Environmental and Microbial Biotechnology

Naga Raju Maddela
Luz C. García Cruzatty
Sagnik Chakraborty *Editors*

Advances in the Domain of Environmental Biotechnology

Microbiological Developments in Industries, Wastewater Treatment and Agriculture

Springer

Environmental and Microbial Biotechnology

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Preface

The global population is steadily increasing, which greatly demands the optimal use of biota (e.g. plants, animals, bacteria, fungi, and algae) in the surroundings of human beings to produce energy (renewable), food, and nutrients. This is achievable when 'waste' becomes a 'feedstock' in a synergistic integrated cycle of profit-making processes. However, due to these processes, there should not be an imbalance in the environment, also such profit-making processes should not disturb the mutual relationships that already exist in the environment. 'Science' and 'technology' are updated on a regular basis and play pivotal roles in the establishment of a sustainable environment. Therefore, it is necessary to review the latest insights in the 'science and technology' for their successful applications and implications. This is our main intention behind the commencement of this volume, we do want to provide recent updates in microbiological science towards industries, water/wastewater treatment, and agriculture. This book in its present form has been designed in such a manner so that any enthusiast can be well aware about the very recent works that have been commenced since a long time. It can serve as a 'handbook' dealing with modern technologies that evolved recently.

The chief target audience for this book are the professors who are associated with environmental biotechnology, chemical engineering, and other interdisciplinary fields. Graduate students of the aforesaid fields too can be beneficiated by this book. In order to meet our objectives, we paid special attention while inviting the chapter contributors. Key contributors that have participated in this book have a solid research background, most of them are research professors, scientists, postdoctoral research scholars, and doctoral students, who have proven track-record internationally. Overall, the book portrays a very clear idea about the evolving modern technologies and also to direct young minds in the same path. This book has been designed to serve as a kind of information hub about modern sciences of environmental biotechnology. It will also serve as a ready reference by practicing students, researchers of biotechnology, environmental engineering, chemical engineering, and other allied fields likewise. Distinctive features of this book are lucid language, updated information, discussion on modern technologies related to the persisting

field which is absent in available books in the market, and clear figures and illustrations.

To reflect the title of this book, the chapters have been provided under three different parts. Part I is about *Industrial Biotechnology*, there will be much focus on the role of lactic acid bacteria for the production of platform chemicals, solid-state fermentation its use of agroindustrial residues, microemulsified systems and their environmental advantages for the oil industry, importance of microbial exopolymeric substances, and biodegradable polymers for food packaging and active food packaging. Part II is about *Environmental Biotechnology*, which emphasized on 3D printing technology in the environment, renewable energy from marine sources, modified or functionalized natural bioadsorbents and their applications, electrochemical biosensing of algal toxins, bio-inspired superoleophobic materials for oil–water separation, biotechnological advances in the treatment of agroindustrial wastes, multicriteria analysis in the selection of agroindustrial waste for the production of biopolymers, mathematical modelling challenges associated with waste anaerobic biodegradability, anammox in wastewater treatment, conventional wastewater treatment processes, analytical techniques/technologies for studying ecological microbial samples, and bioremediation approaches for the treatment of heavy metals, pesticides, and antibiotics from the environment. Finally, Part III consists of *Agricultural Biotechnology*, which provides the latest insights related to sustainable agriculture, example rhizobium diversity is the key to efficient interplay with *Phaseolus vulgaris*, Algae as environmental biotechnological Tool for monitoring the health of aquatic ecosystem, contribution of the environmental biotechnology to the sustainability of the coffee processing industry in developing countries, plant–microbial inoculants and their impacts on plant biology, applications of microalgae, and marine resources with potential in controlling plant diseases.

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We greatly acknowledge the support of the chapter's contributors for their valuable contribution and timely responses during this project. Without their enthusiasm and support, this volume would not be ready in the scheduled time; therefore, we really appreciate their cooperation and collaboration. Special thanks to Springer Series (Environmental and Microbial Biotechnology) Editor Prof. Ram Prasad for his invitation to be part of this prestigious project as editor and for his encouragement throughout this journey. Without his motivation, this project would not be achieved. We also thank anonymous reviewers for their constructive criticism, which would help us in improving the quality of this book by inviting the experts to contribute to the additional chapters. We greatly acknowledge the Springer Editorial and Production team for their valuable support, without their guidelines, this project would not have been completed in such a very short time. It is our honour to work with them, honestly. Finally, yet importantly, we are very much thankful to the colleagues at Universidad Técnica de Manabí (Ecuador) and Jiangsu University (PR China) for their unconditional support and for the provision of valuable suggestions at the time of book proposal and final book preparation.

