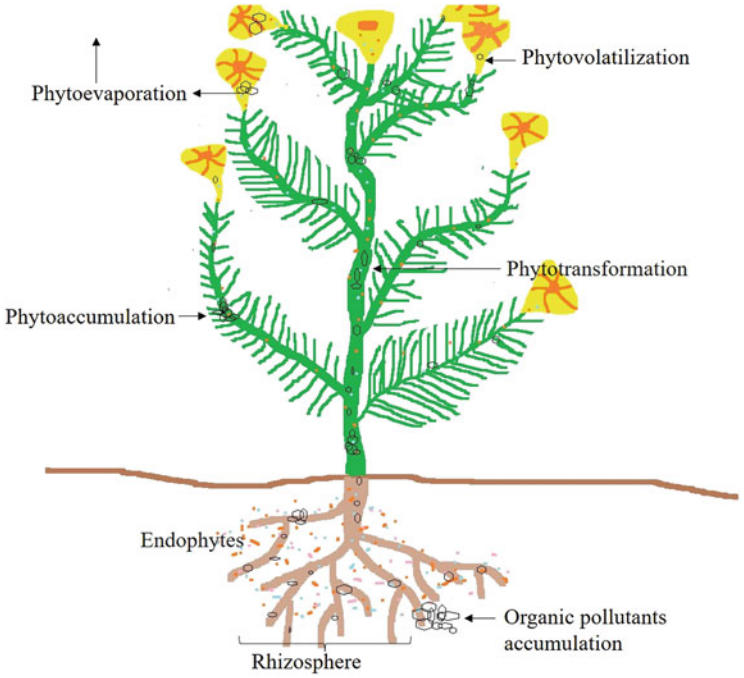

Fig. 8.3 Phytoremediation is diverse as it involves: phytoaccumulation, phytotransformation, phytovolatilization, photoevaporation, and rhizoremediation

(Cline and Zak 2014). These mechanisms are not solely responsible for uptake, accumulation, and translocation of contaminants in plants (Babu and Reddy 2011; Vivas et al. 2006). The inoculation of plants (poplar and willow) with endophytic bacteria enhances the degradation of organic contaminants and plant growth (Newman and Reynolds 2005). Application of endophytes can be applied for mass scale clean-up of soil contaminated with organic compounds or polluted with heavy metals. As we know that heavy metal contamination slows or inhibits plant growth and biodegradation as well but the endophytes enhance the plant growth, organic compound degradation, and reduce the phytotoxicity of heavy metals (Sandrin and Maier 2003; Weyens et al. 2010a). Endophytic bacteria can be manipulated genetically to enhance organic pollutant degradation and heavy metal resistance in soil (Dixit et al. 2015). In addition, to reduce the soil contamination, plant–microbes association can also be applied for the consummation of organic waste polluted water. Endophyte, assisted plants to show potential for organic compound degradation when growing hydroponically in contaminated water (Weyens et al. 2009c). They decrease the plant toxicity and transpiration of evaporative organic waste elements. Both, the endophytes and plant have the potential to suppress or promote each other's growth and we can predict their relationship by using the integrated omics (Bell et al. 2014a, b). Endophytic bacteria manipulation, by transformation

Table 8.4 Enzyme-mediated catalysis of organic pollutants

S. no.	Enzymes source	Enzyme family	Catalytic action	Reference
1.	*Trametes Versicolor*	Laccase	Aromatic compound degradation	Novotný et al. (1997)
2.	*Coriolopsis polyzona*	Laccase	Aromatic compound degradation	Novotný et al. (1997)
3.	*Pseudomonas* sp.	Dioxygenase	Aromatic compound degradation	Pieper et al. (2004)
4.	*Mycobacterium* sp.	Dioxygenase	Aromatic compound degradation	Pieper et al. (2004)
5.	*Xanthobacter autotrophic*	Dehalogenase	Hydrolysis of aliphatic (e.g., TCE) and aromatic hydrocarbons (e.g., DDT)	Mena-Benitez et al. (2008)
6.	Hybrid poplar (*populous* sp.)	Dehalogenase	Hydrolysis of aliphatic (e.g., TCE) and aromatic hydrocarbons (e.g., DDT)	Susarla et al. (2002)
7.	*Sphingobium chlorophenolicum*	Dehalogenase	Hydrolysis of aliphatic (e.g., TCE) and aromatic hydrocarbons (e.g., DDT)	Cai and Xun (2002)
8.	Horse radish (*Armoracia rusticana*)	Peroxidase	Reductive dehalogenation of an aliphatic hydrocarbon and aromatic compound degradation	Susarla et al. (2002)
9.	*Phanerochaete chrysosporium*	Peroxidase	Reductive dehalogenation of aliphatic hydrocarbon and aromatic compound degradation	Novotný et al. (1997), Aken et al. (2000), Khindaria et al. (1995)
10.	*Phanerochaete laevis*	Peroxidase	Reductive dehalogenation of an aliphatic hydrocarbon and aromatic compound degradation	Bogan and Lamar (1996)
11.	Willow (*Salix* sp.)	Nitrilase	Cyanide groups cleavage from aromatic and aliphatic nitriles	Susarla et al. (2002)
12.	*Aspergillus niger*	Nitrilase	Cyanide groups cleavage from aromatic and aliphatic nitriles	Kaplan et al. (2006)
13.	*Comamonas* sp.	Nitroreductase	Reduces and/or removes nitro groups/N on nitroaromatic compounds (e.g., 2,4,6-TNT)	Liu et al. (2007)
14.	*Pseudomonas putida*	Nitroreductase	Reduces and/or removes nitro groups/ N on nitroaromatic compounds (e.g., 2,4,6-TNT)	Caballero et al. (2005)

(continued)

Table 8.4 (continued)

S. no.	Enzymes source	Enzyme family	Catalytic action	Reference
15.	Hybrid poplar (*Populus* sp.)	Nitroreductase	Reduces and/or removes nitro groups/ N on nitroaromatic compounds (e.g., 2,4,6-TNT)	Susarla et al. (2002)
16.	Giant duckweed (*Spirodela polyrhiza*)	Phosphatase	Cleave phosphates from organophosphates (e.g., pesticides)	Susarla et al. (2002)
17.	All plants, fungi, and many aerobic bacteria	Cytochrome P450 mono-oxygenase	Aromatic and aliphatic hydrocarbons hydroxylation	Sandermann (1992), Urlacher et al. (2004), McLean et al. (2005)

techniques, may lead to the improved potential for clean-up of polluted water and contaminated soil (Doty 2008). A genetically modified strain of *P. Putida* reported for degrading the TCE in poplar plants while it simultaneously enhanced plant growth (Weyens et al. 2010b). The inoculation of genetically modified endophyte was shown useful enough for appropriate remediation of aliphatic, volatile, and aromatic pollutants present in wastewater. Engineered endophytes can be used for enhanced degradation and remediation of organic compounds polluted water. In *Arabidopsis thaliana*, the synergistic effect of AtPCS1 and GSH1 was recorded for enhanced accumulation, tolerance of As and Cd, and increased level of Phytochelatins PCs (Guo et al. 2008). This indicates that modification in the genetic material of plants can enhance the efficacy of stress tolerance, detoxification, and metal bioaccumulation. Coontail (*Ceratophyllum demersum* L.) also shows PCs mediated tolerance against Pb (Mishra et al. 2006). Based on the above studies we can say that endophyte–plant interaction will be an ideal approach for remediation of organic and toxic waste aggregates in field, land, soil and prevent the exposure of crops from the toxic compounds and provide reliable food products. Therefore it is a sure and confident approach to improve phytoremediation efficiency, bioaccumulation of heavy metals, and enhance plant biomass. Endophyte's physical activities in the endosphere and rhizosphere of populous and *Pisum sativum* can be monitored by the marker gene like gusA and gfp (Germaine et al. 2006). Green fluorescent protein-tagged strains were examined in various parts of Populus and *Pisum sativum* plants (Germaine et al. 2004).

8.7 Factors Impacting Endophytic Bacteria Remediation Activities

Degradation of organic pollutants occurs at a much slower rate than the culture environment, and it may be due to biotic or abiotic factors and inter-microbial competition (Bell et al. 2014a, b). Abiotic and biotic factors including physical

and chemical properties of soil, types of soil and plants concentration of pollutant, inoculation methods, bacterial strains, plants metabolites, interaction of endophytes inside plants affect endophytes colonization and metabolic activities in the different organs of plants (Anderson and Dawes 1990; Afzal et al. 2013). Studies have shown that the concentration of different hydrocarbons in the soil also impact colonization and expression of pollutant-degrading genes (Andria et al. 2009). Soil type and texture may be the main factors that affect pollutant degrading genes. Alkane-degrading alkB expression is high in rye-grass cropped in the loam field, while the same gene expression is lowest when cultivated in sandy soil (Pandotra et al. 2018). This could be possible as sandy soil contains low cation exchange, low organic matter, and have a minimal surface for microbial adherence. The studies indicate that soil type undoubtedly affects the endophytes colonization as well as their physical feature, metabolic activities, and molecular mechanisms. In recent studies, some plants (Italian ryegrass, birdsfoot trefoil, and alfalfa) have been inoculated with the alkane-degrading endophytes, and they showed a high level of colonization in plants root. Expression of the degrading gene noticeably differs among different plant cultivars, plant compartments, and their developmental stages. Inoculation methods could also affect phyto-endophyte adherence, accumulation, adsorption, and metabolic activities in soil contaminated with petroleum products (Hutchinson et al. 2001; Merkl et al. 2005). Biotic factors also play an important role in phytoremediation, which includes the difference in metabolism and genetic makeup of organisms involved. The heat shock proteins (HSP's) can repair the heavy metal stress in plants. These HSP's (metallothionein and phytochelatins) binds to the metal ions and helps in degradation (Singla-Pareek et al. 2006). Some bacteria have metal resistant genes, which create the possibility for creating the transgenic plants with resistant bacterial genes like merA, merB, merP, merT, merC, merE, and merG (Milner and Kochian 2008). In *Arabidopsis thaliana*, GSH (Glutathione synthetase) helps in sequestration and chelation of heavy metals, detoxification of xenobiotics, cell division, cellular defence, apoptosis, and flowering (Jutsz and Gnida 2015; Yadav 2010; Foyer and Noctor 2005; Pastore et al. 2003; Cotgreave and Gerdes 1998). While simultaneously, Cadmium can stimulate glutathione biosynthesis (Bruns et al. 2001). With the help of glutathione S-transferase and γ-glutamylcysteine synthetase, the metal conjugates, further transported to the vacuoles for further metabolism and protect the intracellular machinery (Jutsz and Gnida 2015). Genes such as glutathione synthetase (GSH2), phytochelatin synthase (PCS), serine acetyltransferase (SAT), cystathionine synthase (CTS), γ-glutamylcysteine synthetase (GSH1), ATP sulfurylase (APS), glyoxalase I, glyoxalase II, and glutathione reductase (GR) have been found to regulate the GSH and PCs levels for heavy metal stress tolerance. GSH starts the biosynthesis of phytochelatins (PCs), in response to heavy metals especially, e.g., Zn Cd, Ni, Cu, Hg, and Pb, to chelate the metallic components within the plants and resist the cellular damage (Mehra and Mulchandani 1995; Hirata et al. 2005).

8.8 Phytoremediation Application Approaches Through Omics

Omics refers to a field of study in biology that includes the knowledge of computers such as genomics, proteomics, or metabolomics (Singh et al. 2014). These branches of modern science have established their importance in the insights of the genome of both prokaryotes and eukaryotes. Now, it is easy to identify the specific part of genetic components for the prediction of a variety of products. To produce useful omics data for phyto-endophytic remediation, we have to isolate uncultivable bacteria by finding unknown factors needed for their survival in in vitro conditions (Bomar et al. 2011). Once we observed the unculturable microbes, their host plant should be identified with the highest bioremediation potential (Bell et al. 2014a, b). These endophytes can be genetically manipulated by using the information of the best known degrading gene through omics (Fig. 8.4) for the better bioremediation output (Tanaka et al. 2007; Shukla 2019; Jaiswal et al. 2019). Systems biology, in addition to genetic manipulation, may also be applicable to plant growth promotion and exploring the vast area for further research (Basu et al. 2018).

We can also identify the cryptic improvements for remediating through microbial communities by using comparative omics (Mason et al. 2012). We should also have

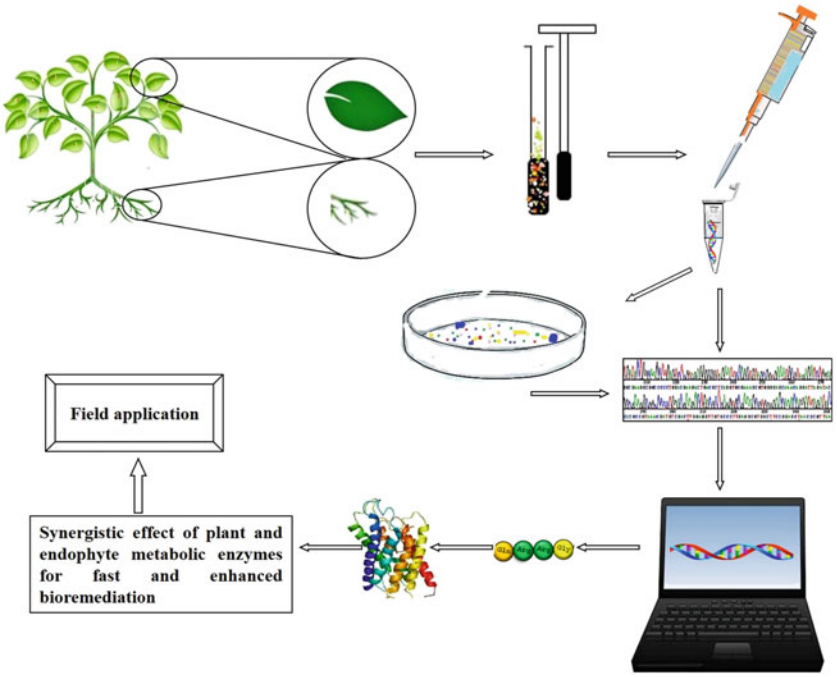

Fig. 8.4 Use of proteomics and metabolomics for potential phytoremediation

to screen the soil metagenomes for the identification of microbes that are not present at a particular site of soil for developing new strategies of bioremediation at a specific contaminated site (Cline and Zak 2014).

8.9 Conclusion

Bacterial endophytes showed significance in the remediation of the organic pollutants. In association with plants, these endophytes are able to decontaminate the water and soil resources. Wastewater plants can develop the technology and methodologies utilizing these endophytes for water treatment against heavy metals (e.g., Zn Cd, Ni, Cu, Hg, Pb) and organic pollutants (e.g., TCE, DDT, benzene, etc.). Phytoremediation is proved to be an eco-friendly approach comprising the utility of microbial enzyme (e.g., Laccase, Dioxygenase, Peroxidase, Nitrilase, Nitroreductase, Phosphatase, and Cytochrome P450 mono-oxygenase Dehalogenase) in association with plants for the uptake and oxidation and reduction processes for the degradation of pollutants. The factors affecting the above degradation processes are studied by various researchers and more study will be needed to understand the metabolic activities of bacterial endophytes. Different types of soil, plants, concentration of pollutant, inoculation methods, bacterial strains, plants metabolites, interaction of endophytes inside plants, and metabolic activities in the different organ of plants are supposed to be the key parameters which affect the degradation processes. Moreover, omics-based information is also needed by the researchers for studying the degradation applicability of microbes and to explore the systems biology for the better understanding of chemical pathways and biology of degradation pathways.

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9 Biotechnological Interventions for Arbuscular Mycorrhiza Fungi (AMF) Based Biofertilizer: Technological Perspectives

Punit Kumar and Kashyap Kumar Dubey

Abstract

The growing population demands more food for their survival and decreasing agricultural land has put pressure on farmers and agricultural scientists to enhance the crop productivity. This has led to excessive use of chemical fertilizers and chemical pesticides to enhance agricultural productivity. Arbuscular mycorrhiza (AM) symbiosis with rooted plants is considered one of the oldest symbiotic interactions in the nature. Arbuscular mycorrhiza approximately colonizes 80% of the terrestrial plant species. These uptake the nutrients from the soil and transfer to host plants in exchange of carbon sources. Indeed, AM fungi provide plant's protection against biotic and abiotic stresses, thus play a key role in sustainable agriculture. Such properties of the AM fungi make them important for biofertilizers as chemical based fertilizers are not good for healthy soil. This chapter focuses on drawback of current agricultural practices, problems associated with chemical fertilizers and beneficial role of mycorrhizal association to symbiotic host plants. Furthermore, the roles of arbuscular mycorrhiza as biofertilizers are also discussed.

Keywords

Crop productivity · Chemical fertilizers · Biofertilizers · Arbuscular mycorrhiza fungi · Root architecture · Bioprotection · Biocontrol

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9.1 Introduction: Fertilizers and Agriculture

It is easy to understand that basic requirements for the population are food, water, land and medicine, etc. Including this, the growing population requires the sustained availability of these materials of acceptable quality. It is a well understand fact that ability of a country to feed its citizen depends on various factors such as availability of agricultural land, water resources and population. It is estimated that world population has increased with the annual rate of 1.9% since 1960 and the growth of future population will take place in developing countries which have limited resources (Byrnes and Bumb 1998). In this scenario, there will be requirement of more input (fertilizers, irrigation facilities, technological development) to enhance the agricultural production while maintaining the soil fertility. Food and Agriculture Organization (FAO) has estimated that global population will be about 9.1 billion in 2050 and for this population, cereals production must increase to more than 3 billion tons and food production have to be increased by 70% (FAO 2020).

Higher population and limited resources create situation of hunger or malnutrition (undernutrition) in developing economies. As per the reports of World Health Organization (WHO) that 462 million people are underweight, and 47 million children of age below 5 years are wasted, 14.3 million are severely wasted, and 144 million are stunted. Including this, approximately 45% of deaths among children under the age of 5 years are associated with undernutrition (Malnutrition, Key Facts, WHO 2020). In another report it was suggested that world population is going to rise by approximately 35% over the next 40 years (Stewart and Roberts 2012) and to feed the increasing population, agricultural productivity must be increased which requires substantial increase in fertilizer production capacity. Including this, more population will also require more land for housing and other associated infrastructure facilities, which will further cause water and land scarcity. It is also assumed that land and water supply will influence from population growth (Schneider et al. 2011). Thus, it must be clear that in future mankind will need more agricultural output from fewer land and limited water resources.

Agricultural production should increase significantly for the growing population using existing farms, new farmland and implementation of new approaches such as innovative food formulations, nutrigenomics, food engineering, etc. (Stewart and Roberts 2012; Tian et al. 2016). There is significant enhancement in food production due to development of improved crop varieties, disease-resistant varieties, the expansion of irrigated agriculture land and the increased use of pesticides and chemical fertilizers. In order to enhance the agricultural production the increasing population significantly affected the use of fertilizer for agricultural activities. A study conducted for 77 countries during the period of 1970 to 2011 demonstrated that population pressure put impact on fertilizer use. It was observed that 1% increase in population pressure caused 0.118% increase in fertilizer use (Xiang et al. 2020). It has been reported that appropriate combination of fertilizers (NPK fertilization compared to PK fertilization) enhanced yield by 19–41% for rice and 61–76% for rapeseed of 2 years of rice-rapeseed rotation experimental study in Honghu, Shayang and Jingzhou in China (Yousaf et al. 2017). Another study

revealed that the use of fertilizers enhanced average percent of yield from 40% to 60% in temperate climates and this enhancement was found higher in the tropical climate (Stewart and Roberts 2012). These studies suggested that inorganic fertilizer plays significant contribution to world food security, but further enhancement in the yield may be achieved by application of both inorganic and organic nutrients in appropriate combination. Further, adoption of 4R principles (right nutrient, right time, right rate and right place) in application of fertilizers plays a key role in efficient use of available nutrients and enhancement in crop productivity (Stewart and Roberts 2012).

9.1.1 Chemical Fertilizers for Crops

Sustainable development may be described as development of society with justified use of resources to fulfil the demand of present population and maintaining the resources available for the future generation. The continuous increase in population will put natural resources under pressure. Increasing emphasis on agriculture production becomes critical with high yielding varieties as these varieties require chemical fertilizers and water in high amount. Including this, lack of micronutrients and macronutrients in soil also provoked utilization of fertilizers in agriculture (Patra et al. 2016). These nutrients are supplied to soil in right proportion and right time for optimum agricultural production (Fig. 9.1).