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Part II Environmental Biotechnology

Editors and Contributors

About the Editors

Naga Raju Maddela received his M.Sc. (1996–1998) and Ph.D. (2012) in Microbiology from Sri Krishnadevaraya University, Anantapuramu, India. During his doctoral program in the area of environmental microbiology, he investigated the effects of industrial effluents/insecticides on soil microorganisms and their biological activities and worked as a Faculty in Microbiology for 17 years, teaching undergraduate and postgraduate students. He has received 'Prometeo Investigator Fellowship' (2013–2015) from Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (SENESCYT), Ecuador, and 'Postdoctoral Fellowship' (2016–2018) from Sun Yat-sen University, China. He also has received external funding from 'China Postdoctoral Science Foundation' in 2017, internal funding from 'Universidad Técnica de Manabí' in 2020, worked in the area of environmental biotechnology, participated in 19 national/international conferences, and presented research data in China, Cuba, Ecuador, India, and Singapore. Currently, he is working as a full-time professor at the Facultad de Ciencias de la Salud, Universidad Técnica de Manabí, Portoviejo, Ecuador. He has published 3 books (Springer), 2 chapters (InTech Open/Springer), and 42 research papers.

Luz C. García Cruzatty is graduated with a Ph.D. in Forest Sciences from the Universidad Austral de Chile (UACH), Valdivia, Chile in 2014. Since 2014, she has been a full-time Professor at the Faculty of Agronomic Engineering, Universidad Técnica de Manabí (UTM), Portoviejo, Ecuador. She has also worked as a Director of Institute of Investigation at UTM since 2017. Her line of research is 'Conservation and Improvement of Plant Genetic Resources', she directs a program of 'Ecophysiology, Conservation and Genetic Improvement of Theobroma cacao' in which higher genotypes are being developed with better production, disease tolerance, drought tolerance, and excellent organoleptic characteristics. To her credit, there are more than 20 scientific publications and participated as a speaker in several National and International conferences. She has received some distinctions and

awards, such as best graduate of the doctoral program (UACH), scholarships from MECESUP (Programa de Mejoramiento de la Calidad y la Equidad de la Educación Superior, Govt. of Chile, Chile), and SENESCYT (Secretaría de Educación Superior, Ciencia, Tecnología e Innovación, Govt. of Ecuador, Ecuador) for her doctoral study, awards as a leading researcher at the Universidad Técnica Estatal de Quevedo, Ecuador (2014–2016) and at the UTM (2017–2019), best teacher of the Faculty of Agronomic Engineering 2019, II Place in National MAGAP (Ministerio de Agricultura, Ganadería, Acuacultura y Pesca, Govt. of Ecuador, Ecuador) Agri-Economic Contest, GIZ (Gesellschaft für Internationale Zusammenarbeit).

Sagnik Chakraborty completed his M.Sc. in Biotechnology (2006–2008) from Bangalore University and Ph.D. from National Institute of Technology, Durgapur in 2014 Department of Biotechnology, India. His research work focused on the removal of the dyes by biomass derived from waste materials and removal of the dyes from an industrial outlet of the textile plants—biomass derived from the various waste. Until now, he has published forty papers according to the research findings. He has received several awards during his research career. He has mentored undergraduate and postgraduate students for their projects. Furthermore, he completed his Postdoctoral Research 2015–2018 from Hebei University of Technology, Tianjin, PR China, the fund approved by the Ministry of Education, PR China. He has received several intramural projects funds in his research domain. Moreover, he received an International Young Scientist Award from the National Natural Science Foundation of China in 2019 with grant aid for further research. He received the Brand Ambassador Award in 2019 from The Bentham Science Publisher and won several outstanding reviewing awards from Elsevier. Lately, he is engaged as Senior Researcher at Jiangsu University in the School of Environmental Science and Safety Engineering.