The green revolution caused positive impact on increase of significant agricultural production. To enhance the production of agriculture, farmers used excessive water, unbalanced use of fertilizers and chemical pesticides (Patra et al. 2016).

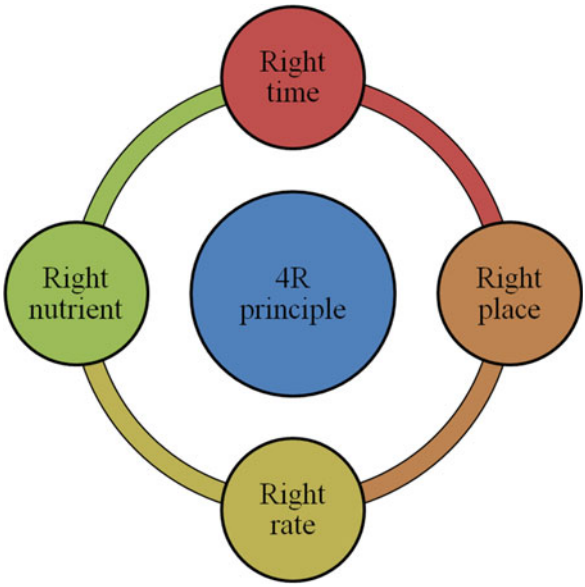

Fig. 9.1 4R principle of fertilizer application for efficient utilization of nutrients and enhancement in agricultural productivity

Though the use of chemical fertilizers has significantly enhanced the agricultural production but excessive use of chemical fertilizers caused the change in nutrient composition in soil, destruction of useful microflora, insects and worms in soil, degradation of soil, ground water contamination, etc. (Kopittke et al. 2019; Singh et al. 2020; Srivastav 2020). Moreover, the excessive use of chemical fertilizers and chemical pesticides causes severe damage to environment such as (World Agriculture: Towards 2015/2030)

1. Pollution and increase of nutrients in water bodies which resulted decrease in dissolved oxygen of water bodies and caused associated effects (Srivastav 2020).
2. Release of greenhouse gases into environment by excessive application of nitrogen fertilizers contributes to climate change (Chai et al. 2019).
3. Effect on biodiversity due to loss of insects and herbs by the use of insecticides and herbicides. These affect biodiversity–ecosystem function relationships.
4. Acidification of soil which affected soil productivity (Zhu et al. 2020).
5. Reduction in availability of minerals and nutrients in food items.

9.1.2 Biofertilizer

Thus it may be assumed that excessive use of chemical fertilizers enhanced the production of crops but it also deteriorated the soil health and caused environmental problems (World Agriculture: Towards 2015/2030; Patra et al. 2016). The excessive use of fertilizers changed the microbial community of soil and reduced crop productivity of soil due to improper management of soil. Moreover, chemical fertilizers are also expensive and unhealthy in nature (Righi et al. 2005; Sahoo et al. 2013). Use of chemical based fertilizers cannot be omitted because these are key players in crop productivity but alternatives for chemical fertilizers must be explored for sustainable agriculture. Soil is important resource for agricultural activities and reservoir for microbial community. Thus, it may be assumed that proper maintenance of soil is required for optimized crop production (Sahoo et al. 2013). In this regard, biofertilizers including PGPR have been recognized as an alternate and ecofriendly approach to enhance the fertility of soil and crop productivity by including the tools for bioinoculant improvements (Reddy et al. 2020; Yadav and Sarkar 2019; Chaudhary et al. 2020; Chaudhary and Shukla 2019a, b; Basu et al. 2018).

The definition of biofertilizer has been proposed by Vessey (2003) and Malusá et al. (2012) as substances containing living microorganisms which, after application to plant surfaces, seed, or soil, perform colonization to the rhizosphere or the interior of the plant, modify the morphology of the roots, promote beneficial plant–microbial interaction, reduce the disease, and promote growth of plant by enhancing the supply or accessibility of nutrients to the plant (Vessey 2003). Today 'Biofertilizer' is a broad team and well known term for the farmers and agricultural scientists. Biofertilizers do not comprise single component or extract, while these contain efficient strains of microorganisms and composted waste of plants which provide all the required nutrients to the growing crops and as well as soil. The combination of

constituents of biofertilizers is selected to enhance the fertility of soil and to increase productivity.

9.1.3 Microorganisms Used in Biofertilizer

Microorganisms present in biofertilizers perform growth and proliferation around rhizosphere and exhibit benefits to host plant (Igiehon and Babalola 2017; Dal Cortivo et al. 2020). Microbial content of biofertilizers plays a key role in conversion of plant material and unavailable form of nutrients into available form. Including this, microbial populations play a key role in the supply of nutrients such as nitrogen, phosphorus and sulphur to the plants by several mechanisms (Sahoo et al. 2013). Microbial culture also helps to control plant diseases, degradation of pesticides and other degradable materials, production of plant growth stimulators like plant hormone, auxin and cytokinin, and protection against abiotic and biotic stress (Sturz et al. 2000; Sahoo et al. 2013). Common microorganisms used in biofertilizers are categorized into arbuscular mycorrhiza fungi, plant growth-promoting rhizobacteria (PGPR) and nitrogen fixing rhizobacteria. These microorganisms are commonly called as plant growth-promoting microorganisms (PGPM) (Malusá et al. 2012). There are some PGPM which comprise auxin-producing bacteria to promote root elongation. The research outputs in this field have further contributed to enhance the efficiency and consistency of microbial consortium for biocontrol and biofertilization. The common inocula for biofertilizers are mycorrhizal fungi, *Rhizobium* sp., *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Glomus* sp., *Yarrowia lipolytica,* etc. It is reported that coinoculation resulted in efficient uptake of nutrients and enhanced the growth of the plant (Gryndler et al. 2002; Artursson et al. 2006) and efficient inocula were developed using combination of microbial strains most commonly mycorrhiza and bacteria.

9.2 Arbuscular Mycorrhiza Fungi

9.2.1 General Properties of Mycorrhiza

Arbuscular mycorrhiza fungi (AMF) are the one of the general form of symbiotic association existing between a fungus and the roots of approximately 80% of vascular plant (Jin et al. 2012). Approximately 150 species of AM fungi have been identified which possess wide host range (Harrier and Watson 2004). In this symbiotic association the hyphae of fungal partner penetrate into host plant cells and develop branched morphological structures, which allow vast exchange of materials between both organisms. These fungi are able to provide nutrient to host plant under low soil nutrient conditions. The fungal mycelium surrounding the roots of the host plants works as extension of roots and explores more soil for the nutrients uptake (Jin et al. 2012).

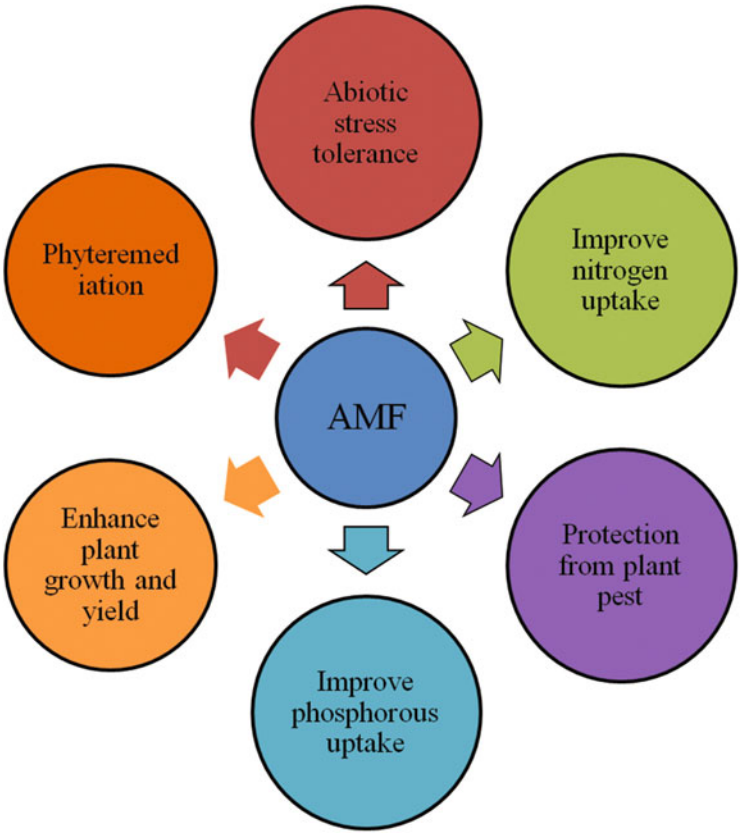

Fig. 9.2 Role of Arbuscular Mycorrhiza Fungi (AMF) in sustainable agriculture

AMF are obligate biotrophs, macrosymbionts and soil microorganisms which are considered as natural biofertilizers. AMF create important link between plants and soil mineral nutrients and these are found associated with many beneficial activities such as phytoremediation, abiotic stress tolerance, improved nutrient uptake, protection from pest and enhance growth and yield of crops (Fig. 9.2). These fungi belong to one of the widely distributed group of fungi which are economically and ecologically important. These fungi are member of order *Glomales* and are member of phylum *Glomeromycota* (Schüßler et al. 2001). These fungi obtain their nutrient through obligate symbiotic relationship with the vascular plants. These fungi interact with soil and other organic material via mycelium. In this association the symbiotic interface enables the exchange of the nutrients where fungi help in uptake of inorganic nutrient by plant and fungus obtains sugars from the host plant. Including the symbiotic relationship with vascular plants these fungi also influence plant biodiversity, help to control pests (e.g. nematodes) and fungal pathogens and affect the health of plants in polluted environments. Moreover, the plant–fungal relationship has been recognized important for huge nutrient transfer at global level,

universal carbon sequestration and stabilization of soil (Parniske 2008). Thus it may be considered as AMF play a key role (direct or indirect), on growth of vascular plant on land (Schüßler et al. 2001).

9.2.2 Classification of AMF

The origin and evolution of these fungi are associated with evolution of land plants. It is assumed that AMF were originated more than 460 Mys ago and thus these fungi exhibited ancient phylogenetic clade within the fungi (Redecker et al. 2000). Initially AMF were kept in family *Endogonaceae* (*Zygomycota*) but unlike other members of the family AMF did not produce zygosporangia. Further, cladistic analysis based on morphological characters led to the formation of new order *Glomerales* with two suborders. The phylogenetic analysis was carried out on the basis of SSU rRNA of these fungi and it was suggested that AMF may be separated into monophyletic clade and it has no association with zygomycetous group while it possibly shared common ancestry to *Ascomycota* and *Basidiomycota* clade. Therefore, AM and related fungi were placed into new phylum *Glomeromycota* (Schüßler et al. 2001). On the basis of aseptate and septate mycelia these fungi were also grouped. Here, aseptate endophytes have been grouped into *Glomeromycota* and septate is grouped into *Asco* and *Basidiomycota* (Bonfante and Genre 2010).

These fungi are also classified into two other groups, ectomycorrhiza and endomycorrhiza, which depend on colonization of fungi in intracellular spaces in roots or develop inside the cells (Fig. 9.3). The ectomycorrhiza belongs to Asco-Basidiomycetes. In ectomycorrhiza fungal mycelium grows and surrounds the root tip and also performs development between root epidermal cells but this mycelium does not penetrate into cells of epidermis and cortex as in endomycorrhiza. Further distribution of endomycorrhiza has been performed into categories known as orchid, ericoid and arbuscular mycorrhiza (Bonfante and Genre 2010).

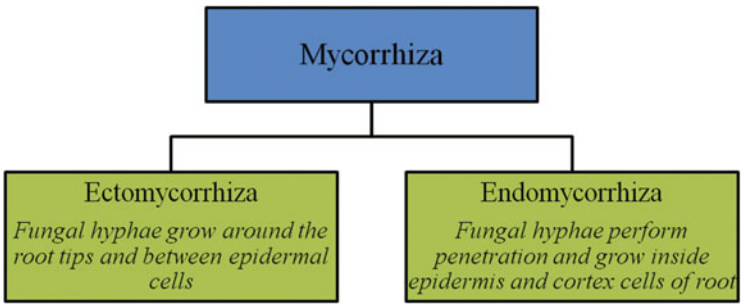

Fig. 9.3 Mycorrhiza are categorized into two categories; Ectomycorrhiza and Endomycorrhiza

9.2.3 Life Cycle of AMF

The beginning of life cycle of AMF is considered from fungal spore germination and this leads to growth of fugal hyphae. The growing fungal hyphae then perform growth towards the roots of host plant. The association of fungal hyphae with host plant enables stimulation of signals that are responsible for physiological changes in the host plant. These physiological changes mainly include the suppression of plant immune responses (Denison and Kiers 2011). The suppression of plant immune system makes the intracellular conditions favourable for the growth of the fungal hyphae in plant cell. These hyphae may be called as internal hyphae. The hyphae enter into cortex parenchyma of roots of the host plant and branching of the hyphae takes place. These branched hyphae are called arbuscules (coils) which are involved in nutrient exchange between plant and fungi. Including this, external hyphae of fungi colonize in the soil. The external hyphae collect the nutrients (nitrogen, phosphorus and other micronutrients) from the soil and provide these nutrients to the plant cells. It is suggested that mycorrhizal roots possess different kinetics for phosphate absorption and also contain lower threshold values as compared to nonmycorrhizal roots. The external hyphae cover the large area of soil surrounding the roots and efficiently absorb the phosphate even beyond the depletion zone. Colonization of mycorrhiza has also been reported for enhanced of copper and zinc (Hayman 1983). It is suggested that a well developed transport system plays an important role in transport of nutrients from external hyphae to internal hyphae and these nutrients are provided to the host by intracellular arbuscules (Gianinazzi-Pearson and Gianinazzi 1983). In exchange of these nutrients, internal hyphae collect host derived sugars from the cortical cells which is further stored in energy rich vesicles, and support vegetative growth of fungi (Denison and Kiers 2011; Bonfante and Genre 2010). It is also suggested that hyphae growing from both host roots and spores may perform colonization in new plants.

In case of arbuscular mycorrhiza (AM), it had been observed that host plants can survive even if fungal symbionts are absent, while fungus cannot grow in the absence of host. This relationship illustrates that fungi are obligate biotroph as host is required for growth and development. Arbuscular mycorrhiza functions as true partner microbes which promote overall plant fitness and assist in absorption of soil nutrients, enhance plant biomass and contribute to improved resistance of host plant to pathogen and stress (Bonfante and Genre 2010; Čatská et al. 1997).

9.2.4 Molecular Evidences of Fungal and Plant Symbiosis

The hyphae of AMF contain large population of genetically distinct nuclei, which performs different way of reproduction. Though symbiotic AMF are supposed to be evolved about millions of year ago, and divergent allelic nucleotide sequences were supposed to be present in mycorrhizal fungi. Individual fungal offspring is believed to receive different nuclei from parents thus genetically distinct nuclei should get

evolved in one individual. It has been analysed by DNA–DNA in situ hybridization that different nuclei co-exist in hyphae belonging to particular fungi.

Further, polygenetic analysis revealed that genetic variation in individual AMF though (through mutations and some infrequent recombination (Kuhn et al. 2001). This theory represents two implications

1. Proliferation of certain nuclei may be affected by availability of nutrients in local environment or environmental factors. Possibly, these factors trigger the development of unique mycorrhizal networks which enhances their flexibility with respect to both space and time (Denison and Kiers 2011; Sanders and Croll 2010).
2. Significant evolution may be possible in the absence of new mutations. It has been found that there is functional diversity within individual AMF species probably due to presence of distinct nuclei within individuals. This led to possibility that in asexually reproducing organisms, genetic information can be exchanged, like sexual reproduction (Munkvold et al. 2004).

9.2.5 Host Root Recognition

It is evident that root colonization is crucial step for the growth of AMF and thus as spore performs germination, it catabolizes the stored lipid for the growth of hyphae and search for the host roots in the soil. If hyphae are not able to find out the host, then it retracts its cytoplasm back into spore and growth is arrested. The spore becomes dormant and again performs germination and searches for host roots. This process is repeated over and over. As due to wide host range, association with the host is not a very frequent problem in nature but this situation revealed the important aspect of obligate biotrophy of AMF (Heupel et al. 2010).

Though the process how fungi are able to detect the host plant is not well understood but it has been identified that host root releases some signalling molecules or branching factors (sesquiterpene lactones) such as strigolactone, strigol (member of the strigolactone family, Fig. 9.4), 5-deoxy-strigol, GR24 (Fig. 9.5), etc. which induce branching in hyphae of germinating AM spores (Akiyama et al. 2005). It is reported that strigolactone rapidly and strongly regulates cell proliferation at very low concentration 10^{-13} M (Besserer et al. 2006). It was also suggested that strigolactone stimulates mitochondrial activity and respiration (Besserer et al. 2006). Moreover, the GR24 (a strigolactone analogue) has been found associated with enhancement in the concentration of NADH, NADH dehydrogenase activity, and ATP present in fungal cells (Besserer et al. 2006). It is identified that natural strigolactone possesses C-D ring structure, which is considered essential for its biological activity (Yoneyama et al. 2009). In another report it was suggested that ABC scaffold is required for functioning as branching factor. It was also reported that B ring is not strictly (essentially) required for activity (Zwanenburg et al. 2016). Due to importance of strigolactone in plants, these compounds have been considered as new class of plant hormones which inhibit branching in shoots. The other branching factors are (+)-5-deoxystrigol, strigol, orobanchol, sorgomol which are

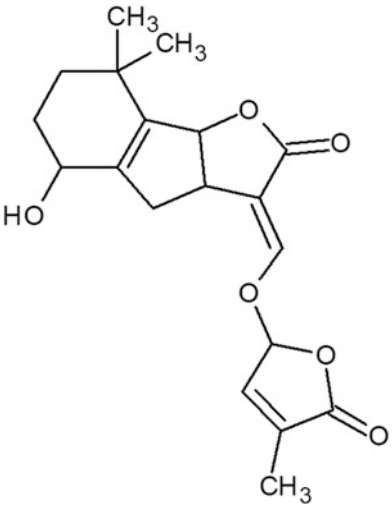

Fig. 9.4 Structure of Strigol: host root exudates associated with AMF colonization

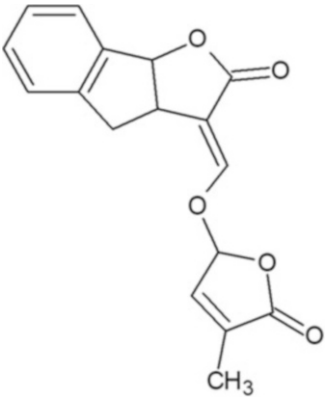

Fig. 9.5 Structure of GR24: derivative of plant root exudates associated with AMF colonization

produced by chemical modifications of strigolactones (Yoneyama et al. 2009; Zwanenburg et al. 2016).

It is observed that AM fungi are not inactive at the time of their presymbiotic growth. Though key molecule involving in symbiotic relation is not identified but it is suggested that fungal partner secretes signalling molecules known as 'Myc factors' which are involved in root symbiotic relationship. These bioactive compounds possess size smaller than 3 kDa and are lipophilic (Navazio et al. 2007). The Myc factor is believed to be recognized by host plant even in the absence of physical association with fungi and induces expression of membrane protein which involves in mycorrhization. Lysophosphatidylcholine is also reported as mycorrhizal signal which is lipophilic in nature and it initiates expression of phosphate transporter gene using P-controlled transcriptional process. This process is involved in uptake of orthophosphate which are secreted by mycorrhizal fungi (Bucher et al. 2009). In one study dual cultures experiment was performed using

culture of AM fungi and *Medicago truncatula* which were separated by using a physical barrier between them. It was demonstrated that hyphae of germinating spores release a diffusible factor which is perceived by roots of the tested plant without having physical contact in direct form. This factor induced the expression of the Nod factor-inducible gene, i.e. MtENOD11. This study has been conducted with many AM fungi such as *Gigaspora margarita*, *Gigaspora gigantea*, *Glomus intraradices* and *Gigaspora rosea* and all of these tested fungi showed similar response (Kosuta 2003).

Researchers also have suggested the Ca^{++} mediated responses in the host plant in response to AMF. This study demonstrated the role of calcium ions as intracellular messenger involved in symbiosis between plant and the AMF. It has been identified in *Glycine max* (soybean) cell cultures analysing the expression of the aequorin (bioluminescent calcium indicator) with respect to the intracellular calcium changes associated with exposure of germinating spores of *Gigaspora margarita* in culture medium in the absence of the plant partner. It was found that that diffusible signals secreted by the AMF are perceived by calcium mediated signalling in the host plant cells. It was also observed that induced Ca^{+2} signature was not modified while coculturing the germinating spores with the host plant cells (Navazio et al. 2007).

9.3 Properties of Mycorrhiza to Be Use as Biofertilizer

9.3.1 Phosphate Absorption

Phosphorus is considered as macronutrient required for the growth and it plays important role as structural element in many compounds such as genetic material (DNA and RNA), plasma membrane, coenzymes and many enzymes. Phosphate is involved in many activities such as energy metabolism, activation of molecular factors and metabolic intermediates, cellular signalling and regulation of activity of many enzymes. Phosphorus also contribute about 0.2% of dry weight and is one of the nutrients which is difficult to acquire by plants. Thus phosphorus is recognized as important molecule in cell and maintenance of cellular phosphorus homeostasis is necessary requirement (Karandashov and Bucher 2005). The phosphorus is considered as one of the limiting nutrients in natural habitat and affects agricultural productivity at global scale (Bucher 2007). Despite the presence of phosphorus in significant amount in soil it is poorly available to plants due to low solubility of phosphates of different salts resulting into the very less soil concentration and low mobility (Smith et al. 2011). It is found that the rate at which absorption of phosphate (orthophosphate: Pi) takes place by growing roots is much greater than the rate at which soil phosphate diffusion takes place and this difference in rates causes formation of a zone of depletion at the root system level which results in limitation of the phosphorus supply to the plant. In this situation, symbiotic mycorrhiza fungi assist in supply of soluble phosphate to plant roots by accessing new sources for phosphate (Fig. 9.6).

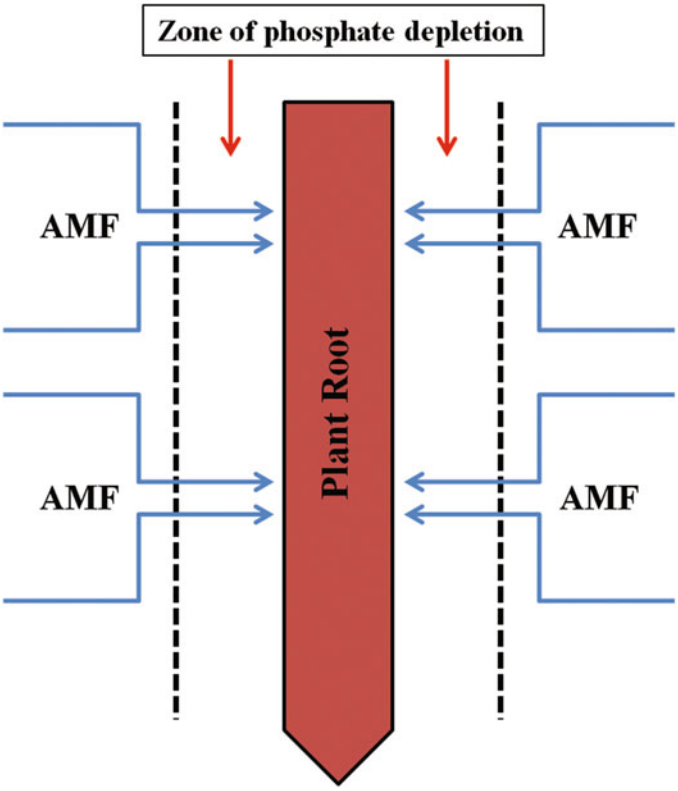

Fig. 9.6 Mechanism of phosphate uptake by plant roots assisted by AMF colonization

It is suggested that phosphate is absorbed by the root cells against the concentration gradient as concentration inside the cell is about 1000-fold higher than outside. Thus this transport is energy driven which utilizes phosphate transporters (Pht1 family) and proton ATPases existing as transmembrane proteins (Bucher 2007). Accordingly plants have developed different strategies to enhance phosphorus uptake or to enhance the availability of phosphorus in the soil. Two ways of phosphate absorption by plants may be assumed; one is absorption by formation of dense cluster of roots which release organic anion involved in Pi release from poorly available inorganic form and second is mycorrhizal mycelium dependent symbiotic phosphate absorption (Lambers et al. 2008). It is also observed that during phosphate deficiency the corresponding genes for phosphate transporters are constitutively expressed or over expressed to enhance AM fungal colonization (Karandashov and Bucher 2005; Smith 2003). Including this many constitutively expressed genes have been found at epidermis of roots (root hair and root tip).

Till now many phosphate transporters have been reported and characterized for their role in phosphate uptake in fungi and host roots. Role of phosphate transporters (Pht1) in direct uptake of phosphate has been elucidated by loss of function in mutant

Pht1 transporter in *Arabidopsis* which further caused approximately 75% reduction in phosphate uptake (Shin et al. 2004).

Two phosphate transporters (MtPT1 and MtPT2) have been reported in *Medicago truncatula* roots which were not identified in leaves (Liu et al. 1998) suggesting their expression only in roots. In the AMF, two phosphate transporters, i.e. GvPT from *Glomus versiforme* and GiPT from *Glomus intraradices* have been identified (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001) involved in phosphate uptake. The genes for these transporters have been reported as primarily expressed in the extraradical fungal mycelium which was exposed to micromolar concentrations of phosphate and it can be assumed that these transporters are involved in phosphate uptake at soil–fungus interface. The absorbed phosphate is then utilized in its biomolecules synthesis and stored in the form of polyphosphate. These phosphate biomolecules are transported to intraradical fungal mycelium (Ezawa et al. 2004). It is also suggested that transfer of phosphate in fungal mycelium through fungal membrane is governed by concentration gradient and it is facilitated by channels, pumps and ion-specific carriers. The phosphate is released into root cortex cells by the phosphate transporters.

Moreover, expressions of phosphate transporters in plant roots are also found under the influence of symbiotic fungi. Researchers have reported plant phosphate transporters which are induced by AMF and these transporters participated in transfer of fungal delivered phosphate. The examples of such transporters are *MtPT4* in *M. truncatula*, *OsPT11* and *ORYsa*;*Pht1*;*11* in rice, *StPT3* and *StPT4* in potato, *SiPHT1;8* and *SiPHT1;9* in *Setaria italic*, and *GigmPT* in *Gigaspora margarita* (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002; Glassop et al. 2007; Ceasar et al. 2014; Xie et al. 2016).

It is also found that during mycorrhizal uptake of phosphate there is upregulation in mycorrhizal phosphate transporters and downregulation of the phosphate transporters involved in direct uptake pathway. As it is well explored that most phosphorus is absorbed by mycorrhiza thus plant phosphate transporters upregulated by mycorrhiza play a key role in plant health and productivity (Karandashov and Bucher 2005).

Recent study also suggested that high phosphate level negatively affects AM symbiosis and favours nonsymbiotic uptake of soil phosphorus by roots. Interestingly, in high phosphate soil environment, root exudates did not contain strigolactone and supplementation of strigolactone did not stimulate root colonization in high phosphate conditions (Balzergue et al. 2011, 2013).

9.3.2 Nitrogen Absorption

Arbuscular mycorrhiza (AM) fungi are also found to enhance the uptake of nitrogen from organic material. But this contribution of AMF is under debate as biomass of the plant total nitrogen did not increase (Thirkell et al. 2016). Investigators have reported *Glomus* species transferring nitrogen to host plants (*Plantago lanceolata*) from the organic material. Studies have revealed that host plants colonized with

G. intraradices showed higher amount of nitrogen than controls. It was suggested that uptake from organic nitrogen is an important contribution during AM symbiosis for both partners and some AM fungi may also uptake inorganic nitrogen from organic sources (Leigh et al. 2009). It was observed that mutual benefit in symbiotic relationship is independent on the stoichiometry of carbon, nitrogen, phosphorus (Thirkell et al. 2016). Researchers have performed compartmented microcosm study to analyse the role of fungi in nitrogen uptake. In this study mycorrhizal plant was placed in compartment having nitrogen-rich (N-15 labelled) patch of organic matter which is accessible to AMF hyphae only. It was found that AMF performed sufficient proliferation and transferred nitrogen to host plant. It was also observed that accession of organic matter by hyphae increased in total nitrogen and phosphorus content of the plant and biomass of the plant was also increased (Thirkell et al. 2016). This it may be suggested that AM symbiosis increases decomposition of organic matter and uptake of nitrogen from organic matter. Including this, proliferation of hyphae also enhanced uptake of nutrients independent of host plant (Hodge et al. 2001).

Studies have demonstrated the hyphal transfer of inorganic nitrogen to plants. Study conducted using labelled (N-15) organic sources (^{15}N-Gly and ^{15}N-Glu) inorganic sources ($^{15}NH_4$, $^{15}NH_4^+$, $^{15}NO_3$, $^{15}NO_3^-$) of nitrogen have demonstrated the role of AMF in nitrogen transfer. It is suggested that amino acids, ammonium and nitrate are important sources of nitrogen (Hawkins et al. 2000; Santi et al. 2003). Nitrate is present in agricultural soil in abundant form and amino acids present in the soil are organic source of nitrogen. Alanine, asparagine, glutamine, glutamate are one of the common amino acids which are present in the soil within in the range of concentration as 1–10 μg/g. Studies have revealed molecular level that ectomycorrhiza possesses the capacity to import inorganic and inorganic nitrogen sources such as ammonium, peptides, nitrate from soil using urea permease or ammonium transporter. The nitrogen compounds are accumulated at mantle and Hartig net and transported to plant. Investigators have observed that glutamine and ammonium are one of the common of the nitrogen compounds which are transferred through membrane transporters (Martin and Nehls 2009; Lipson et al. 1999).

Though mechanism of nitrogen transfer is not well elucidated between AMF and host plant but energy driven mechanism is assumed for nitrogen transfer. Expression of some associated genes also has been reported associated with nitrogen uptake and transfer. Researchers have demonstrated the role of nitrate transporter gene (*ZmNRT* 2.1) and root plasma membrane H^+-ATPase gene in maize. In this study it was suggested that the activity of ATPase resulted enhanced transport of nitrate. It was also suggested that higher proton pumping activity is required at increase of nitrate flow and nitrate may be triggering expression of H^+-ATPase (Santi et al. 2003).

It has been demonstrated that mycorrhizal hyphae can enhance the uptake and transfer of organic and inorganic nitrogen to plant roots. AMF increase access of nitrogen sources which is not accessible to plant roots alone. It is reported that nitrogen is immobilized into external hyphae before uptake by plants. Though it was also identified that amount of nitrogen transferred by hyphae did not contribute much to nitrogen nutrition of the plant (Hawkins et al. 2000).

Now there are clear evidences that AMF help in uptake of nitrogen and transport to the host plant. Studies have revealed that nitrogen transfer by AMF is associated with carbon flux as nitrogen flux is only stimulated in a situation when carbon is transported by plant as mycorrhizal surface and it is not taken place if carbon is directly supplied to extraradical mycelium of fungi in the form of acetate (Fellbaum et al. 2012). This study also indicated that stoichiometry of carbon and nitrogen is involved in exchange of nutrients between AMF and host plant roots. It was also suggested if both nitrate and ammonium are available as nitrogen source the AMF will preferentially use ammonium as nitrogen source and extraradical hyphae transferred ammonium to roots of the host rather than nitrate which suggests that it is more energetically favoured to assimilate ammonium (Toussaint et al. 2004). It is suggested that ammonium is transferred by transporters such as *GintAMT1* and *GintAMT2*. Transporter genes have been reported in *G. intraradices* which encode ammonium transporters having high affinity for ammonium and participate in the transfer of ammonium to mycelium (López-Pedrosa et al. 2006; Pérez-Tienda et al. 2011). Nitrate transport by AMF is associated with H^+ symporter and it causes alkalinization of mycorrhizosphere. High affinity nitrate transporter from *G. intraradices* (GiNT) has been reported involved in nitrate transfer. It has been reported in one study that nitrate possibly absorbed and assimilated by AMF and not directly uptake by plant roots (Tisserant et al. 2012).

It was reported that about 21% of total nitrogen absorbed by AMF is transferred to host plant (Toussaint et al. 2004). However the transfer of nitrogen by AMF is dependent on the efficiency of the fungal strain, physiologlogical conditions and type of available nitorgen source. It was observed that in water limiting conditions, *Rhizophagus arabicus* improved drought tolerance of host plant as compared to *Rhizophagus irregularis* (Symanczik et al. 2018).

9.3.3 Abiotic Stress Tolerance

Arbuscular mycorrhiza (AM) fungi have also been involved in maintaining and actively participating in water uptake under abiotic stress. It is assumed that AMF proliferate in soil and cause changes in soil structure, soil aggregation and soil hydraulics (Rillig and Mummey 2006). It is found that AM association with plants is beneficial in plant growth during many types of abiotic stresses such as drought, salinity, soil compaction and heavy metals (Miransari 2010). Different mechanisms have been suggested working in different stresses. For example, stress is caused by drought in host plant by controlling the uptake of water and nutrients (Khalvati et al. 2005).

The role of AM in water uptake is not well understood but there are many reports suggesting the importance of AM fungi in water limiting situations. The possible mechanism for water uptake may be due to increased growth of external mycelia providing higher exploration of soil and higher surface area for absorption. It was suggested that AM symbiosis with host plant caused improved osmotic adjustment in roots of host plant which maintained favourable water potential in roots for

transfer of water from soil into roots during drought conditions (Porcel and Ruiz-Lozano 2004). Enhanced nutrient transfer by mycorrhizal strain (*Rhizophagus arabicus*) to host plant (*Sorghum bicolor*) is also observed in water limiting conditions (Symanczik et al. 2018). A study conducted with barley host plant and *Glomus intraradices* (AMF) in drought conditions revealed that drought resistance was enhanced in mycorrhizal host plants as compared with nonmycorrhizal plants. Though only small amount of mycorrhizal transport of water was detected but it was suggested that larger water uptake was done by AMF hyphae in root compartment (Khalvati et al. 2005). Study conducted in AM association with soybean plants in drought conditions indicated that AM plants showed sustained growth in drought conditions and showed enhanced shoot biomass, enhanced leaf water potential, more accumulation of proline in roots, lower lipid peroxides and protection against oxidative damage (Porcel and Ruiz-Lozano 2004).

AMF association with plant roots is also found providing resistance in high temperature. Study conducted with AMF symbiotically associated with maize revealed that primary photochemical reactions were higher in high temperature. Study conducted in mycorrhizal symbiotic association with sunflower revealed that AM fungi also increased plant defense mechanisms against drought or high temperature. It was found that symbiotic association with ectomycorrhizal fungi resulted higher activity of antioxidant enzymes under drought conditions which resulted in reduced cellular damage and enhanced plant growth than nonmycorrhizal plants (Alvarez et al. 2009). It was observed that AM association induced activities of enzymes such as glutathione S-transferase (GST), guaiacol peroxidase (POX) and polyphenol oxidase (PPO) which are associated with high temperature tolerance of sunflower (Mayer et al. 2017). AMF also caused enhanced magnesium uptake which resulted higher chlorophyll content which enhanced photosynthesis and accumulation of biomass while growing the plants in high temperature environment (Mathur et al. 2018).

In another study conducted with symbiotic association of ectomycorrhiza (*Pisolithus tinctorius*) with cork oak revealed that roots growth was significantly enhanced during drought conditions and these plants represented lesser drought associated symptoms compared to nonmycorrhizal symbiotic plants. It was also observed in one study that many factors were not affected in AM symbiotic plants such as osmotic adjustment, or drought-induced ROS production suggesting that these factors are not involved in drought tolerance but other many parameter of plants were improved in AM symbiotic plants such as shoot basal diameter, shoot biomass, plant height and root growth (Sebastiana et al. 2018).

There are many studies which have suggested the role of aquaporins in fungi and increased root hydraulic activity which are involved in water absorption in ectomycorrhizal plants while many studies revealed no effect of ectomycorrhizal association on water flow properties (Lehto and Zwiazek 2011). It is also suggested that the structure of mycorrhiza (root architecture, hydrophobicity of cell wall and more membranes to cross) may also cause reduction in movement of water (Lehto and Zwiazek 2011). Recent study has revealed the involvement of Jasmonic acid (JA) in water uptake and hormonal regulation of tomato plants having AMF

symbiotic association. It was found that JA affected uptake of water by controlling stomatal conductance, and control expression of aquaporins. It was observed that concentration of hormones (JA, ABA, salicylate) involved in stress tolerance is influenced by AM symbiosis (Sanchez-Romera et al. 2018). One study reported that salinity increases the production of strigolactone which promoted symbiosis in lettuce and tomato plants.

The role of AM fungi symbiotic association has also been found associated with alleviating salt stress in host plants. It was found that relative water content, stomatal conductance and net photosynthetic rates were reduced in saline soil but this reduction was less in AM symbiotic plants. It was also observed that AM symbiosis enhanced photosynthesis, K^+ content and reduced Na^+ content. Molecular study revealed that there was enhanced expression of chloroplast genes and membrane transport proteins participating K^+/Na^+ homeostasis and expression of aquaporins in presence of soil salinity (Chen et al. 2017). It was also reported that AM symbiosis alters hormonal concentration and plant physiology to alleviate effect of salt stress (Aroca et al. 2013; Ruiz-Lozano et al. 2016).

One study has revealed the alleviation of cadmium phytotoxicity and enhanced cadmium tolerance in *Phragmites australis* (Cav.) Trin. ex Steud. by AM symbiosis (*Rhizophagus irregularis*) by cadmium uptake and cadmium distribution (Huang et al. 2018). It is assumed that metal transporters participate in heavy metal homeostasis and genome wide analysis has reported the genes associated with expression of transporters of metals (Zn, Fe, Cu) which are involved in transport of metals and provide resistance to plants against metal toxicity (Tamayo et al. 2014).

9.3.4 Biocontrol of Plant Pathogens

Biocontrol may be described as the mechanism by which protection against pathogenic organisms is provided by biological sources. Due to adverse side effects of chemical pesticides, the biocontrol of pathogens may be considered a tool for sustainable agriculture (Azcón-Aguilar et al. 2002). AM fungi are also found to provide protection against root pathogens. There are limited report about role of AMF against biotic stresses. It is also assumed that this association reduces the damage to plant caused by pathogens and thus enhances the resistance to these pathogens. Though exact mechanism behind this activity is not well explained but this activity enabled the applications and development of AM fungi for sustainable agriculture (Harrier and Watson 2004). However, degree of protection is dependent on type of pathogen, type of soil and environmental conditions. It is assumed that plant and fungi symbiosis cause modulation in plant defense responses thus symbiotic association provide protection against pathogens (Jung et al. 2012; Azcón-Aguilar and Barea 1997). Investigators have reported bioprotection to host plant against many soil borne pathogenic organisms like species of *Thielaviopsis*, *Sclerotinium*, *Rhizoctonia*, *Pythium*, *Phytophthora*, *Macrophomina*, *Fusarium*, *Cylindrocladium*, *Aphanomyces*, *Verticillium,* and many nematodes (Harrier and Watson 2004). In one study root infection caused by *Fusarium oxysporum* was

controlled in the presence of AM fungi in susceptible plant species (*Setaria glauca*) (Sikes 2010). Recent study conducted with *Funneliformis mosseae* and *Rhizophagus irregularis* (AM fungi) with host apple plant revealed that symbiotic association enhanced the resistance of apple plant against fungal pathogen, i.e. *Neonectria ditissima* (Berdeni et al. 2018). In another study the symbiotic association between AM fungi (*Glomus mosseae*) and tomato roots provided protection against soil borne pathogen, i.e. *Phytophthora parasitica* (Pozo et al. 2002). Meta-analysis study conducted on 106 articles to analyse effect of symbiotic association with the pathogen (fungal and nematode). In this study, it was observed that AM fungi reduced the growth of fungal and nematode pathogens. It was observed that AM fungi reduced 44–57% growth of fungal pathogens and 30–42% growth of nematode pathogens. It was also assumed that life style and type of the pathogen did not affect the pathogenic suppression activity of AM fungi and AMF provided similar protection to host plants from different pathogens. Moreover, type of AM fungi, *Glomus mosseae* was found most effective contributing pathogen resistance to plants (Veresoglou and Rillig 2012).