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Part I
Industrial Biotechnology

Chapter 1
Lactic Acid Bacteria for Production of Platform Chemicals: A Dark Horse in the Field of Industrial Biotechnology

Kumar Pranaw, Debjani Dutta, Surender Singh, and Sunil Kumar Khare

Contents

Abstract Climate change, environmental security, and sustainability of petrochemical resources along with depletion of fossil fuels are the current immerging issues all over the world. The industrial production of commodity chemicals from agricultural

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biomass provides a noteworthy contribution to solving the above mentioned environmental problems. The use of nonrenewable energy or petrochemical resources for the bulk chemical production can be completely saved with use of present-day state-of-the-art industrial microbiology and biotechnology. The industrial microbiologists are always in search of a robust organism which can utilize cheap, renewable agro-wastes and sustain several stresses for the production of commodity chemicals. Almost all of these important and necessary characteristics are available in lactic acid bacteria (LAB), which makes them a dark horse in the field of industrial biotechnology. Till date, due to widespread industrial practice and immense economic value, LAB have been extensively studied, and ample data on their physiology, metabolism and genetics has been generated over the years. This information is fully implemented in different successful application and completely exploited in food industry. However, during the last few decades, exponential advancement in technology created the platform for LAB to be explored for industrial applications beyond its regular use in food industry. Here in this book chapter, we discussed about the importance of platform chemical, viz. lactic acid, different substrates for lactic acid fermentation and their limitation. We also discussed the metabolic engineering studies with LAB and recent strategies to expand their cellular traits for the biobased production of commodity chemicals.

Keywords Lactic acid bacteria · Biorefinery · Metabolic engineering · Platform chemicals · Lignocellulosic biomass · Polylactide (PLA) · Industrial biotechnology

1.1 Introduction

The dwindling fossil resources coupled with sustainability and environmental challenges have focussed attention on biomass-based production of platform chemicals. Lactic acid, a C3 platform chemical, has an enormous worldwide market due to its versatile applications in different industrial sectors, viz. food, cosmetic, tanning, brewery, biomedical, and pharmaceuticals. The most sought after chemicals, viz. pentanol, acrylic acid, lactate ester, glycol, 2,3-pentanedione, propylene, acetaldehyde, biodegradable solvent ethyl lactate, and pyruvic acid, are produced from it, which makes it a high-value metabolite with vast demands. Its usage for the production of polylactide (PLA)-based biopolymers has made it indispensable for textile, automotive, and biomedical sector. PLA polymers with their distinctive thermal and mechanical properties and biodegradability serve as ideal base for fabrication of biomaterials such as tissue engineering scaffolds, absorbable sutures, coatings of implantable devices, and orthopaedic plates and stents. The global production capacity of biobased plastics is expected to reach 7.85 million tonnes in 2019 and PLA will be among the leads due to its expanding markets (Wee and Ryu 2009; Liang et al. 2014; Wang et al. 2015; Ahring et al. 2016; Klotz et al. 2016). The current research efforts are extensively oriented towards development of

PLA-based materials as a viable and sustainable alternate to fossil derived plastics by synthetic route. Thus, for the fabrication of biobased polymers, production of biomonomers like lactic acid by microbes through fermentation becomes critically important (Van Wouwe et al. 2016; Zhu et al. 2016; Zia et al. 2016).

The lactic acid production can be carried out either by chemical synthesis or fermentation at commercial scale. The chemical route is based on using different petrochemical feedstock, viz. lactonitrile, acetaldehyde, hydrocyanic acid, or, more recently, biobased substrates such as trioses, hexoses, monosaccharide sugars, glyceraldehyde, and glycerol (Dusselier et al. 2013). However, the use of different harsh homogeneous and heterogeneous catalysts under stringent reaction conditions with high energy consumption favours towards fermentation method. The production of racemic mixture of lactic acid by chemical synthesis is another bottleneck, which is avoided by microbial fermentation as many microorganisms synthesize enantiomerically pure lactic acid. The raw materials constitute 60–80% of the total cost of lactic acid production. Hence, the choice of appropriate fermentable feedstock and microorganism is critical for effective bioproduction at industrial scale (Grewal and Khare 2018).