Though mechanism of plant protection against pathogens using AM fungi is not well understood but it is suggested that fungi contribute to resistance by production of hydrolytic enzymes against cell wall of fungal pathogens and various other different mechanisms and there are evidences to accept them or disprove them. These mechanisms are mentioned following (Wehner et al. 2010).

9.3.4.1 Improved Nutrient Supply to Host

AM fungi caused nutrient (nitrogen and phosphorus) rich condition of the host plant by uptake of more nutrients and enhanced nutrient uptake is associated with higher tolerance against fungal pathogens. It has been found that root colonization of AM fungi (*Glomus mosseae*) with eggplant and tomato caused higher uptake of nutrients (nitrogen and phosphorus) and reduction in pathogenic effect of *Verticillium dahliae* (Karagiannidis et al. 2002). In another study similar results were obtained where AM fungi enhanced the growth of cotton plants and reduced the growth of *Verticillium dahliae* (Zhang et al. 2018). In another study, test tomato plants were provided phosphorus supplementation without AM fungi to analyse role of AM fungi in disease protection. It was observed that AM fungi (*Glomus mosseae*) enhanced the resistance against *P. nicotianae* var. *parasitica,* while host plant supplied with phosphorus did not show protection against disease (Trotta et al. 1996) suggesting involvement of other factors contributing protection against pathogens.

9.3.4.2 Competition with Pathogenic Fungi for Resources

In ecosystem interactions exist between nematodes, fungal pathogen, soil microbiota, AM fungi and plants which affect the establishment of ecosystem. It is assumed that symbiotic association of AM fungi enhanced the competition with pathogenic fungi for nutrients in roots, infection sites, rhizosphere, etc. which caused reduction in the growth of pathogenic fungi in the presence of AM fungi (Graham 2002; Wehner et al. 2010; Yang et al. 2014).

9.3.4.3 Structural Changes in the Root System

It has been assumed that AM fungal association causes development of branched root architecture and structural changes in root such as enhanced lignification of roots which caused formation of thicker roots. It has been reported that symbiosis with AM fungi enhanced the lignification in cortical cells of roots which possibly suppressed the infection and growth of *Verticillium dahliae* (Matsubara et al. 1995). It has been reported that AM fungal (*Rhizophagus irregularis* CD1) colonization with cotton plant inhibited growth of pathogenic fungi (*Verticillium*) through enhanced expression of pathogenesis-related genes and lignin synthesis associated genes (Zhang et al. 2018).

9.3.4.4 Stimulation of Defense Mechanism of Host Plant

AM fungi symbiosis is also supposed to induce the defense mechanism of host plant to provide protection against pathogens through many approaches such as improved nutrient supply, competition with pathogen for resources, change in root architecture, stimulation of plant defense system, etc. (Fig. 9.7). Though the role of AM fungi in stimulation of plant's defense mechanism is not much clear about but researchers have identified a correlation between physical stimulation of plant cell surface and gene expression for defense of host. Host plant expresses certain set of specific genes and production of some chemical compounds which provide protection against plant diseases. In another study mycorrhizal symbiosis with host roots exhibited induction of enzymes such as β-1,3-glucanase, chitosanase, chitinase and superoxide dismutase (Pozo et al. 2002) which are involved in protection against pathogens. In a related study, expression of chitinase, and peroxidase (pathogen associated genes) have been found in tomato roots with response to *Rhizoctonia solani* (Taheri and Tarighi 2012) and expression of DMI3 protein was found associated with pathogen protection (Genre et al. 2009). In another study, expression of pathogenesis-related antifungal protein (PR-5) has been identified with response to fungal infection. This protein is also involved in phytoalexin production (El-kereamy et al. 2011).

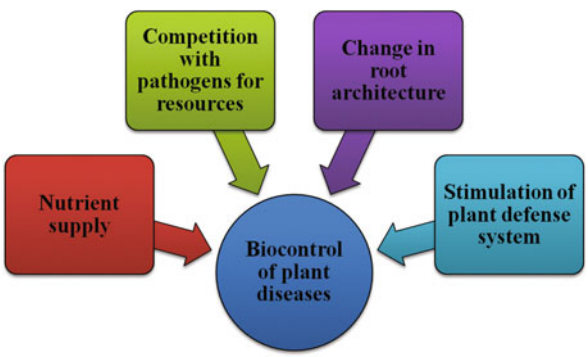

Fig. 9.7 Different ways by which AMF work as biocontrol agent for plant diseases

9.3.5 Phytoremediation and Environment Protection

Phytoremediation is well explored property of microorganisms including AM fungi. It is reported that insecticides affect the growth of spore germination and mycelial growth of arbuscular mycorrhiza (AM) fungi thus these fungi may be explored as bio-indicator of pesticides (Giovannetti et al. 2006). Studies indicated that arbuscular mycorrhiza (AM) fungi have been reported to perform bioremediation of pesticides in soil caused by enzymes secreted by AM fungi (Li et al. 2006). The AM fungi (*Rhizophagus intraradices*) have been found exhibiting heavy metal tolerance and alleviate oxidative stress caused by cadmium in *Glycine max* (Molina et al. 2020). The fungi (*Glomus intraradices*, *G. Mosseae*) are reported to decrease the amount of organophosphate in crops and degradation of organophosphate (phoxim) in soils (Wang et al. 2011). Another study has revealed the benefit of AM fungi in bioremediation (rhizodegradation) of polycyclic aromatic hydrocarbons (PAH) (Leyval et al. 2002). It has been found that mycorrhiza enhanced the uptake of PAH in host plant roots but the amount was reduced in shoots (Gao et al. 2011). One recent study has demonstrated the role of mycorrhiza in reduction of total petroleum hydrocarbons (Guarino et al. 2020).

AM fungi also have been reported for the reduction in emission of greenhouse gases. It has been reported that AM fungi controlled the emission of N_2O (potent greenhouse gas) into environment by enhancing its assimilation. Including this, genes were also found associated with production of N_2O (*nirK* which expression was reduced) and consumption of N_2O (*nosK* which expression was enhanced) linked with abundance of AM fungi (Bender et al. 2014).

9.4 Use of Mycorrhiza as Biofertilizers

AM fungi are key biological soil components which are involved in many beneficial activities such as uptake of nutrients (phosphorus, nitrogen, sulphur, potassium and many micro) under the low nutrient content in soil and growth of plants under biotic and abiotic stresses. Thus re-establishment of association of AM fungi with plant roots in soil may be a good approach for sustainable agricultural practices as its use may decrease the use of agrochemicals but this requires detailed knowledge about the adaptation of fungi in soil and ecosystem and events leading to establishment of functional symbiosis (Berruti et al. 2015). There are following aspects associated with use of AMF as biofertilizers.

9.4.1 Production of AM Inoculum

As biofertilizers, inoculum of AM fungi is transferred to soil where these fungi grow and establish symbiosis with roots of host plants. The preparation of inoculum is challenging as AM fungi are obligatory symbionts which requires host during cultivation for propagation. Moreover isolation of pure culture and analysis of

level of host root colonization are also very challenging. There are many methods reported by which preparation of inoculums may be performed (Ijdo et al. 2011; Berruti et al. 2015; Kumar et al. 2017). These are soil and substrate based, substrate free inoculum production and in vitro cultivation;

1. Spores of AMF isolated from soil are used for inoculum production.
2. Roots of host plant having symbiosis with AMF and contain AMF spores, hyphae and root fragments. Though it is associated with possible risk of transfer of soil borne pathogens and weed seeds.
3. Crude inoculum is obtained from host plant colonized with AMF and is grown in sterilized medium having constituents and culture conditions optimized for AMF production.

The other conventional methods for AMF production are recognized as growth and maintenance in pots having sterilized soil, hydroponics, aeroponics and in vivo methods based on greenhouse, etc. but these methods are also prone to contamination. Studies have recommended use of root organ culture for AMF production (Kumar et al. 2017).

Researchers have used different substrates for production of AMF inoculum. Researchers have reported the production of AMF inoculum (*Dentiscutata heterogama*, *Claroideoglomus etunicatum*, *Acaulospora longula*, and *Gigaspora albida*) using organic matter (vermicompost, coir dust and Tropstrato) with sand and vermiculite. It was found that vermicompost enhanced the sporulation of *A. longula* compared to another substrate and AMF. The substrate Tropstrato® inhibited the growth of *D. heterogama* but growth of *C. etunicatum* was not affected. The inoculum of *A. longula* enhanced the accumulation of biomass in maize plants (Coelho et al. 2014). A study has demonstrated the farm production of AM fungi using vermiculite, perlite and peat based potting media with a host plant *Paspalum notatum Flugge* (bahiagrass). In this study vermiculite was observed as more suitable medium (Douds Jr. et al. 2010). Another study has demonstrated the use of sucrose-agar globule (SAG) for enhanced production of mycorrhizal spores of *Glomus fasciculatum* and *Gigaspora gigantea*. This study suggested the use of sucrose-agar globule with root exudates inoculum for enhanced production of fungal spores (Selvaraj and Kim 2004).

9.4.2 Use of AMF Inoculum in Field

The extensive use of AMF inoculum to agricultural field is costly hence make the agriculture cost sensitive and thus different approach may be used by which AMF biodiversity is maintained in the field which persist for long time in the soil. This approach is a good alternative to large scale production of inoculum which requires low cost. Industrially produced inoculum comprises pure culture or combination of more than one AMF species. It is observed that many times commercial inoculums

do not produce expected results thus evaluation of AMF inoculum is required with target crops before field utilization (Faye et al. 2013; Tarbell and Koske 2007).

9.4.3 Analysis of Enhancement in Crop Production Using AMF Biofertilizers

There are many studies supporting the use of AM fungi associated with increase in crop production at laboratory level and field level. In laboratory trial, the use of AM fungi in barren soil caused improvement in quality of soil and increased plant growth of wheat (*Triticum aestivum* L.) (Pal and Pandey 2016). There are many reports which indicate that crop production was improved further when AMF were supplemented with nutrients. Field trail was conducted with garlic and AMF with different level of phosphorus in soil revealed that garlic production was enhanced when plants were inoculated with AMF and intermediate level of phosphorus (Al-Karaki 2002). In a long term field study conducted with wheat and AMF (*Glomus caledonium*) revealed that AMF inoculation enhanced biomass, crop yield, enhanced uptake of phosphorus and activity of soil alkaline phosphatase with NPK treatment (Hu et al. 2010). In another field study conducted with AMF and sweet potato (*Ipomoea batatas* (L) *Lam*) revealed that AMF inoculation enhanced the production of sweet potato and biofertilizers supplemented with nitrogen and potassium showed higher yield than AMF alone and suggested use of starter nutrient in case of nutrient depleted soil (Mukhongo et al. 2017).

9.5 Problems Associated with AMF Biofertilizers

It is evident that all plant root and soil fungi associations are not mutually beneficial for both plant and fungi. Rather, other interactions are also established which are strongly pathogenic to parasitic, neutral or beneficial. Analysis of association including many fungal species revealed that only AMF are involved in symbiotic phosphate transport taking place at plant–fungi interface and it provides functional attribute to phylum Glomeromycota.

Though biofertilizers seem to be useful for the plant growth and productivity but investigators have observed inconsistency in outcomes in laboratory, greenhouse and field trials. It was suggested that probably it was due to lack of detailed study of the relationships between the host plant, the inoculum and the available environmental conditions of the soil (Artursson et al. 2006). There are many other factors which affect adversely to AM fungi such as interruption with hyphal networks by tillage, excess use of pesticides, fungicide and chemical fertilizers and selective breeding of crop varieties (Berdeni et al. 2018). It is also found that commercial inoculums did not produce expected results in filed and during the production of inoculum, there are chances of contamination by soil borne pathogens thus pre-evaluation of AMF inoculum is recommended (Berruti et al. 2015).

In addition, it was also observed that absence of right formulations and the costly and time-taking procedures of registration are also the issues associated with the use of PGPM on a wider scale (Malusá et al. 2012).

9.6 Conclusion and Future Prospective

It is estimated that up to 2050 the world's population will be more than 9 billion, thus agriculture production needs sustainable enhancement in crop production and lesser dependence on chemical fertilizers and chemical pesticides (Rodriguez and Sanders 2015; FAO 2020). Including the enhancement of crop production, AM fungi may be explored in bioremediation of pesticides and toxic metals, use as bio-pesticide, indicator of pollution and control of soil erosion (Igiehon and Babalola 2017).

AMF has positively enhanced the crop production in a sustainable way and simultaneously reducing the dependence on chemical fertilizers and pesticides. AMF symbiosis with host roots assists in uptake of nutrients and provides protection against abiotic and biotic stress to host plants. At molecular level it has been demonstrated that AMF have phosphate transporters for transport of phosphate and associated genes involved in the transport of nitrogen (ammonium and amino acids) from soil to host (López-Pedrosa et al. 2006; Cappellazzo et al. 2008; Hijikata et al. 2010).

The use of AMF as biofertilizers for sustainable agriculture seems promising but long journey need to be covered. More scientific analysis is required to reveal the importance of the role of AMF in crop production. Though genomic and proteomic analysis with respect to AMF also have further enhanced the understanding of the plant fungi relationship. Investigators have used next generation sequencing approaches to elucidate the interaction of AMF with host roots and other soil microorganisms but more analysis is required to understand the establishment of symbiosis. Including this, mechanisms behind the uptake of nutrients, regulation of abiotic and biotic stress, bioremediation, control of soil erosion, etc. are not well explored. Moreover, production of AMF inoculum at industrial level is also not easy task and further research studies are required in this direction.

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Probiotics and Prebiotics: Techniques Used and Its Relevance

10

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Abstract

Modernism in older techniques has led to expansion in modern strategies for studying probiotics and prebiotics. These strategies reduce the difficulties in identifying the unknown species and manipulations of the rumen microbial ecosystem. These high-throughput techniques can quickly achieve maternal links of fetus; it can help in knowing the density and amount of bacteria throughout the neonatal gut. Advances in "omics" have led this to new levels, where the relationships between food supplements and gut microflora can be known easily. Further, knowing these inter-linkages can act as a mediator in applying modern techniques such as encapsulation, immobilization, etc. In this chapter, all these techniques, including in vivo and in vitro strategies have been discussed in detail along with the modern strategy including modeling and other omics techniques for better understanding of both prebiotics and probiotics. Moreover, knowing these techniques in detail can help in improving the efficiency and efficacy of both of these.

Keywords

Probiotics · Prebiotics · Synbiotics · Synthetic biology · Computational tools

10.1 Introduction

Probiotics and prebiotics are the products that can benefit humans by inducing a positive effect on health. The global market of them is consecutively increasing by the years because of their multifunctional properties (Davison and Wischmeyer

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2019). The common effects of them are deleting the unwanted microbes from the gut and increasing the digestive functions, e.g. bowel functions, nutrients absorptions, controlling pH, etc. The big pool of information can be derived from the omics approach, which can further enhance the new global market of beneficial microbes and their related potential products. The analytical methods for studying these are deplorable and cannot explain the proper function of these in metabolic disorder and health (Andersen et al. 2019). The traditional method in disease management, i.e. the use of dairy products for lactose intolerance has some gaps in defining the proper target actions of these beneficial microbes (Huynh et al. 2019). For this, we can also use non-dairy probiotic products based on vegetables and fruits, which further help as a natural remedy against such metabolic disorders (Yadav and Mandeep 2019). In addition to this, advances in techniques such as metagenomics, transcriptomics, and proteomics had led this to a new echelon (Selim et al. 2019). They relatively target the proper functions of the gut microbiome and their target actions such as immunomodulation, pathogen inhibitions, nutrients absorptions, etc. Knowledge of all this information can lead to the mechanisms involved in modulating the compositions and activities of the gut microbes (Mamuad et al. 2019). This had devoid the scientific interest in advanced nutritional styles that enhance intestinal functions, mainly food mediated probiotics, and prebiotics. For example, a zootechnic field involves poultry farms and other products such as cereals, and dairy products baked foods, fermented milk, including a specific amount of synbiotics into it (synbiotics are mixtures of probiotics and prebiotics) (Sagheddu et al. 2019). For consuming such type of food items, proper assessments are required, traditional in vivo and in vitro methods which have basic criteria of removing pathogens and virulent strains are not enough. In addition to this, the final selected strains and products should be properly accepted for therapeutic use against different metabolic health-related disorders (Raman et al. 2019).

This chapter will brief about strategies adopted for proper authentication of both prebiotics and probiotics along with their limitations. In addition to this, some new aspects and techniques for increasing the shelf life of synbiotics in health enhancement have also been discussed. The drying method is generally used to enhance the stability of probiotics for a longer period. This is an important concern for manufacturers towards the probiotic shelf life (Vivek et al. 2020). In this process, moisture or water content of probiotics is reduced via different drying technologies such as spray drying, vacuum drying, fluidized bed drying, and freeze-drying. Among all these, spray drying is the most rapid and cost-effective method, whereas freeze-drying or also known as lyophilization, is the most common and broadly used method (Verlhac et al. 2019). For example, microencapsulation, immobilizations, etc., can be used nowadays for metabolic disease management (Khangwal and Shukla 2019a).

Moreover, other methods such as protein–oligosaccharide conjugates, modeling based on omics data, and other bioactive synbiotics can be proved as better techniques for predictions as well as interpretations of the gut microbiome. These high-level techniques can help in identifying the proper interactions between the gut microbiome and other oligosaccharides, which can further help in knowing the exact

mechanism of their actions in the diseased conditions (Lordan et al. 2019). Next-generation probiotics can help in reducing the risk of commonly known disease such as insulin resistance, e.g. *Prevotella copri* and *Christensenella minuta* also; it can reverse the effect of obesity, e.g. *Parabacteroides goldsteinii*, *Akkermansia muciniphila*, and *Bacteroides thetaiotaomicron* (Yu-Ling et al. 2019). In addition to this, other NGP that protects against intestinal diseases, e.g. *Faecalibacterium prausnitzii* and other anticancerous and anti-inflammatory NGF, e.g. *Bacteroides fragilis* are nowadays considered as new agents for disease targeted therapies which are discussed here (Mailing et al. 2019).

10.2 Prebiotics, Probiotics, and Their Proposed Mechanism of Action

Probiotics and prebiotics have different action sites based on their mode of transmission and function performed by them. Gut microflora gets adversely affected by various metabolic disorders such as obesity, diabetes, and other intestinal diseases (Pascale et al. 2019). Synbiotics directly modulate the gut microflora and increase the beneficial microbes in the gut which can reduce the risk of disorder or help in the treatment and prevention of various health diseases. Studies have been going on from the last few decades in knowing the evidences which can relate the gut microbiota with dysbiosis in the gut in the diseased conditions (Pascale et al. 2019; Szychlinska et al. 2019). Based on the site of action, they can have different revelation such as immunomodulatory effect, pathogen inhibition, adhesion and absorption of nutrients. They when introduced to the gut can induce the other beneficial microbes present in the gut and get attached to the intestinal membrane (Khangwal and Shukla 2019b). These reduce the current active sites for the harmful microbes and elute out the pathogens by creating a competition for host binding sites (Sagheddu et al. 2019). Glycosidic proteins such as glycolipids, immunoglobulins, and electrolytes produce a viscous fluid which protects the inner layer of intestine from other pathogen and mechanical damage. Probiotics bacteria such as *Lactobacillus* and *Bifidobacterium* consist of surface layer associated proteins (SLAPs), lipoteichoic acid (LTA), and mucin binding proteins (Mubs) which interact with these intestinal proteins and help in adhesion of bacteria to the intestine (Monteagudo-Mera et al. 2019). Further, some LAB contains surface adhesive proteins such as mucus-binding proteins including Mub or MucBP (MUCin-Binding Protein) domains, which link to peptidoglycan cell wall by LPxTG motifs. In addition to this, surface layer proteins (SLPs) and fibronectin-binding proteins (FBPs) act as an important role in the adherence mechanism of beneficial bacteria and dribbling out the pathogen (Selim et al. 2019). Not only the adhesion, other action sites of these products are immune cells which further target in immunomodulation benefiting the host cells. Generally, immunomodulatory effects are promoted by M cells from the Peyer's patches or dendritic cells (DCs) which can respond to the occurrence of other pathogen cells, this can be appraised by

co-culturing probiotic consortium and immune cells which further can be estimated by the sensing cytokines (Hajjar et al. 2019).

10.3 New Generation Techniques

Synthetic biology, recombinational DNA technology, evolutionary engineering, and computational engineering are emerging technologies used for the enhancement of probiotics and prebiotics (Berlec and Strukelj 2019). The engineered microorganisms are intended for use as probiotics or feed supplements to commence with the development of probiotics targeting enteric pathogens (Dlamini et al. 2019). According to the studies engineered probiotics have high nutritional quality and therapeutic measures to improve human health (Yadav and Shukla 2019a). Probiotics developed by using synthetic biology involves biological part libraries for expression systems and gene editing tools (Fig. 10.1). The emerging model for synthetic biology is *Lactococcus lactis* (Mays and Nair 2018). Synthetic constitutive promoter libraries were generated in *Lactobacillus plantarum* and *L. lactis* with the help of promoter mutagenesis. In alteration to this, inducible promoters can be used

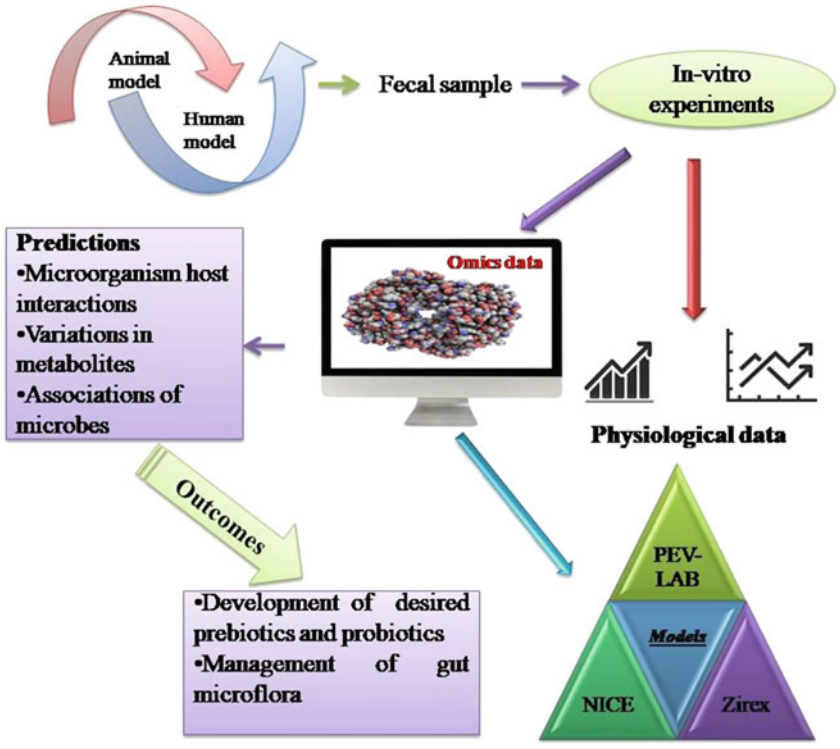

Fig. 10.1 A snapshot of in silico modeling for corroboration of in vitro experiments

such as in nisin controlled expression (NICE) system. It is most commonly used for auto regulatory production of antimicrobial peptide nisin and drawback of this system is leaky basal expression. This is not beneficial for expression of noxious proteins or applications that require tight control (Xiong et al. 2020). For such circumstances zinc-regulated expression (Zirex) and PZn-zitR expression system can employ concurrently with the NICE system to endow with tight co-expression. Chromosomal integration can be achieved by both knock-in and knock-out functionalities. Single gene manipulation can be done by these tools but for the genome scale mutation we need additional tools because it requires pathway engineering or gene circuit engineering. In lactic acid bacteria (LAB), chromosomal manipulation has been done by using the pathway engineering vehicle for lactic acid bacteria (PEVLAB) system (Mays and Nair 2018; Yadav and Shukla 2019a). It is a species-specific plasmid copy control system. This shows high-copy numbers in *E. coli* but single-copy availability in *L. lactis* for smaller edits approximately 10 base pairs using recombination (Kong et al. 2015). The Clustered regularly interspaced short palindromic repeats (CRISPRs)—and CRISPR-associated (Cas) CRISPR/Cas9 technologies provide specific gene editing within the organisms. This can be used to control gene expression and modulates the production of proteins and metabolites. By using transformation, conjugation, and transduction we can deliver CRISPR-Cas system in the targeted bacteria either by in vivo or in vitro strategy (Bikard et al. 2013; Schilling et al. 2018). The in vitro screening is a preferable choice because of the simplicity, squat cost of such approaches, and ability to screen multiple strain simultaneously. This study is adopted to select the strains based on their survival ability in different cubicles of the gastrointestinal tract (Papadimitriou et al. 2015). Engineered probiotic *E. coli* strains Nissle 1917, express enzymes to disperse biofilms, antimicrobial compounds, antigens, quorum sensing molecules to control pathogen virulence and specific metabolic functions (Hwang et al. 2017). For example, genetically modified bacteria *Lactobacillus* species, *E. coli*, *Lactococcus* species producing anti-TNF nanobody, IL-35 and IL-10-, IL-27-, HO-1, respectively, alleviate the mucosal inflammation efficiently by encouraging a homeostatic immunologic profile (Vandenbroucke et al. 2010; Zhang et al. 2018; Mishima and Sartor 2019). Nowadays biotech companies are developing engineered *Lactobacilli* as targeted therapies against a broad range of diseases including inflammatory bowel disease, oral mucositis, bacterial and viral infections (Singh et al. 2017). By using such editing tools we can produce specific bacteriocins (Bikard et al. 2014). Bacteriocins are the ribosomally synthesized antimicrobial peptides produced by bacteria having ability to influence human health positively (Dobson et al. 2012). Bacteriocins derived from LAB are utilized as topical, oral antibiotics, or disinfectants. Promising probiotic candidate of bacteriocin production is *Lactobacillus salivarius* (Thomas et al. 2019).

Moreover, omic studies also play a vital role in the augmentation of probiotic action. Metagenomics, proteomics, metabolomics, metatranscriptomics, fluxomics, etc., are used as key tools to enhance the effectiveness and efficiency of probiotic bacteria (Blanco-Míguez et al. 2017). Metagenomics allows the identification of genetic changes via whole genome sequencing, analysis of genetic structures,

Table 10.1 Encapsulation strategies used for various probiotic strains and matrix involved in it

S. No.	Matrix used	Bacteria or probiotic strain involved	Method adopted	Reference
1.	Alginate–goats' milk–inulin	*Bifidobacterium animalis* subsp. *lactis* BB-12	Encapsulation with compact structured capsules	Pradeep Prasanna and Charalampopoulos (2019)
2.	Inulin as coating agent	*Lactobacillus acidophilus* La-5	Microencapsulation by spray drying	dos Santos et al. (2019)
3.	Maltodextrin and arrowroot starch	*Lactobacillus plantarum*, *Weissella paramesenteroides*, *Enterococcus faecalis*, and *Lactobacillus paraplantarum*	Freeze-dried and encapsulated	Samedi and Charles (2019), Pech-Canul et al. (2020)
4.	Mannan and sodium alginate	*Lactobacillus plantarum*	Encapsulation	Jahari et al. (2019), Amira et al. (2020)
5.	Maltodextrin and gum Arabic (GA)	*Lactobacillus Acidophilus* (NCDC 016)	Microencapsulation with powdered probiotic by spray drying	Arepally and Goswami (2019), Pech-Canul et al. (2020)
6.	*Eleutherine americana* oligosaccharide extract	*Lactobacillus plantarum* TISTR1465	Encapsulation with capsules	Phoem et al. (2019)
7.	Maltodextrin and sucrose or sorbitol	*Saccharomyces cerevisiae* (strain KTP) and *Issatchenkia occidentalis* (ApC)	Microencapsulation by spray drying	Suryabhan et al. (2019)
8.	Apple matrix alginate mandarin juice	*Lactobacillus salivarius* spp. *Salivarius*	Encapsulation by vacuum homogenization and hot air drying	Ester et al. (2019)
9.	Alginate biopolymer of cabbage and kimchi	*Lactobacillus plantarum* KCC-41	Microencapsulation	Song et al. (2019)
10.	2%-alginate beads cells released from beads	*Lactobacillus casei* Lc01 and *Bifidobacterium bifidum* Bb02	Microencapsulation	Bevilacqua et al. (2019)
11.	Agarose-based hydrogel particles	*Bifidobacterium pseudocatenulatum* CECT 7765	Food-grade encapsulation	Alehosseini et al. (2019)
12.	Methoxyl pectin	*Bifidobacterium breve* beads	Encapsulation by ion-cross linking	Li et al. (2019a)

13.	Alginate microparticles	*Lactobacillus acidophilus*	Encapsulation by external ionic gelation followed by freeze-drying	Poletto et al. (2019a)
14.	Ca-alginate/cryoprotectants/ cellulose composite (ACFP) capsules	*L. plantarum*	Encapsulation by vacuum freeze-drying process	Li et al. (2019b)
15.	Sodium alginate, chitosan	*Bifidobacterium longum*	Emulsification and internal gelation is an encapsulation	Ji et al. (2019)

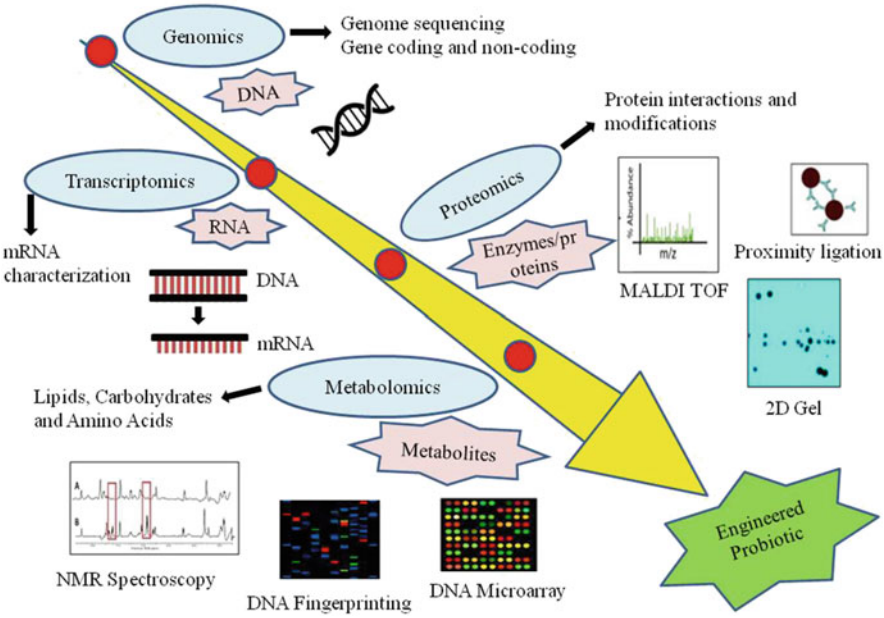

Fig. 10.2 A diagrammatical illustration for bioengineering of probiotics using different omics tools

biosynthesis of secondary metabolites, and microflora establishment process in new born (Mora et al. 2019). Comparative genome hybridization (CGH) approach can be used to conclude the phylogenetic relationships. Still, the limitation of this study is that only genes present on DNA microarray are detected whereas new genes cannot be detected (Wels et al. 2019). Proteomics and transcriptomics studies have been executed to determine the bile tolerance in probiotic strains, analysis of surface proteins, interactions between host cell and microorganisms, and revealed the catabolite control proteins in *L. acidophilus* A4 (Siciliano et al. 2019; Lee et al. 2010). Ultimately this will facilitate the in silico assessment of potential probiotic candidates. Metabolomics helps us to determine the metabolic changes occurred in the host. In *L. acidophilus* and *L. gasseri* species-specific biochemical qualities are characterized by comparing their metabolomic and lipidomic profiles via ultra-high-performance liquid chromatography–high resolution mass spectrometry (Chamberlain et al. 2019; Yadav and Shukla 2019b). Fluxomic analysis endows with the closest depiction of a cell and it is broadly used in the development of various strains. This will facilitate the estimation of bioenergy and redox production of the metabolic networks as well as formulate the quantitative relationship between nitrogen metabolism and central carbon fluxes (Wu et al. 2019; Yadav et al. 2018). All these bioengineering techniques provide the possibilities of probiotic strains improvement (Fig. 10.2). This will help to control the expression of genes, improve the functionality of genes, addition of required genes, increase resistance to enhance

the survival in the gastrointestinal tract, allow the delivery of vaccine and antimicrobial peptides to the gut (Martínez-Fernández et al. 2019). The first commercialized genetically modified probiotic product is Zbiotics.

10.4 Encapsulation and Immobilization

These are the new techniques in this modern era for transferring the synbiotics products in a better manner to host cells for advancement in health (Vallianou et al. 2019). This directly increases the shelf life of these products which makes them endures in the harsh environment of intestine to promote health and increase the number of beneficial microbes in the gut, removing the pathogens. In addition to this external gelation is one of the commonly used method for high cellular viability, it is cheap simple and have mild formulations (Laffleur and Strasdat 2019). Prebiotics can be added to probiotics in the form of microparticle formulations. The addition of different prebiotics such as inulin, maize rice bran alginate to probiotic strains using ionic gelation technique increases the power of these and made them incredible compound to resist in the harsh environment of the intestine (Ozaltin et al. 2019). For example, the encapsulating matrix of inulin with highest efficiency can be consider as best to be used for the transfer of microparticles of size ranged from 79.7 μm (alginate) to 117.70 μm (rice bran) (Poletto et al. 2019b). To increase the viability and activity of these symbiotics microencapsulation is a good approach recently to protect their life before reaching to their target site. It can be easily done by covering the live cells with a semipermeable coating protecting them from the unfavorable conditions and delivering them to the specific site of the body such as colon (Jain 2020).

The most important requisite of these particles is that they should resemble the context and texture of that compound or food so that these can be identified by the other gut microbes and can be utilized properly (Taneja et al. 2019). To fulfill these basic necessities some new strategies such as electro-hydrodynamic atomization (EHDA) or electrospray technology has been introduced; it has advantage over other encapsulation techniques such as spray drying and freeze-drying (Zaeim et al. 2019). In this technique, active materials having a polymeric solution are eluted out by a nozzle which is then atomized into ultrafine droplets by sturdy electrical forces. The droplets are then solidifies into hydrogel particles after immersing them in a gelling bath, a non-toxic and biodegradable wall matrix can also be crucially used for better protection of live cells (de Silva et al. 2019). The most commonly used material for encapsulation is Alginate, it is cheap and compatible; also, it can easily make hydrogel formulations (Pravinata and Murray 2019). In addition to this, these polysaccharides are very stable and can be degraded at low pH; also, the presence of chelating agents can erode their hydrogel networks. Usually these alginate capsules are covered by the layer of chitosan which can make strong polyelectrolyte complexes for improving the stability (Niizawa et al. 2019). Moreover, co-encapsulation of probiotics can be done with high molecular weight prebiotics in Ca-alginate/chitosan microcapsules by EHDA which can be proved

as a good combinatorial product for the exposure to gastrointestinal conditions (Kumar et al. 2019).

10.5 Conclusions

Probiotics and prebiotics coalesce have a great impact on human health, so the validation of these as food products is itself state of the art, which is urging a great interest in this modern era. Moreover, clear workflow can help in attaining the conclusive and trustworthy proof of their efficient work. Advancement in strategies used for identifying the function of specific strains can lead the story to the peaks. Various in vivo and in vitro techniques can validate many products for food industries; also in silico modifications can develop new products with the help of various techniques such as immobilization encapsulations, etc. Incorporation of these food products into the diet helps in enhancement of gut microflora and prevention of various metabolic or health-related disorders. In addition to this, progression in omics strategies can be useful for knowing the mechanism involved which can further focus on one strain-one function approach.

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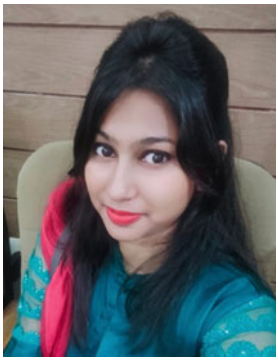

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Evaluation Techniques of the Chemical and Microbiological Water Quality in the Coastal Environment

11

Camila Burigo Marin, Katia Naomi Kuroshima, Ana Paula Stein Santos, and Marcus Adonai Castro da Silva

Abstract

Historically, many people live in coastlines, the social importance of the coastal environments has increased especially because of economic resources. The water quality monitoring is necessary because the environment health is equivalent to human health. The common chemical and microbiological parameters for water quality analyses include the physicochemical indicators; inorganic nutrients; organic compounds; trace metals; chlorophyll concentration; biochemical oxygen demand; and fecal indicator organism (FIOs) to evaluate the influence on the physiology of aquatic organisms, reactivity, trophic state of the ecosystem and to infer pathogenic risk assessments of coastal waters. Different indexes can help scientists to explain many parameters of analysis in a single representation of the water quality but the use of a unique index in different locations around the world will become increasingly distant, especially because of the technological development and the population growth to produce new and different substances. For a better assessment it is necessary to understand what activities are developed and which are the water uses to monitor these environments to choose parameters that help indicate the environmental health, to scale the risks and to show these results to the population and the government for planning ways to mitigate impacts.

Keywords

Water quality · Water quality index · FIOs · Emerging pollutants

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11.1 Coastal Environment and Pollution

The coastal environment is the area where atmosphere, land, ocean, and society meet (Angulo 2004). The term coastal environment is used to refer to environments that are subject to the action of tidal mixing, waves, marine currents (Philippi Jr 2010), for example, estuaries, coastal lagoons, bays, mangroves, wetlands, rocky coasts, and beaches. These environments are fragile and dynamics, highly stressed by human activities and anthropogenic inputs (Angulo 2004) and your social importance has increased over the last few years, supporting the human population growth, tourist activity and moving economic resources (WHO 2003).

Currently, more than 40% of the world population lives around coastal environments, especially within 100 km of the coastline (Zheng and Klemas 2018). For instance, the population growth and tourism development increased serious environmental impacts and damages, it can disrupt the balance between natural forces, increase water and energy consumption, production of sewage, waste and marine debris in large quantities (Vasconcelos and Coriolano 2008).

Coastal environmental and water quality are influenced by many natural factors interacting in the drainage basins, geological, hydrological, and geological characters (Marin et al. 2016), but closely reflect human activities in densely populated areas too (Billen et al. 2001).

The pollution in coastal environment occurs when substances added to the environment changes its natural characteristics, this situation is reported by many papers around the world, common pollutants are pathogenic microorganisms (Boehm et al. 2009; Henry et al. 2016; Kacar and Omuzbuken 2017), inorganic nutrients (Barletta et al. 2019; Marin et al. 2016), organic compounds (Sanganyado et al. 2018), trace metals (Nour et al. 2019), and recently reports about emerging pollutants (Peña-Guzmán et al. 2019) and microplastics (Farady 2019; Marin et al. 2019; Saley et al. 2019).

These anthropogenic contaminants originated mainly from the release of domestic and industrial wastewater (sewage), agricultural activities, combustion of fossil fuels, and poor waste management (WHO 2003; Marin et al. 2016), the effect depends on the quantity and composition of the pollutant as well as the environment depuration and dispersion capacity.

Untreated sewage is a potential source of several anthropogenic substances to the coastal areas being considered the major source of a wide variety of pollutants (Saeed et al. 2015). A large part of the nutrients and pollutants carried along the river course accumulate in the lower energy zones located in the estuaries. These environments receive, therefore, all polluting load, coming from the most diverse sources (Gopal et al. 2018). At the same time, it is in these areas that the largest population densities are found, needing these waters for their supply. Moreover, it is in these transitional ecosystems that there are a variety of organisms that use them as nurseries (Vasconcelos et al. 2011). Thus, the evaluation of the quality of these waters becomes of fundamental importance. Wastewater presence in coastal areas can cause several impacts on the environment, including effects on aquatic organisms, especially benthic biota, eutrophication, oxygen-depletion, bioaccumulation and biomagnification of carcinogenic and persistence compound.

The main provider of non-point source contamination is the agricultural runoff causing, the depletion of the environmental quality of water bodies (Chen et al. 2017). The excessive use of herbicides, insecticides, fertilizers, and soil regulators ends up to introducing large amounts of nutrients (especially nitrogen and phosphorus) (Yazdi et al. 2019). This increase in nutrient loading has detrimental effects on the coastal environment, especially on the exacerbated growth of primary production, naturally limited by the low phosphorus concentration, which may lead to eutrophication (Harrison et al. 2019). Furthermore, soil remobilization by tillage turns out to be carrying much of the particulate matter of soil and the contaminants adsorbed to organic matter (Chen et al. 2016).