The lactic acid is produced by both fungal and bacterial fermentation. The fungi belonging to *Rhizopus* genus have been majorly used for lactic acid production due to release of extracellular hydrolytic enzymes and ease of separation of mycelium for downstream processing (Trakarnpaiboon et al. 2017). *Corynebacterium glutamicum*, *Escherichia coli*, *Bacillus* strains, and lactic acid bacteria (LAB) are the promising bacterial producers of lactic acid. These microorganisms in both wild-type and engineered forms are being researched for cost-effective lactic acid production (Zahoor et al. 2012; Hasunuma et al. 2013). Lactic acid bacteria (LAB) are emerging as major bioresource due to their GRAS (generally recognised as safe) status and production of many antimicrobial and immunomodulatory compounds. LAB can produce lactic acid by either homofermentive or heterofermentive pathway resulting in different yields (Bosma et al. 2017; Sauer et al. 2017).

1.1.1 Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) comes from a highly heterogeneous group of Gram-positive, nonmotile, non-sporulating organisms that ferment a variety of sugars to produce lactic acid as a major end product (Franz and Holzapfel 2011). LAB are facultative anaerobic microorganisms; it implicates that they do not need oxygen for growth, and oxygen has generally been demonstrated to exert negative effects on their growth (Van De Guchte et al. 2002). Although many genera of bacteria produce lactic acid as a primary or secondary end-product of fermentation, the term lactic acid bacteria are conventionally reserved for seven genera, out of which *Enterococcus*, *Lactococcus*, *Pediococcus*, and *Lactobacillus* have been used worldwide for industrial production of commodity chemicals like lactic acid, food ingredients, nutraceuticals, antimicrobial products, and other high-value-added metabolites.

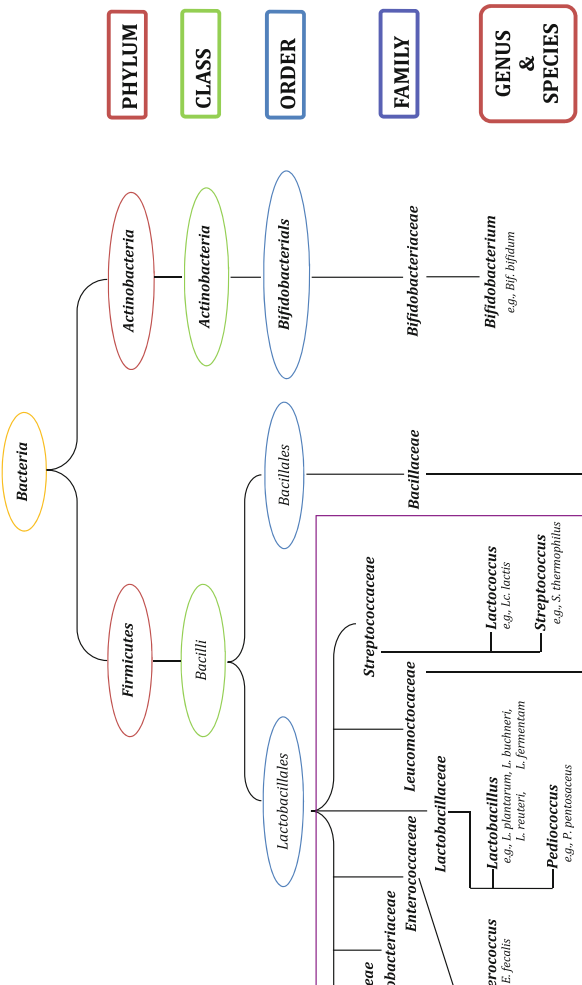

Fig. 1.1 Classification of lactic acid bacteria (Adapted from Von Wright and Axelsson (2011))

Taxonomically, LAB belong to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales* (Fig.1.1) (Von Wright and Axelsson 2011).