Urban runoff has also been an important source of pollutants to coastal water bodies. With increasing urbanization and increasing automotive fleet, vehicles contribute with several chemicals into the environment due to brake and tire wear and fuel combustion, leaking of oil and tailpipe exhaust (Agrill et al. 2017). These substances accumulate along the impervious surfaces, such as streets, roadways, and parking lots, and are transported to rivers by storm water runoff in rain events (Chow et al. 2019). Furthermore, cities produce large amounts of waste that, if improperly disposed of, it also ends up being transported and flushed into water bodies.

Industrial wastewater is an important pollution source, depict some of the most challenging water treatment because it owns a diversity of chemicals such as phthalic acid, nitrogen, chemical oxygen demand, heavy metals, ammonium nitrogen, organic compounds, hydrocarbon, and other hazardous substances varies among different industries (Li et al. 2011; Raper et al. 2018)

The environmental health impacts directly on the quality of human life, studies about the link between water quality and human diseases in coastal environments are very important, especially because these are recreational areas.

Coastal waters may offer diverse health hazards, exposing users to chemical, biological or physical agents can bring risks to human health depending on the concentration and time exposition to pollutants (WHO 2003). Contact with contaminated water sources is responsible for 2.5 million deaths per year around the world (Henry et al. 2016).

11.2 Common Chemical and Microbiological Parameters in Coastal Environment

Due to the high population density and industrial activities increase along coastal regions, the deterioration of coastal waters is growing. The absence of planning in the use of water resources causes pollution of watercourses, erosion of surrounding areas affecting biota, human health, and quality of life.

To evaluate the impacts of a substance on the ecosystem is essential to take into consideration the concentration of the chemical in the water or sediment, their toxicity of the chemical, the exposure time to this, the tolerance of the organisms to exposure, and bioaccumulation potential. Even a low concentration of a chemical compound with high bioaccumulative potential could be lethal to the organism.

The presence of some substances in the environment may cause changes in the organism's metabolism, causing damages in the feeding, social, and reproductive behavior. These damages can make changes in the hole trophic chain. Also, some compounds may be bioaccumulated, increasing the vulnerability of the ecosystem. Biomagnification is another effect of some substances that increases their concentration following the food chain up, affecting directly the humans by the consumption of high trophic organisms.

Quality of the coastal environment, especially water quality can be evaluated in accordance with the substances contained, common pollutants include biological parameters (fecal indicator organisms-FIOs, phytoplankton, and zooplankton), chemical parameters (inorganic and organic compounds, oxygen demand, trace metals, emerging pollutants, marine debris), and physicochemical parameters (water temperature and turbidity (Baumgarten et al. 2010; Henry et al. 2016; Marin et al. 2016, WHO 2003).

Water quality can be evaluated through physicochemical indicators, biological parameters, indicators, and the effects caused by the variation of these parameters in the environment. Allowing to assess the condition of the environment at the time of collection, providing bases to detect trends and cause–effect relationships. These parameters are selected according to the potential pollution activities in the coastal environment or monitoring parameters that imply human or environmental health. The common pollutants can be seen in Tab.11.1.

The introduction of domestic and livestock wastes in the environment causes an increase in the number of enteric bacteria and viruses in coastal water bodies. Many of these microorganisms impose a threat to human health, causing waterborne diseases like cholera and hepatitis (Bitton 2011). Thus, to assess the microbiological quality of coastal environments, the analysis of several groups of microorganisms may be employed, especially fecal indicator organisms (FIO) (Rodrigues and Cunha 2017).

Probably, the most important FIO of water quality is the coliform bacteria group. The bacteria belonging to this group are defined as gram-negative and facultative anaerobic bacilli which can ferment lactose producing gas at 35 °C (Rodrigues and Cunha 2017). This group includes several genera of bacteria belonging to the family Enterobacteriaceae. A subgroup of the coliform group is the fecal or thermotolerant coliform, which comprehends three genera of bacteria, *Escherichia*, *Klebsiella*, and *Enterobacter*, that are capable of fermenting lactose at 44.5 °C. Although these three genera are usually inhabitants of the gastrointestinal tract of warm-blood animals, only the species *Escherichia coli* is exclusively fecal (WHO 2012).

Another group of fecal organisms that may be used as an indicator of water quality is the fecal streptococci group, in particular, the genus *Enterococcus*. The enterococci are differentiated from other fecal streptococci on its ability to grow at a pH of 9.6, at 45 °C, and in 6.5% NaCl (Scott et al. 2002). *Enterococcus* has been suggested as the most appropriate microbial indicator for the assessment of marine environments, due to a better correlation between these microorganisms and the incidence of waterborne diseases (Cabelli 1983).

Table 11.1 Description of chemical and microbiological parameters

Groups	Parameters	Justifications	Reference
Physicochemical	pH	Hydrogenionic potential has a direct influence on the physiology of aquatic organisms, especially at ion exchanges. Furthermore, alters the solubility and reactivity of several compounds and their bioavailability, such as trace metals and nutrients	Boyd et al. (2011)
	Dissolved oxygen	Low oxygen concentrations (<4 mg/L) in the aquatic environment can be lethal to most fish species, affecting migration and biological cycles, and even alter the chemical blast of these ecosystems	Coffin et al. (2018)
	Suspended particulate matter	May vary according to the nature of the environment and adjacent inputs. Additionally, suspended solids tend to adsorb toxic substances such as pesticides, fertilizers and metals. The concentration of these is closely related to the increase of turbidity, hindering the radiation to pass along the water column	Krepsky et al. (2019)
Inorganic nutrients	Nitrogen	In general, the main nitrogen source for rivers is domestic sewage, typically in the form of protein and ammonia. Some industrial effluents and runoff from agricultural areas may also contribute to the enrichment of the element in the aquatic environment	Adyasari et al. (2018)
	Phosphorus	High levels of this element indicate the input of effluents rich in organic matter and triggering high primary productivity rates	Liang and Xian (2018)
Organic compounds	Pesticides	Due to their widespread use, low affinity, and substantial soil accumulation, pesticides are generally leached through runoff to water bodies. It has high mutagenic, carcinogenic and teratogenic potential. It can cause mortality or genetic/reproductive problems in the fauna	EPA (2002)
	Oils and fats	Oil layer can cover the water surface thus prevents the oxygen dissolution. When mixing with water causes increases the oxygen demand and may cause several damages to aquatic biota and	Hanisah et al. (2013)
	Polycyclic aromatic hydrocarbons	Arising in the water bodies from natural (volcanic activity, natural burning...) and anthropogenic processes (industrial activities, domestic sewage, drilling and maritime navigation). EPA and the European Union categorized sixteen PAHs as priority pollutants, due to their teratogenic and carcinogenic properties	Tong et al. (2019), Kuśmierz et al. (2016)

(continued)

Table 11.1 (continued)

Groups	Parameters	Justifications	Reference
Microbiological indicators	Chlorophyll A	Chlorophyll a level in an aquatic environment is related to phytoplankton biomass and can be used to evaluate the trophic state of the ecosystem	Dodds and Whiles (2017)
	Biochemical oxygen demand	Low oxygen levels may indicate the input of effluents with high organic load, as bacterial decomposition increases, consuming dissolved oxygen in the water	O'Boyle et al. (2009).
	Fecal indicator organism—FIOs (*Escherichia coli*, *Enterococci*, etc...)	To infer and quantify pathogenic risk assessments of coastal waters	Henry et al. (2016)
Trace metals	Mercury	The main supply of mercury to coastal environments is through atmospheric deposition and inappropriate discharge in water bodies. Are classified as neurotoxic, mutagenic, teratogenic and carcinogenic, listed as a high-priority environmental pollutant	Eisler (1987), Colla et al. (2019)
	Arsenic	As sources of As can be considered farming, agriculture, wood preservation, mining, fossil fuels burning, among others. Fish and seafood are responsible for 90% of humans As exposure. Due to their high toxicity and carcinogenic potential, it is also listed as a high-priority environmental pollutant	Baeyens et al. (2019), Liu et al. (2019a, b)
	Lead	Lead concentrations in the coastal environment have been heightened by anthropogenic activities (industrial emissions, leaded paint, vehicular exhaust, as an example). This metal is considered one of the most hazardous heavy metals because of their high toxicity and bioaccumulation potential	Espejo et al. (2019), Marcirella et al. (2019)
	Chromium	Due to their widespread use in industrial applications Cr is among the hazards trace elements in the coastal environment. Chromium is a redox-sensitive metal, presenting a high solubility in Cr(VI) form. Long-term exposure to this can cause gastrointestinal and allergic effects even cancer	Almeida et al. (2019), Yang et al. (2019)
	Cadmium	Used mostly for the batteries' production, followed by plating, alloys, coatings and plastics' stabilizer. Densely industries populated areas exhibit high average concentrations of cadmium in the atmosphere	International Agency for Research on Cancer (2012), Zhang and Reynolds (2019)
Ecotoxicological risk	Toxicological risk to organisms	Toxicity tests evaluated the pollutants lethality in different species have been used to understand about lethal concentration, toxicity conditions and changes in environmental	European Community (2008)

Several other bacterial groups have been suggested as FIO. These include the species *Clostridium perfringens* and the bifidobacteria. *C. perfringens* is an anaerobic, gram-positive, spore-forming bacterium that causes gastroenteritis in humans. Although not widely used as a microbial indicator, it is recommended as such when the prediction of viral pathogens is desired (Scott et al. 2002). The bifidobacteria is another group of gram-positive and anaerobic bacteria that live in the gastrointestinal tract of humans and other warm-blooded animals. As stated by Ashbolt et al. (2001), the sensitivity of the bifidobacteria to oxygen has limited its use as indicators of water quality.

Some viral groups may also be used as FIO in the assessment of coastal water quality. This is important since the traditional bacterial indicators used do not correlate well with the presence of pathogenic viruses (Jurzik et al. 2010). To indicate the presence of these pathogens in the aquatic environments, it might be more appropriate to use viruses as bacteriophages, viruses that infect bacteria, as indicators of water quality, since they share several characteristics with human pathogenic viruses (Rodrigues and Cunha 2017). Among the bacteriophages suggested as microbial indicators the F(male) specific RNA bacteriophages, somatic coliphages, and phages of *Bacteroides fragilis* may be cited. Besides its use as indicators of water quality in general (Benjamin-Chung et al. 2017), these bacteriophages may also be used to track the sources of fecal contamination (Ravva and Sarreal 2016).

11.3 Index for Chemical And Microbiological Evaluation in Coastal Environment

The physical, chemical, and microbiological characteristics of water can be defined by assessing all parameters listed in Table 11.1 and discussed above. But, the evaluation of the quality of these waters can be a little more complex, because it depends on the ecosystem to which these waters belong and mainly of their use for the anthropic activity. At the same time, the different anthropic uses of this resource may alter its characteristics, often requiring the evaluation of numerous parameters for its characterization and assessment. Each of these parameters has specific physical and chemical properties, interacting in a unique way in the aqueous environment, in addition to the different interactions with the organisms present, controlling in a very particular way their behavior in the ecosystem in question, making it difficult to evaluate water quality (Soumaila et al. 2019).

Thus, the Water Quality Indices (WQI) were developed to facilitate the evaluation of coastal environment quality, especially the water quality, based on chemical, physical, and microbiological parameters. It is a very well-known method, which in addition to facilitating evaluation, allows reproducibility and reapplication in aquatic environments responding very well to their different uses. But for the proper use of this tool, it is necessary to have prior knowledge of the parameters that will be applied, as well as the biogeochemical processes that control them in each of the evaluated environments (Bharti 2011).

The WQI converts each physicochemical, chemical, and biological parameter that characterizes the water quality, with its different scales in numerical values through specific mathematical equations and accumulatively converges on a single scale of values, synthesizing the quality of the water as a whole (Sánchez et al. 2007; Bharti 2011). In other words, the main objective of a WQI is to provide a unique value of water quality by translating the sample constituents and concentrations of other evaluation methods (Noori et al. 2019; Soumaila et al. 2019). It can summarize large amounts of water quality data in simple qualitative scales (e.g., excellent, good, bad, etc.) to report to public and user management in a simple but consistent way (Kieling-Rubio et al. 2015).

The first index to evaluate the quality of water was developed by Horton in 1965 (Brown et al. 1970), later, a similar index was adapted by the National Sanitation Foundation, called NSFWQI (Bharti 2011; Noori et al. 2019; Oliveira and Pinhata 2008). The most commonly used in the various environmental evaluation studies of that time for the elaboration of this index are: percentage of dissolved oxygen saturation, pH, temperature variation, total solids, biochemical demand of oxygen, turbidity, nitrate, total phosphorus, and fecal coliforms. Each of these parameters was converted into a sub-index with different weights, ranging from 1 to 4, proportional to its impact and importance in the determination of water quality. The final index score was obtained by the weighted sum of the sub-index of each parameter divided by the sum of their respective weights, as shown in Eq. (11.1):

$$\text{NSFWQI} = \frac{\sum_{i=1}^{n} WiQi}{\sum_{i=1}^{n} Wi} \tag{11.1}$$

where Q_i and W are, respectively, the sub-index and the weight coefficient of parameter i, and n is the number of water quality parameters, in this case, this number is nine (Noori et al. 2019).

This index is considered well detailed and generally applicable for a classification of surface water resources of a variety of anthropic activities, and thus, it has been used for a long period and continues still in use not only in America but also in a lot of other countries, like Morocco (Barakat et al. 2018), India (Gupta et al. 2017), Croatia (Tomas et al. 2017), Indonesia (Effendi and Wardiatno 2015; Effendi 2016), and China (Tian et al. 2019).

Noori et al. (2019) reported that the inadequate use of several parameters of this index showed different values of WQI for the same environment. One of the main errors reported by them is the use of different substances in substitution to original compounds during the index calculation, for example: the use of orthophosphate in substitution to the total phosphorus; or, the use of "total dissolved solids" or "total suspended solids" in substitution of "Total solids," and the use of Fecal Coliforms by maximum probable number (MPN) using multiple tube fermentation in substitution to colony-forming unit (CFU) using membrane filtration techniques. These substitutions have generated different values of WQI due to the use of non-original parameters. In most cases, the water was classified as more favorable using these parameters changed, overestimating the real environment (Noori et al.

2019). Thus, the proper use of the parameters for calculating the water quality indices is fundamental for obtaining the exact indices, allowing not only its evaluation but also allowing a comparative evaluation between different sites with the use of the same index.

Despite the frequent use of these indices, some authors have begun to adapt NS FWQI to their region of study including other more important parameters or by altering the weight of each parameter (Abrahão et al. 2007; Moretto et al. 2012; Tomas et al. 2017; Ejoh et al. 2018; Mladenovic-Ranisavljevic and Zerajic 2018; Aguilar et al. 2019; Oliveira and Pinhata 2008; Samsudin et al. 2019; Tian et al. 2019). Several researchers have followed the same trend and continued to work on the elaboration of several WQIs and their use has been strongly suggested by agencies responsible for water supply and control of water pollution. Bharti (2011) describe the history and evolution of these indices in great detail, analyzing mainly eight of these indices, as to the parameters used, the mathematical structure for the elaboration of indexes, and their failures. Later, Kachroud et al. (2019) described another critical analysis regarding the use of the various WQI. They reported the development and evolution of the use of a variety of IQA on all continents. The inclusion of substances such as Alkyl Benzene Sulphonates in the index developed by Prati in 1971, for Europe; the inclusion of pesticides and Polycyclic Aromatic Hydrocarbon in the WQI developed by the Lower Great Miami Watershed Enhancement Program in Dayton (USA) and the emergence of new methods of calculating these indices in the first decade of the 21st century, highlighting the use of method that is based on fuzzy logic. This method allows the inclusion of qualitative parameters, such as odors, that could not be included in the quantitative equations (Kachroud et al. 2019). Briefly, these various existing WQI have a slight variation between the parameters used, but the specific weights and mathematical integrations used for their development vary greatly.

The elaboration of all these indices comprises basically four stages: (1) The selection of parameters, which change according to the different uses and locations of evaluation, (2) the conversion on a common scale due to the use of several parameters with scales and magnitudes different, (3) the attribution and weights and creation of sub-indices, according to the importance of each variable in the evaluated environment, and (4) the development of the mathematical equation to aggregate all sub-indices generating a single representative value of water quality as a whole (Kachroud et al. 2019; Soumaila et al. 2019).

Recently, new WQI models have been developed, with the inclusion of new tools integrating field observations and laboratory determinations with geospatial technologies, including Remote Sensing, Global Positioning Systems (GPS), and Geographical Information System (GIS) providing information continuously, spatial and temporally (Jha et al. 2015; Obade and Moore 2018). The use of multivariate statistics such as principal component analysis (PCA) and cluster for analysis of parameters concentrations and obtaining the final value for WQI (Sun et al. 2016; Liu et al. 2019a, b; Tripathi 2019) or by the use of specific software developed for this purpose (Mladenovic-Ranisavljevic and Zerajic 2018) are also methodologies to obtain this index.

Kachroud et al. (2019) report in their review the existence of 20 WQI in 1982, rising to more than 30 WQI in 1997, and currently, this number should already be higher, all working with the same principle of WQI but varying in the number of parameters used, weights and calculations techniques for obtaining the final value. All these WQI refer to river environments and vary according to the use of their waters, the activities that are exercised in their watersheds and the quality of the waters refer mostly to the use of humans. Few indices are determined for environmental quality, for the ecological equilibrium of the environment. Therefore, there are few indices observed for coastal waters. Exception is observed for coastal areas of environmental protection, such as the determined index for the coral reef regions belonging to the Marine Protected Area of the Rosario Islands in Colombia (Tosic et al. 2019) or in the coastal area of Mallorca Island, Northwest of Mediterranean (Alvarez-Berastegui et al. 2014).

Thus, since the first WQI developed by Horton in 1965, many other indexes have been developed, with specific purposes for each area according to their uses. Technological development to produce new substances incorporating industrial activities, as well as their introduction to aquatic environments and their analytical detection, will certainly make the WQI number continually increase and uniformity in a single WQI to facilitate evaluation in different locations around the world will become increasingly distant.

Another index that we can highlight is based on the resistance of bacteria to antibiotics, it was developed and used to express the degree of multiantibiotic resistance (MAR) of a sample or area (Krumperman 1983). This index for a sample is calculated by the formula MAR index $= a/(b*c)$, being a the total number of resistant scores of all bacterial strains from the sample, b the number of antibiotics tested, and c the number of bacterial strains isolated from the sample. A threshold of 0.20 has been used to separate samples more contaminated (MAR index >0.20) from samples with less antibiotic exposure (Krumperman 1983; Titilawo et al. 2015).

The MAR index may be applied in the microbial tracking of animal and human sources of fecal contamination in aquatic environments. Guan et al. (2002) compared three methodologies to discriminate the source of fecal contamination concluded that the MAR index, along with the analysis of 16S genes, provided moderate to high degrees of correct classification of strains of *E. coli* from human and animal sources. The authors also stated that the MAR index is more appealing since it is an easy and low-cost method of evaluation.

The MAR index was also suggested by Chitanand et al. (2010) as a way to recognize high-risk contamination sites. These authors observed that the samples collected downstream to the city of Nanded (India), which were more contaminated, had higher MAR index (MAR = 0.43, average of three sites) that samples collected from upstream sites (MAR = 0.15, average of three sites). This application was also evaluated by Titilawo et al. (2015) who observed that the MAR index of rivers samples, calculated from the examination of 300 strains of *E. coli*, ranged from 0.50 to 0.80. This result indicated that the strains evaluated were originated from high exposure to antibiotics.

The index is very useful, classification of the waters is used to transform many parameters in a single representation of the water quality (Sánchez et al. 2007). The WQI is usually applied to safe drinking water or freshwater environment evaluation (Abbasi and Abbasi 2012; Adimalla and Qian 2019; Wu et al. 2018), but its application in marine waters or estuarine zones is also possible (Samsudin et al. 2019). The WQI application is usually due to the risks that water offers to human health, but we need to use them carefully. The water quality evaluation is the most important to understanding the environmental health, to scale the risks, plan ways to mitigate the impact and to show these results to the population.

11.4 Best Available and Emerging Techniques for Anthropic Contamination

Sundry chemical markers can be used as indicators of anthropogenic pollution, may be naturally present in the human body, or may come from our consumption of food or products. The population growth and the chemical industry development make available a variety of chemicals to coastal environments and it can be used as indicators of human contamination beyond conventional indicators.

11.4.1 Chemical Markers (Sterols)

The microbiological markers (total coliforms, fecal coliforms, and fecal Streptococci) have been used as an indicator of sewage pollution due to the fast and simple application (Da Costa and Carreira 2005). Moreover, fecal pollution occurs from an assortment of sources, and just these microbiological markers do not allow us to discriminate this variety of sources by themselves (Leeming et al. 1996). Many studies have highlighted the use of sterols as environmental biomarkers, due to their stability and permanence in the depositional environment, preserving specific structural characteristics (Da Costa et al. 2018; Volkman 1986).

Owing to their importance as cells membrane constituents, sterols are commonly found in coastal waters and sediments. This lipid family compounds are naturally present in plants and animals (Lim et al. 2017). Phytosterols (Campesterol, stigmasterol, and β-sitosterol) compounds, with 28–29 carbon chain, are commonly attributed to terrigenous markers by their presence in the composition of higher plants (Derrien et al. 2017). Long chains sterols, such as C_{28}, C_{30} and dinosterol, are predominantly deriving from phytoplankton (Mackenzie et al. 1982). Cholesterols could be attributed to a range of sources, including phyto and zooplankton and terrestrial inputs (He et al. 2018).

After passing through the intestinal tract of warm-blooded animals, sterols can be converted to stanols by enteric bacteria. As a consequence, the presence of stanols is inherently associated with feces presence. The most commonly mentioned stanols in sewage contamination studies are coprostanol and epicoprostanol, as these are predominant sterols in human feces and are not found in non-impacted marine

sediments (Leeming et al. 1996). Coprostanol is a well-known biomarker for anthropogenic sewage input to marine environments, making up about 40–60% of the human wastewater sterol content, and combining with other sterols work as indicators of abundance in sediments (Nakagawa et al. 2019; Jeng et al. 1996; Cabral et al. 2018).

Some ratios between some sterols and stanols can be used to subsidize the indication of the level of domestic sewage contamination.

Sterols and stanols ratio	Wastewater contamination	Reference
Coprostanol/cholesterol	>1.0	Quérméneur and Marty (1994) Peng et al. (2005)
Coprostanol/cholestanol	>0.5	Leeming et al. (1997) Davene et al. (2019)
Coprostanol/(cholestanol + cholesterol)	>0.06	Writer et al. (1995) Lim et al. (2017)
Coprostanol/(coprostanol + cholestanol)	>0.7	Leeming et al. (1998) Grimalt et al. (1990) Wayland et al. (2007)
Coprostanol/epicoprostanol	>1.5	Martins et al. (2014) Fattore et al. (1996)

Sterols and stanols, which acts as chemical markers, have been successfully employed as organic molecular markers to evaluate and distinguish between sources of either anthropogenic contamination or natural organic matter inputs (marine and terrigenous sources) (Eganhouse 1997). Coastal environments are particularly prone to sewage contamination due to the singular hydrodynamics condition, high population density around water bodies, and improper or illegal waste disposal into the environment (Cabral et al. 2019).

11.4.2 Emerging Contaminants

In the last few years, the interest about the presence and risks associated with the presence of emerging contaminants in coastal environments have been increasing. Substances used in day-to-day life have generated a series of new contaminants that are called emerging pollutants. More than 1000 different emerging substances from anthropogenic activities are identified and classified into different groups: Personal care products, UV filters, pharmaceuticals, endocrine disruptors, metabolites, food additives, hydrocarbons, illicit drugs, fire retardants, and pesticides (Peña-Guzmán et al. 2019). The great majority of these contaminants enter into the water bodies by the human use and disposal, insufficient municipal sewage treatment, landfill leachate, urban runoff, and industrial waste.

Unfortunately, conventional water treatment and sewage treatment plants are not designed to treat or eliminate these substances, these pollutants do not eliminate them easily leading to human and environment exposure (Ebele et al. 2017; Jardim et al. 2012). The coastal environment is particularly susceptible to emerging

pollution, especially because of population growth and tourism (Peña-Guzmán et al. 2019).

Analysis of emerging pollutants is very important because these contaminants may persist in the environment, bioaccumulate, are capable of altering the metabolism of a living being and as potentially causing adverse effects such as endocrine-disrupting effects, mutagenicity, carcinogenicity, and higher toxicity on ecosystems and/or humans (Ebele et al. 2017; Ribeiro et al. 2017).

Studies concerning emerging pollutants have increased for the last years, but it is necessary to understand the occurrence of different pollutants in various components of the coastal environment. The analysis of emerging pollutants is not easy must be carried out with selective, sensitive, precise, and with methods applicable in a wide range of matrices and many different compounds (Lorenzo et al. 2018). This substance detection only has been possible with recent studies and technological advances in analytical techniques (Gogoi et al. 2018).

11.4.3 Personal Care Products

Personal care products and pharmaceuticals make up the largest group of emerging pollutants, affecting soils, and aquatic ecosystems surrounding treated or raw sewage inputs. A great mix of chemicals takes part in soaps, lotions, cosmetics, fragrances, sunscreens, and other composition (Montes-Grajales et al. 2017). There are the great concern about the presence of these kinds of products in the aquatic environment, because their toxic effects, persistent character, and bioaccumulative potential.

UV filters are largely used (about 10,000 tons annually), especially with the awareness of the dangerous effects of UV radiation has been one of the most personal care substances found in surface waters, wastewaters, and tap water also. Exhibited a lipophilic character tend to accumulate in organisms such as fish, mussels, sea urchin, and mammals (Molins-Delgado et al. 2017). The effects of continuous exposure to these compounds in marine biota are not well elucidated. However, some types of UV filters have shown estrogenic effects, affecting reproductive metabolism (Weisbrod et al. 2007).

The most common active substances in mosquito repellents are N,N-diethyl-m-toluamide (DEET) (Brausch and Rand 2011). It is considered an emerging pollutant due to its continuous input in the aquatic environment, persistence and toxic effects evidence (Celza et al. 2011). DEET has been widely found in aquatic environments (Pietrogrande and Basaglia 2007). According to Costanzo et al. (2007), this repellent shows a low bioconcentration ratio, nonetheless, it has been efficient to cross the dermal barrier and can accumulate in fat deposits.

Triclosan and triclocarban are common antimicrobial agents in personal care products from toothpaste to disinfectant (Chen et al. 2018). Due to their high hydrophobic character, they have a great tendency to retain soil or organic matter (Durán-Álvarez et al. 2012). These compounds have been showing toxic and bioaccumulative effects on biota, especially to the benthic fauna (Perron et al.

2012; De Lorenzo et al. 2008). The presence of triclosan in fish and even breast milk has been reported (Adolfsson-Erici et al. 2002).

11.4.4 Pharmaceuticals Substances

Even at low concentrations, pharmaceuticals can induce correspondent specific biological responses in the aquatic biota, similar to clinical effects expected for humans. Recent field and laboratory studies have been shown that fish submitted to a low concentration of pharmaceuticals present an analogous reaction to those expected clinical effects (Galus et al. 2013).

While in a concentration lower than human use doses, due to the bioaccumulation potential and chronic exposure, some pharmaceuticals have been distributing effects to the coastal environment.

Estrogens and hormones directly affect organism growth and development (Dey et al. 2019). Due to their long-term persistence, exposure during the organism's critical life stages can affect reproductive development. Gonadal intersex and altered sex ratios in fish have been reported by Vajda et al. (2008), Woodling et al. (2006), and Niemuth and Klaper (2015). A commonly used oral contraceptive is Ethinylestradiol.

11.4.5 Antibiotics

The indiscriminate and incorrect use of antibiotics over several decades, along with the disposal of animal and human wastes, resulted in an increment of the concentration of these drugs in the aquatic environments. Le and Munekage (2014), for instance, detected the presence of four antibiotics in all samples collected at shrimp ponds in a mangrove area in Vietnam. Higher concentrations were observed in sediments, breeding zones, and adjacent outlets. As a consequence of these high concentrations of antibiotics in the environment, the incidence of resistant bacteria among the members of the microbial communities may increase (Martinez 2009). Thus, the evaluation of the resistance of bacteria isolated from the aquatic environment to antibiotics may be used as a complementary tool in the assessment of the water quality.

Banerjee and Farber (2018) investigated the resistance of Vibrio strains isolated from mollusks of an estuarine area to several antibiotics. The bacteria evaluated show single and multi-resistance to five commonly used antibiotics: ampicillin, cephalothin, erythromycin, kanamycin, and streptomycin. In another study conducted at the Southeast of Brazil, Oliveira and Pinhata (2008) reported the dominance of *Enterococcus faecalis* among the species of the genus *Enterococcus* in water and sand samples of two beaches. These authors also reported that the frequencies of multi-resistant strains were higher in the more polluted beach in both water (66.7%) and sand samples (61.5%), in comparison with the less contaminated one (35.7% and 31.25% in sand and water samples, respectively).

One of the suggested applications of the analysis of antimicrobial resistance to the assessment of aquatic environments is to discriminate between animal and human sources of fecal contamination. As an example, Edge and Hill (2005) evaluated the resistance of 462 strains of *E. coli* from surface waters and fecal samples to 14 antibiotics. Antibiotic resistance was more common among wastewater strains than from those isolated from bird feces. The authors used this method to investigate the contribution of birds to fecal contamination of the Bayfront Park beach, concluding that this animal source may at times be the main source of *E. coli* to this environment.

11.4.6 Microplastic

In the last years the problems associated with the presence and impacts of plastic marine debris have emerged in our coastal environment and oceans (Farady 2019) because the plastic pollution is ubiquitous and a transfrontier problem, goes far beyond being an environmental nuisance, it is also a big trouble to public health (Avio et al. 2017; Carbery et al. 2018).

The index to assess the coastal environment and water quality excludes the microplastic analysis. A few approaches are using the plastic or microplastic as an indicator parameter, but the indexes were created to measure just the pollution by marine debris and not by integrated approaches.

These tools classified the beaches according to their degree of debris pollution (Marin et al. 2019) such as Pellet Pollution Index—PPI (Fernandino et al. 2015) and Clean Coast Index—CCI (Alkalay et al. 2007). PPI is calculated using the number of collected pellets found in square meter (Fernandino et al. 2015) and included the one type of primary plastic, up to 5 mm. While CCI is used to determine the cleanliness level of the coast is calculated using just the plastic items with a size greater than 2 cm found in the sample area (Alkalay et al. 2007).

The impacts of microplastic or plastic debris are much bigger than visual pollution or a punctual inconvenience causing damages such as: develop diseases or animals death, dispersal of invasive species, high ecological risk. Plastic has the capacity to transport and interact with chemical compounds such as metals, emerging pollutants and act as a hotspot for virus, bacteria and other microorganism (Derraik 2002; Ivar do Sul and Costa 2014; Yukie et al. 2001).

The persistent pollutants like microplastics are recognized as a spreading vector of human pathogens representing a serious risk to users of the coastal environment and a threat to human ingest of marine derived-foods (Imran et al. 2019).

11.4.7 Plasticizers

Plasticizers are additives used in various material alloys to provide flexibility and pliability to several kinds of products such as building materials, food packages, home furnishings, cosmetics, medical equipment, and others (Fromme et al. 2002).

Common examples of plasticizers are bisphenol A and phthalates (Sauvé and Desrosiers 2014). Both compounds can cause damage to development and reproductive systems, especially to mollusks, crustaceans, and amphibians (Oehlmann et al. 2009).

Phthalates began to be used as chemical additives in 1926. During product manufacturing and throughout the use, phthalates can evaporate or desorbed from final products, these losses can be carried to the various environments. These compounds can be found in the atmosphere, surface water, stormwater, wastewater, sediment (Clara et al. 2010). Evidence shows that phthalates can present chronic and acute toxic effects in aquatic fauna and humans. Typical symptoms are effects on aquatic embryos organisms (tail curvature, necrosis and death), cardiac diseases, endocrine-disrupting (Gao et al. 2018).

Bisphenol A (BPA) is the plasticizer with the largest production volume in the world with high potential persistence in the environment (Horn et al. 2004). Large amounts of BPA are missed into the environment in manufacturing processes and daily applications (Munguia-Lopez et al. 2005; Pang et al. 2019), had been found in food, drinking water, atmosphere, surface water, stormwater, wastewater and sediment (Graziani et al. 2019). The human exposure to bisphenol A has consequently generated the determination of the compound in samples of fat, blood, urine, breast milk and even in fetal and placental tissues (Vandenberg et al. 2007). BPA has hazard reproductive effects, in amphibious can alter egg production and sexual maturity in females, in fish can induce female-biased sex ratios and gonadal intersex and gonadosomatic distribution (Vajda et al. 2008). A reported effect is also adipogenesis and insulin level changes (Qui et al. 2019; Casals-Casas and Desvergne 2011).

11.4.8 Food Additives

Chemical additives are often added to human and veterinary food products aiming at increased durability, nutritional enrichment, increased palatability (Singh and Mondal 2019). Emerging pollutants associated with food activity are often found in coastal environments, associated with anthropogenic contamination, such as caffeine and artificial sweeteners.

Caffeine is present in the composition of several beverages, foods, and drugs, and therefore the predominant input in the coastal environment is by domestic wastewater (Dafouz et al. 2018). Daily human consumption of this substance is more than 450 tons (Di Lorenzo et al. 2019). Thus, caffeine has demonstrated to be a good anthropogenic marker sewage contamination (Buerge et al. 2003; Ferreira et al. 2005), may be found in surface waters, rivers, estuary, reservoir. The presence of the substance has been reported in drinking water, in the coastal environment around the world (Silva et al. 2014; Benotti and Brownawell 2007; Gheorghe et al. 2016; Valcárcel et al. 2011). There are not many caffeíne acute effects portrayed in the literature, otherwise, aquatic ecosystems are susceptible to chronic exposure (Rodríguez-Gil et al. 2018).

Artificial sweeteners are food additives applied to flavored foods, drinks, medicines, etc. A high concentration of sweeteners is used in the industry daily in products to humans and animal food (Lou et al. 2019). Just as caffeine, the exclusive source of artificial sweeteners to the river and water bodies is from wastewater contribution, it can be applied as a chemical marker to assess the presence of treated or untreated sewage inputs (Blackstock et al. 2019). Sucralose, acesulfame and cyclamate its largely described in the literature, widely found in most diverse environment (Tollefsen et al. 2012; Gan et al. 2013; Fakadu et al. 2019; Sharma et al. 2019; Cant et al. 2019; Cantwell et al. 2019). Literature reports that environmentally relevant concentrations (0.05–155 μg/L) can cause damage to *Cyprinus carpio* blood cells (Heredia-García et al. 2019), gills, muscle, brain, and liver (Saucedo-Vence et al. 2017). Other studies show that sucralose has low toxicity and has no significant bioaccumulative effect on aquatic organisms (Wiklund et al. 2012; Huggett and Stoddard 2011).

11.5 Conclusions

The water quality evaluation is complex because natural and anthropogenic interferences required multiple analyses using a miscellaneous of indicators. It is very difficult to standardize index and sometimes choose the indicators once each environment has its own specificity, so it is necessary to understand the developed activities and the water uses. Water quality index—WQI techniques are important to facilitate water quality interpretation, on the other hand, the simplification is dangerous when the method does not elect the crucial parameters.

The water quality in coastal environments is very important and is not possible to have any uncertainty. The importance of water is increasing day by day, we need water in quantity and with good quality too, to contact applications, drinking, industry, irrigation, and supply life across the world.

With the advancement of analytical techniques, it becomes possible to use various chemical compounds to determine the anthropic influence on coastal environments. The so-called emerging compounds are increasingly being studied.

The technology development provided the creation of new substances, their use, and we still do not know their effects on the environment, their interactions, or the possibility of generating even more harmful byproducts. Also, we often do not have the technology to detect them.

These facts highlight the importance of constant monitoring not only of chemical and microbiological parameters but also of activities that are regionally developed to update the monitored parameters and to seek for more sensitive and effective analytical methods.

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Enzyme Engineering Techniques for Biotechnological Applications

12

Mandeep, Guddu Kumar Gupta, and Pratyoosh Shukla

Abstract

Enzymes are natural biocatalysts, found in all living organisms, which play a vital role in the biochemical reactions as inside or outside the cell. Wide ranges of enzymes are utilized in different industrial applications including medical, agriculture, food, and environment. Traditional enzymes have high cost, non-immobilization, low temperature and pH instability, and non-re-usability which are deliberated barriers to their industrial applications. Thus, enzyme engineering and designing of enzyme has capability to enhance the stability of temperature and optimal pH, improvement in the catalytic activity for the substrate recognition, and so on. Enzyme engineering uses enzyme designing tools such as rational and semi-rational design, directed evolution, molecular dynamics and homology modeling, random mutagenesis, DNA shuffling, cell surface display technique, peptidomimetics, flow cytometry/cell sorting, and de novo enzyme engineering. Among these, enzyme immobilization on nanoparticles has significant characteristics for the enzyme engineering process. Some recent techniques of enzyme designing process such as computational design, de novo design, and rational and semi-rational design will be consider as future trends to create the new catalytic site with newer amino acids residues.

Keywords

Enzyme engineering · Computational design · De novo design · Rational and semi-rational design · Catalytic site

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12.1 Introduction

Enzymes are the biological macromolecules that catalyze a reaction towards the forward direction. Enzymes catalyze various biological responses based on their substrate specificity (Saini et al. 2020). Enzymes obtained from microorganisms have a high potential to be used in different industries for speedy action and cost-effective production (Yadav et al. 2018). Enzymes are used in various industries such as the paper industry, food industry, leather industry, etc. (Sinha and Shukla 2019).

Enzymes are in high demand for industries as they reduce the level of pollutants released from the industry by dipping the use of synthetic chemicals. Thus there is a requirement of enzymes for eco-friendly development of industries as well (Nigam et al. 2017). But the native form of enzymes is not much efficient to withstand the harsh conditions of the industries. Their compatibility with the industrial conditions needs enzyme engineering (Rothlisberger et al. 2008).

Enzyme engineering uses recombinant DNA technology to identify genes responsible for enzyme production and thereafter the gene modification to improve the stability and function of the existing enzyme (Zhang et al. 2016). Thus enzyme engineering involves genetic mutation by insertion or deletion of nucleotides (Kumar et al. 2018). Enzyme engineering is required to accomplish enhancement of an enzymatic property (Kumar et al. 2017), exclusion of allosteric regulations, and development in the selectivity of substrates increased temperature stability, change in optimal pH, and so on (Xie et al. 2014).

12.2 New Techniques in Enzyme Engineering

Enzyme engineering is a dynamic field (Singh et al. 2017). There is an enormous need for resilient enzymes in industries to bear adverse conditions (Baweja et al. 2016). Enzyme engineering involves enzyme designing (rational and semi-rational), directed evolution, molecular dynamics and homology modeling, random mutagenesis, DNA shuffling, cell surface display technique, peptidomimetics, and de novo enzyme designing (Kellogg et al. 2011). Nowadays, computational tools and artificial intelligence techniques (Dixit et al. 2019a; Dhankhar et al. 2019) are being used for designing best fit models for quick enzyme modeling techniques. Figure 12.1 shows various advanced techniques used for enzyme engineering for enhancement of catalytic activity and stability of enzymes.