LAB are well known for their ability to grow anaerobically in an acidic environment by the production of organic acids. Within the LAB, *Lactobacillus* is a highly diversified and differentiated genus with more than 150 different species exhibiting a large segment of catabolic activities. The LAB genera include many highly important industrial species, widely used in dairy and other food and beverage-related fermentations. The different species are commonly involved in the production of yogurts, cheese, pickles, sauerkraut, olives, fermented sausages, and fish products; they are also employed for fermentation of alcoholic beverages (Leroy and De Vuyst 2004). In dairy products, LAB are known to contribute to the taste, aroma, flavour, and texture. LAB are also recognized for their production of natural antimicrobials such as bacteriocins which inhibit the growth of foodborne pathogens including *Staphylococcus aureus*, *Clostridium botulinum*, and *Listeria monocytogenes* (Leroy and De Vuyst 2004; Cesselin et al. 2011). Their production of organic acids (primarily lactic acid, but also acetic, formic, phenyllactic, and caproic acids) results in rapid acidification of raw material and also plays a role in prolonging the product's shelf life and increasing its microbial safety (Leroy and De Vuyst 2004). Consequently, various LAB species are often employed as food preservatives (Fig. 1.2) (Lücke 2000; Corr et al. 2007; Gálvez et al. 2007).

Due to their long history and use, LAB are generally regarded as safe and beneficial microorganisms. Some of them are even considered to have health-promoting features and are therefore employed in probiotic functional foods. Examples include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Bifidobacterium lactis*, which are used by numerous companies around the world (Upadrasta et al. 2011). On the other hand, however, there are also some LAB species which are human or animal pathogens (Von Wright and Axelsson 2011). For instance, *Streptococcus pyogenes* and *Streptococcus pneumonia* are both recognized to be opportunistic pathogens causing infections such as strep throat, impetigo, pneumonia, scarlet fever, and meningitis (Henningham et al. 2015).

LAB can be found in a variety of ecological niches, from human gastrointestinal tract and cavities, through dairy products, fermented meat, fish, and vegetables, to silage, soil, and decaying plant material (König and Fröhlich 2017). In general, LAB are isolated from nutritionally rich habitats; being evolved in environments rich in sugars, amino acids, vitamins, and nucleotides, they often demonstrate a limited scope of biosynthetic capabilities which is reflected by their complex nutritional requirements. Many researchers have isolated *Lactobacilli* from decomposed lignocellulosic agro-residues, which suggest that it could be remarkable candidates for most appropriate as well as efficient utilizers of the second-generation agro-residues, perhaps even superior to the strains traditionally used (e.g. *Escherichia coli* or *Saccharomyces cerevisiae*) (Olsson and Hahn-Hägerdal 1993; Mussatto and Teixeira 2010; Abdel-Rahman et al. 2011).

Many of the LAB species were already reported for effective utilization of biomass to value-added products like lactic acid. Such a wide spectrum of natural

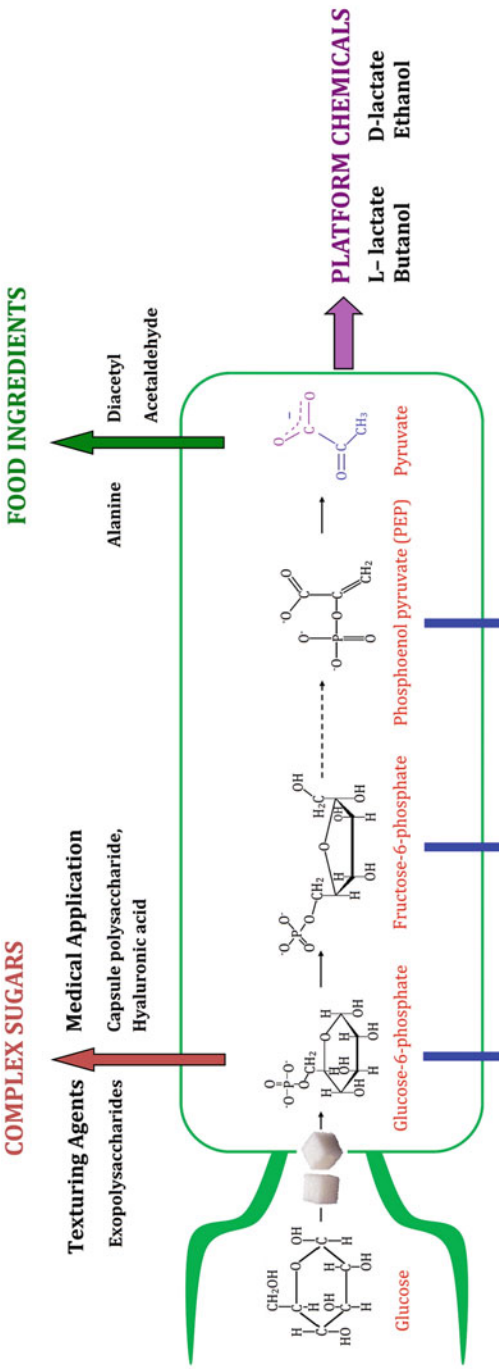

Fig. 1.2 Application of lactic acid bacteria for production of different industrially important chemicals

habitats makes the LAB group highly heterogeneous; yet, it also reflects the scope of their catabolic potential (Abraham et al. 2016; Sauer et al. 2017; Vivek et al. 2017).

1.2 Substrates for Lactic Acid Production by Fermentation

The availability of inexpensive, abundant renewable feedstock is essential to compete with chemical synthesis and ensure feasibility of bioprocessing on economic terms. Thus, replacing traditional feedstocks such as refined sugars or edible crops with lignocellulosic biomass or agroindustrial wastes is a viable alternate. However, the utilization of nonedible waste substrates requires pretreatment process to make them amenable to efficient saccharification for release of reducing sugars. The deployment of different agroindustrial wastes as feedstocks for lactic acid production will lessen the environmental and economic burden by avoiding unsafe disposal practices, reducing greenhouse gas emissions, saving landfill costs, and generating value-added products (Zhang et al. 2014, 2016; Reddy et al. 2016). Thus, the transition from linear fossil-based economy to biobased circular bio-economy relies on optimal harvesting of potential waste biorefineries.

1.2.1 Starchy Biomass as a Substrate for Lactic Acid Production

Starchy substrates derived from various plant sources are an attractive alternate to pure reducing sugars. Starch being a complex carbohydrate composed of amylose and amylopectin requires hydrolysis for release of glucose for further fermentive action. Hence, the action of amylolytic enzymes, viz. α-amylase, β-amylase, and glucoamylase, becomes prerequisite for utilization of starchy substrates. Thus, either concomitant addition of saccharifying enzmyes or secretory amylolytic activity of fermenting organism is required. Most of the studies for lactic acid production from starchy waste rely on use of commercial amylolytic enzmyes for release of reducing sugars. However, few wild-type LAB-possessing amylolytic activity has been reported. Thus, *Lactococcus lactis*, *Lactobacillus amylovorus*, *L. amylophilus*, *L. amylolyticus*, *L. plantarum*, *L. paracasei*, *L. manihotivorans*, *L. fermentum*, *Enterococcus faecium*, and *Streptococcus bovis* have been shown to produce lactic acid from different starchy substrates, viz. potato, sorghum, cassava, rice, barley, and corn starch by using their amylolytic activity. However, screening for more native potential producers is another hotspot research area for development of starch-waste based biorefinery. The construction of recombinant amylolytic strains is another approach to develop consolidated bioconversion process for lactic acid production. *L. plantarum* was genetically modified to replace L-lactate dehydrogenase gene with α-amylase-secreting expression cassette and was used for production for D-lactic

Table 1.1 Recent studies on D/L-lactic acid production from variable lignocellulosic and starchy substrates using wild-type and engineered LAB