12.2.1 Rational and Semi-Rational Designing of Enzymes

In the rational design, we understand the structural and functional knowledge of protein to make their desire protein changes (Gupta et al. 2017). Thus, certain features of the protein can be modified easily. It has the advantage of being technically easy and inexpensive (Ghislieri et al. 2013). To better understand,

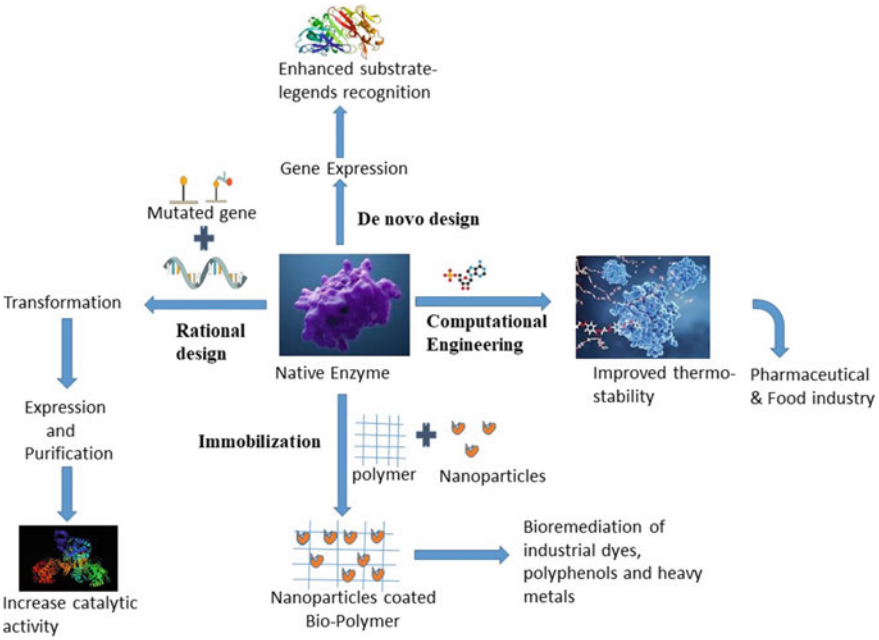

Fig. 12.1 Various advanced techniques used for enzyme engineering

site-directed mutagenesis techniques are well developed. However, the major drawback of it is that the fundamental knowledge of protein is not available, but sometimes even when possible; it is more complicated to predict the effects of various mutations; provided a static picture of a protein (Kille et al. 2011). However, there are various techniques such as Folding@home and Foldit used to gain insight into protein motifs, which are work at the platform on crowdsourcing techniques (Liskova et al. 2015). The rational re-design of the catalytic site can be simply conceivable, e.g. by the interruption in the creative binding site for the undesired structure by the introduction of a large molecule (Pavlova et al. 2009). The detailed structure of proteins can be found via various sources like X-ray and nuclear magnetic resonance (NMR) structure available on the Protein Data Bank (PDB) in the form of indirect structural information about legend or substrate binds (Reetz et al. 2006).

While semi-rational design uses facts about a protein structure, function and sequences in tandem with predictive algorithms (Xu et al. 2013), which used to identify the intended amino acid residues to influence protein function. Sometime these amino acids are mutated and provide enhanced protein properties (Korendovych et al. 2011). This helps to point out the hot spot residues using structural observation with the help of site-saturation mutagenesis test (Moroz et al. 2015) and also to obtain the desired product, the catalytic spot must be complementary in shape to the reaction transition state (Raymond et al. 2015). The detail comparative techniques of rational and semi-rational designs are given in

Table 12.1 List of rational and semi-rational designs with their uses to analysis of different parameters for enzyme designing processes

Designs	Parameters and features	Representative citation
Rational design		
Visual inspection, docking, ISM	Substrate specificity, Stereo selectivity	Ghislieri et al. (2013)
B-Fit	Thermostability and pH optimization of enzyme	Liskova et al. (2015)
IntFOLD	Protein structure prediction	Xu et al. (2013)
Threading ASSEmbly Refinement (I-TASSER)	Protein structure prediction	Moroz et al. (2015)
site-directed mutagenesis (SDM)	Evaluate the structure, stability, substrate–legends interaction of the enzyme	Pavlova et al. (2009)
Semi-rational design		
FoldX	Point mutation detection, determine interaction energy of protein complex and in silico alanine scan	Xie et al. (2014)
Rosetta-ddG	Estimation of protein complex binding affinity, detection of single point mutation	Kellogg et al. (2011)
FireProt	Design of thermostable multiple point mutant	Musil et al. (2017)
B-FITTER	B-factor analysis and molecular dynamics (MD)	Reetz and Carballeira (2007)
Iterative saturation mutagenesis (ISM) and RosettaDesign	Detection and engineering of hotspot flexible sites	Zhang et al. (2016)

Table 12.1. These semi-rational design techniques provide powerful and compelling new strategies to manipulate the properties of biocatalyst more than rational design technique (Burton et al. 2016).

12.2.2 Computational Engineering for Fast Designing

The primary purpose of computational engineering of enzymes is to create the three-dimension structure of the active site in the transition state of reaction that can be expected to obtain desire catalytic activity. There are several enzyme virtual libraries in silico created by the computational engineering (Kiss et al. 2013a, b). Designs are then assessed and categorized automatically based on energy scoring functions or geometric restraints (Wijma et al. 2014). The use of computational methods with less specific parameters gives poor predictability and suffers disadvantages in case of enzymatic reaction due to less specific substrate binding, orientation of substrate with enzyme, and stereoselectivity (Siegel et al. 2010). Although the computational design is comparatively unexplored to the directed evolution method, so it is

becoming the most useful tool for refining enzyme characteristics (Goldsmith and Tawfik 2012). The computational design also helps for the introduction of 10-20 point mutations in one step, which marked it to obtain unexpectedly huge changes in legend recognition and other catalytic assets (Privett et al. 2012). The secondary structural modification of protein has been possible by using computational enzyme engineering method (Althoff et al. 2012). Additionally, the computational design techniques have approximately 10^2–10^4 designs for the re-design of an entire binding pocket (active site) that contrasts in primary sequence, 3D structure, and potential energy as well (Bjelic et al. 2013). The production of a highly stable design of catalytic site has been suggested the lower potential energy due to the inaccuracies of energy predictions. The designs must be scrutinized and, indeed likely to be enzyme designed alternates will fold, bound with the substrate, and accomplish the preferred catalytic reaction (Hallen et al. 2013). The computational design uses following parameters such as conformational sampling, constraints, search algorithms, ranking, and conformational sampling (Scouras and Daggett 2011).

For the study of a mutation or a conformation in protein design structure, firstly, it must be generated by conformational sampling via using the K^* and FOLDX software (Chen et al. 2009). The mutations can be introduced at lower energy potential, demonstrated by the ORBIT and ROSETTA software (Leaver-Fay et al. 2012). The simple constraints tool being used to avoid the undesired conformations of the designed protein residues in the transition state of reaction, which fitted inside the cavity of the active site (Khersonsky et al. 2011). It also plays a vital role in the speeding up of calculations because there is no longer useless energy remains. In the present space of vast sequences, search algorithms (e.g. FASTER-Fast and Accurate Side-Chain Topology and Energy Refinement) can use pairwise potential to get the lowest energy conformation sequence (Lassila 2010). Another stochastic algorithm (called Monte Carlo) is used to find out the multiple low-energy points and repetitively test random mutation in conformational sequence space. Protein design structures are ranked based on catalytic efficiency and protein stability after the search algorithm. The rank design of the enzyme may be done with the help of molecular dynamics simulations and quantum mechanical methods (Richter et al. 2012). Molecular dynamics simulations predict the accurate structure and dynamic fluctuations of design, and also proved the favorable geometric of active site where the substrate is bound to the catalytic site. The computational designing methods support re-design of the catalytic site according to their substrates. It has been as improvements in the k_{cat}/K_M value or substrate specificity up to three order magnitude (Murphy et al. 2012), for example, adenosine deaminase in the organophosphate hydrolase ($>4 \times 10^3$ k_{cat}/K_M). Besides, computational re-design was noted in the specific recognition site. Homing endonucleases are a powerful tool for genetically modified organisms that can be re-designed. Also, re-design the dimer interface or the DNA-binding site (Musil et al. 2017).

12.2.3 Use of Artificial Intelligence Approach

The artificial intelligence (AI) methods for industrial processes are being designed to meet the aspects such as high cell productivity and selectivity, high enzyme activity, and low operational cost. Artificial intelligence deals with several areas such as natural language, knowledge-based expert systems, robotics, and visions (Richter et al. 2011). Turing acknowledged as a father of the AI, especially traditional, rational, and symbolic AI, was interested in the biological structure which deals with the epistemological and philosophical problems of Al (Francesco 2016). In the therapeutic diagnosis gene-editing tools like zinc finger nucleases (ZFN) and clustered regularly interspaced short palindromic repeats (CRISPR) have been used for the modification of precise components of a genome, thereby creating an innovative opportunity to accurate restoration of defective genes varying from HIV to cancer (Miller et al. 2015). For example, currently, the SB-913 ZEN based genome editing therapeutic tools were approved to diagnose the patients with Hunter syndrome (Lim et al. 2019). AI-generated nano-diamond based biomaterial platform has been reported as relief in injury cure and prevention in reinfection after root canal therapy (Rawson et al. 2019). Among these, another biomaterial has been reported as custom-made 3D printing of polycaprolactone (PCL), being used in the reconstruction of the breast after the tumor removal (Ho et al. 2020). There are lots of several Al-based techniques that have been reported, such as an artificial neural network (ANN), genetic neural network (GNN), genetic algorithm and fuzzy logic for the optimization of high yield production of products and their stability towards the hostile conditions (Hassanien et al. 2013).

12.2.4 De Novo Designing of Enzymes

The development and design of novel enzymes with novel function and structure to produce the desired product can be achieved with the help of de novo design methods. The introduction of large changes in the protein structure is also possible by the de novo design methods. In these techniques, the active site designs that lack the required catalytic site of the protein. The first-time de novo design was performed with quantum mechanical method by incorporation with desired catalyzed reaction (Kiss et al. 2013a, b). The de novo enzyme design methods produce most of the catalysts for various catalytic reactions. This type of design called "ozyme." For example, Diels–Alder reaction, usually form C–C bonds with the production of new carbon atoms presented centrally, which used synthetic chemistry. The enzyme is generated for this reaction by the computational design methods. However, many more proteins are obtained by the de novo design methods, but they act as slow catalysts. However, all the enzymes produced by this method are substantially less catalytic active (k_{cat}/K_M 10^{-2}–10^{2} M^{-1} s^{-1}) than normal enzymes (k_{cat}/K_M 10^{3}–10^{8} M^{-1} s^{-1}) (Smith et al. 2014). Initially, de novo design has been worked on the program Dezymer for the creation of active site His3-Fe-O2 metal, which was introduced into thioredoxin to gain a superoxide dismutase-like enzyme (Hellinga

and Richards 1991). Bolon and Mayo incorporated a single histidine residue into thioredoxin skeleton to deliberate the ester hydrolysis activity. These designed enzymes have modest (k_{cat}/kun$_{cat}$ = 180) catalytic activity compared with 4-methyl imidazole or ordinary esterases (Lassila et al. 2006). To accomplish these Lassila et al. have used a two-layered tactic for the transition-state employment, while Zanghellini and Jiang have used efficient strategy 6D hashing called Rosetta Match (Zanghellini et al. 2006). Among these many, more technologies have been used for the placement algorithm of the new active site such as PRODA-MATCH, Zhu and Lai's vector matching technique, Scaffold Selection and Opt Graft (Lei et al. 2011). The (retro-) Aldolase enzyme designed by the de novo method with the introduction of Lys and H-bond (acceptor/donor) into the Multiple templates (>13) of scaffolds, which has initial catalytic activity of 0.02–0.74 k_{cat}/K_M (M^{-1} s^{-1}) was improved to catalytic activity of 55 k_{cat}/K_M (M^{-1} s^{-1}) (Carbonell et al. 2011).

12.3 Application of Enzyme Engineering

Enzyme modification is a striking and prevailing procedure for the optimization of industrial processes and the production of high valued products, which is usually lacking optimal operational processes (Yim et al. 2011). For example, the generation of the antimalarial drug artemisinin with the introduction of the heterologous pathway in the yeast cell (Arsenault et al. 2008). Another example of metabolic engineering for the production of 1,4-butanediol and isobutanol (Guo et al. 2017). Engineered thermostable enzymes can be used for the maintenance of the high production yield through in vitro metabolic engineering techniques. In this, in vitro, technique, the use of the high cost of the acellular system energy encounters the scaling-up progression. While in the synthetic enzyme modification technology process, it may mitigate the production of the desired product as the enzymes stability (Pan et al. 2017). The several enzyme modification techniques offer the tremendous opening to enhance the bio-manufacturing pathways, reduce the biofuels generation cost, and also decrease the growth limitation risk by hazardous components produced through chemical pretreatment (Ba et al. 2013).

12.3.1 Environmental Applications

The production of the massive amount of hazardous materials from many industries (such as textile, pharmaceutical, leather, and pulp industry), and expulsion of wastewater in the environment have generated hostile effects on community health (Dixit et al. 2019b). However, there is a high demand for engineered insoluble enzymes, i.e. laccases and tyrosinases in the bioremediation treatment of toxic effluent of notorious industries (Fernandez-Fernandez et al. 2013). The natural catalyst compared to engineered catalyst is not up to the mark for the industrial challenges. Thus, the enzyme immobilization and biocatalyst technology have significant approaches to mitigate the uncertainty complications. The laccases

reduce the industrial effluents such as pesticides, chlorophenols, herbicides, dyes, polycyclic aromatic hydrocarbons, antibiotics with the help of free radical mechanism. Its cost-effective process due to only oxygen requires cofactors. Immobilized laccase is reported as the most powerful tool and leads to biotechnological approaches in upcoming areas as they offer thermal stability, extensive pH stability, mechanical stability and use in repetitive. Along with laccase, immobilized tyrosinase has reported as a good bioremediation of industrial pollutants because of a significant number of amino acids present in the tyrosinase enzyme (Voberkova et al. 2018).

Some ligninolytic enzymes have the potential to degrade the dye because the dye has a similar structure as lignin. Due to advancement in biotechnological applications and significant in environmental aspect, the demand of ligninolytic enzymes has increased worldwide in industries. For example, immobilized LiPs and MnP have significant potency to mitigate the rigid aromatic components, oxidize the polycyclic aromatic and phenolic compounds, and ability to decolorize the several industrial dyes (Acker and Auld 2014). Among these, lots of biocatalyst and bio-sorptive enzymes are being used for the mitigation of industrial pollutants. Immobilized biocatalyst such as alginate-carbon materials, nanoparticles, organic gels, and porous glass beads produced either adsorption or covalent binding with support matrix materials. It is more stable than free enzyme biocatalyst materials when making over solid surface (Kellogg et al. 2011).

12.3.2 Food Processing Industry

Due to an increasing population worldwide, the demand for foods is also increasing. Enzyme modification shows an essential part in the food industries. Nowadays, many engineered lipolytic, proteases, and amylases enzymes are used for the food processing industry (Zajkoska et al. 2015). De novo developed lipase enzyme improved efficiency and lipase constancy, which supports in the production of a broad array of esters derivatives such as surfactants by single-enzymatic reactions in the food industry. Enzyme engineering can assist in the production of the maximum quantity of proteins by the help in reducing the operational cost, improved stability with enhancing selectivity and salt resistance. It can also help in the development and maintaining of active components across diverse industries such as cosmetics, food, pharmaceutical, and textile industries (Patel et al. 2016). Generally, catalysts used in the food industries measured as non-hazardous, but some have sensitive and other adverse possessions that cause food spoiling or adulteration. Engineered enzymes have used to mitigate food spoiling due to the production of active toxic products. As compared with the primary enzyme, mutant glucose isomerase derived from *Actinoplanes missouriensis* has significant constancy at optimal conditions (Fenel et al. 2004). There are several enzymes including cellulases, amylases, proteinases, pectinases, lipases, and xylanases which have been used for the high yield production and stability of food products. Among these, xylanases are engineered by designed mutagenesis technique to enhance the optimal pH and

temperature of any ingredients being used in nutriment essences. In the present scenario, lots of bugs such as hyperlipidemia, hypertension, diabetes, and obesity have been reported at a very rate throughout the world (ByungChul et al. 2009). The reason behind this lies in use of an excessive amount of fat and sugars. However, the use of rare sugars and fats designed by several engineering technologies has eco-friendly, cost-effective, and economic aspects. *Agrobacterium tumefaciens* KEase immobilized on the Duolite A568 beads with borate leads produces 441 g/L D-allulose from 700 g/L D-fructose. The production of 33 g/L L-allose from 100 g/L L-allulose over the day can be done by the immobilization of l-ribose isomerase on DIAION HPA25L (Terami et al. 2015). Polyethylenimine polymers were covalently coated with porous support (agarose, polymeric resins) and obtained as strong effective and reversible immobilization of b-galactosidase, lipases, and D-amino acid oxidase. The nanotechnology incorporated with immobilization of enzymes has more significant importance as carrier materials (Singh et al. 2014). The detail list of different industrial applications and their characteristics are given in Table 12.2. It offers lots of opportunities, i.e. enhanced enzyme loading capability, decrease mass transfer limitations, polished diffusional properties, and mitigated fouling (Contesini et al. 2013).

12.4 Advantages of Engineered Enzymes Over Traditional Enzymes

Enzymes are bio-catalyzers that continued several biological reactions, which take place at a very high rate than its absence. The catalyst has highly substrate specificity and catalyzed it from one molecule to another molecule of products (Schoffelen and van Hest 2013). Enzymes are found in both type reversible as well as irreversible reactions but most of them are found in reversible reactions. Additionally, the initial and primary tendency of enzyme engineering technologies was developed for the food industry. Not only now in the food industry, but enzymes are also developed for other industries such as wastewater treatment in bioremediation, detergents, and pharmaceutical synthesis with high specificity, efficiency, and stability. However, the enzymes are produced traditionally from natural resources in meticulous ways (Li and Cirino 2014). Recently, many strategies developed for the improvement of enzymes catalytic activity and new activity with a multi-enzyme activity using novel scaffolding design, de novo design, site-directed mutagenesis methods, direct evolution design, and computational models. In the past decade, enzyme engineering offers new opportunities and perceptions of biotechnology (Singh-Blom et al. 2014). The introduction of new residues and biological molecules to enzymes newly trailed with the help of non-canonical amino acids analogs into the naturally occurring proteins. These new technologies are most suitable for economical and convenient tools for the study of functional improvements of enzymes compared to the previous traditional ones. The traditional aminoacyl-tRNA synthetases show low selectivity towards its anticodon of tRNA. Thus, to overcome this problem, genetic incorporation of non-natural amino acids or alfa-hydroxy acids into amber stop

Table 12.2 Different enzyme engineering technologies and their applications towards various industrial aspects

Type of industries	Products name	Characteristics	Enzyme engineering techniques	References
Beverage and food industry	Alcohol dehydrogenase, glucose dehydrogenase, and phenylalanine ammonia lyase (PAL)	Increased 10- to 13-fold yield production at intermediate products of different metabolic process	Direct evolution and DNA shuffling	Saini et al. (2017)
	Keto-reductase enzyme and superoxide dismutase	Enhancement of thermos-stability and increase the catalytic activity of enzyme; scavenging of free radicals	Rational and computational design with direct evolution methods using B-FIT, SCH EMA, and ProSAR software	Ighodaro and Akinloye (2018)
Pharmaceutical industry	Cyclodextrin, Sitagliptin, etc.	Enhanced 84% production, water solubility and used as preservative of different industrial process, and important role in the treatment of diabetes type 2	Computational design, substrate walking, and mutagenesis followed by the direct evolution methods	Razzaq et al. (2019)
Food industry	Mutant glucose isomerase and Xylanase; immobilized L-ribose isomerase; amylase	Improved stability at different optimal condition; enhanced 33 g/L production; used for the de-sizing of carbohydrates	De novo and designed mutagenesis methods; immobilization technique	Darwesh et al. (2019)
Environmental and textile industry	Immobilized Lac and Tyr; immobilized peroxidase; catalase	Bioremediation of industrial effluents; removal of heavy metals; used for the termination of bleaching process	Immobilization technique; computational engineering	Kaushal et al. (2018)

codon can be achieved by pyrrolysine machinery methods (Wan et al. 2014). The advantage of enzyme engineering is to develope new proteins containing newer properties and improved catalytic functions in primary ones. These can be accomplished by finding out the respective gene and altering the amino acid residues via various enzyme engineering technologies. Enzyme engineering technologies are

required to improve in the kinetic property, removal of allosteric conventions, increment of reactant and substrates selectivity, enhanced thermal constancy and ideal pH values, appropriateness for the practice in biological solvents, and so forth.

12.5 Future Perspectives and Conclusions

Enzymes with innovative and improved properties are significantly essential to accomplish technical necessities. Several useful elevations have been made in proteins via many technologies, such as rational and semi-rational designing, computational engineering, and de novo deigning. In the future, more practice and specific strategies need to be deliberate to evolutionary developments for the incorporation of novel properties in industrial catalysts. Additionally, these and similar tactics might be consenting us positively to the new enzyme design with a variety of unique designers amino acid residues to optimize the catalytic activity and enhance the efficiency and stability of enzyme for the industrial and biosynthetic processes. The development of enzyme engineering is quiet in a growing phase, and it is expected that it would show unexpected outcomes in the upcoming times.

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