Variable lignocellulosic and starchy substrates	LAB species	Lactic acid enantiomer	Lactic acid concentration (g/L)	Reference
Plywood chips	*Enterococcus faecalis* SI	L	59.81	Yuan et al. (2018)
Catfish waste	*L. pentosus*	L	35.70	Shi et al. (2018)
Deoiled cottonseed cake	*L. brevis*	L	–	Grewal and Khare (2018)
Corn stover	*L. delbrueckii* sp. *bulgaricus*	D	18.0	Wang et al. (2017)
Pulp mill residue	*L. coryniformis* subsp. *torquens*	D	57.0	de Oliveira Moraes et al. (2016)
Millet bran	*L. delbrueckii* ssp. *delbrueckii* NBRC 3202	D	25.38	Balakrishnan et al. (2018)
Corncob residue and cotton seed meal	*Sporolactobacillus inulinus YBS1-5*	D	107.2	Bai et al. (2016)
Corn stover and corn steep liquor	*Sporolactobacillus inulinus YBS1-5*	D	70.7	Bai et al. (2015)
Soybean straw	*L. casei*	L	28.0	Wang et al. (2015)
Curcuma longa residue	*L. coryniformis ATCC 25600*	D	91.61	Nguyen et al. (2013)
Curcuma longa residue	*L. coryniformis ATCC 25600*	L	97.13	Nguyen et al. (2013)
Rice straw	*L. brevis*	–	34.2	Kim et al. (2010)
Gelatinized starchy waste from rice noodle factory	*L. plantarum* S21	–	102.0	Unban et al. 2019)
Starch-rich restaurant food waste	*Streptococcus* sp.	L	58.0	Pleissner et al. (2017)
Mixed food waste	*L. casei Shirota*	–	94.0	Kwan et al. (2016)
Sweet potato processing waste	*L. rhamnosus*		10.0	Pagana et al. (2014)
Mixture of rice washing drainage and rice bran	*L. rhamnosus* M23	L	59.0	Watanabe et al. (2013)
White rice bran	*L. rhamnosus* LA04-1	L	123.0	Li et al. (2012)
Cassava bagasse	*L. casei*	L	83.80	John et al. (2006)
Defatted rice bran	*L. delbrueckii* subsp. *Delbrueckii* IFO 3202	D	28.0	Tanaka et al. (2002)

(continued)

Table 1.1 (continued)

Variable lignocellulosic and starchy substrates	LAB species	Lactic acid enantiomer	Lactic acid concentration (g/L)	Reference
Popular hydrolysate	*L. brevis ATCC 367 and L. plantarum ATCC 21028*	D	31.8	Zhang and Vadlani (2015)
Corn stover	*L. brevis ATCC 367 and L. plantarum ATCC 21028*	D	31.2	Zhang and Vadlani (2015)
Corn stover	*L. rhamnosus*and *L. brevis*	D	20.95	Cui et al. (2011)
Casein whey permeate	*L. delbrueckii* ssp. *lactis ATCC 4797*	D	24.3	Prasad et al. (2014)
Corn flour hydrolysate	*Sporolactobacillus inulinus Y2-8*	D	145.8	Ting et al. (2014)
Whey	*L. bulgaricus CGMCC 1.6970*	D	113.18	Liu et al. (2018)
Whey permeate	*L. delbrueckii* and engineered *Lactococcus lactis*	D	44.8	Sahoo and Jayaraman (2019)
Brown rice	*L. plantarum*	D	117.1	Okano et al. (2017)
Corn stover	*L. plantarum* NCIM B 8826	D	61.4	Zhang et al. (2016)
Corn stover	*Pediococcus acidilactici*	D	77.78	Yi et al. (2016)
Corn cob residue, Cottonseed meal	*Sporolactobacillus inulinus* YBS1-5	D	107.2	Bai et al. (2016)
Wheat straw	Engineered *Pediococcus acidilactici* TY112	L	130.80	Qiu et al. (2018)
Hardwood pulp	*Engineered L. plantarum*	D	102.3	Hama et al. (2015)
Waste wood chips	Recombinant *L. paracasei* 7B (ldhD gene deficient)	L	99.3	Kuo et al. (2015)

acid (99.6% optical purity) from brown rice and corn starch (Okano et al. 2009, 2017). Table 1.1 summarizes the lactic acid production achieved from fermentation of starchy substrates by LAB. The starchy substrates especially agro-industrial wastes are highly suitable for lactic acid production via a cost-effective and environment-friendly bioprocess.

1.2.2 Lignocellulosic Wastes Can Serve as Ideal Feedstocks for Lactic Acid

The use of edible carbohydrate substrates for lactic acid production can be challenged on constraints of cost-effectiveness, sustainability, and food security. Thus, lignocellulosic biomass has emerged as key feedstock to unlock the potential of sustainable biobased lactic acid production. The lactic acid production process form lignocellulosic substrates requires (1) pretreatment step for deconstruction of recalcitrance; (2) saccharification by hydrolytic enzymes such as cellulases, xylanases, and cellobiases; (3) microbial fermentation of released monomeric sugars into lactic acid; and (4) downstream processing for pure lactic acid recovery. The pretreatment process is very critical as it requires high energy inputs for making rigid crystalline biomass amenable to enzymatic hydrolysis. The conventional processes employed for loosening of intertwined network of cellulose, hemicellulose, and lignin include acid, alkali, steam, and ammonia fibre explosion (AFEX) pretreatment. Most of the studies on lactic acid production from different lignocellulosic substrates, viz. wheat straw, corn stover, sugarcane bagasse, corncob, cottonseed, cassava bagasse, and paper sludge, have used acid or alkali pretreatment (Abdel-Rahman et al. 2011; van der Pol et al. 2016). The generation of inhibitory toxic compounds, extensive washing after pretreatment, disposal of residual effluents, and environmental concerns are the major drawbacks of these conventional pretreatment methods. Ionic liquids (IL) and deep eutectic solvents (DES) have emerged as new class of green solvents for efficient solubilisation and deconstruction of biomass under mild conditions as compared to conventional methods. The elimination of washing step of these residual green solvents simplifies the cumbersome process with low energy inputs and simultaneously allows coupling of pretreatment and saccharification in a single step. However, the stability of saccharifying enzymes, i.e. cellulases and xylanases in residual IL or DES, is highly desirable for achieving in situ saccharification with high yields. Thus, the development of compatible enzyme-IL/DES systems is one of the thrust research areas for developing lignocellullosic biorefineries. Recently, lactic acid itself has been used as one of the components of DES, and these natural deep eutectic solvents (NADES) have been used for pretreatment of biomass (Bakirtzi et al. 2016; Kumar et al. 2016). Thus, the use of biomass-based lactic acid for pretreatment of agro-waste biomass further potentiates efforts towards renewable circular economy.

1.3 Saccharification and Fermentation for Lactic Acid Production

The enzymatic hydrolysis of pretreated biomass for release of free sugars is achieved by action of exoglucanases, endoglucanase, β-glucosidases, and xylanases which are added in appropriate proportions to achieve high yields. The hydrolysate generated

after saccharification is subjected to fermentation, and this approach referred as separate hydrolysis and fermentation (SHF) has been extensively used for lactic acid production from lignocellulosic substrates (Zhang and Vadlani 2015). However, the inhibitory effect of released sugars on hydrolytic activity of saccharifying enzymes has led to preferential use of SSCF (*simultaneous saccharification and co-fermentation*) approach for lactic acid production. The SSCF process helps to minimize feedback inhibition by continuous microbial fermentation of released sugars. Grewal and Khare (2018) carried out one-pot SSCF process for lactic acid production from lignocellulosic agro-wastes via ionic liquid-stable *L. brevis*. Similarly, both D and L lactic acid productions have been achieved with SSCF of various lignocellulosic substrates, viz. corncob, wheat straw, alfalfa fibre, corn stover, sugarcane bagasse, etc.

1.4 Constraints in Biobased Production of Lactic Acid

1.4.1 Difficult Multistep Processing of Recalcitrant Lignocellulosic Biomass

Though abundant lignocellulosic agro-waste is an attractive substrate for lactic acid production, the inability of microorganisms to use complex carbohydrates (cellulose, hemicellulose) limits its optimal utilization. The separate processing for pretreatment, saccharification, and fermentation leads to high cost and extensive energy inputs. The conventional methods of acid/alkali pretreatment lead to generation of various inhibitory compounds, viz. furfural, 5-hydroxymethylfurfural, levulinic acid, vanillin, sulphate, acetate, formate, and inorganic ions, which either inhibit microbial growth or lower the yield. The lactic acid producing *Pediococcus*, *Lactobacillus*, and *Bacillus* sp. has been shown to be highly resistant to inhibitors generated from lignocellulosic hydrolysates (Boguta et al. 2014; Zhang et al. 2014). Thus, a suitable pretreatment approach, viz. use of ionic liquids and DES, along with selection of microbes tolerant to lignocellulose-derived inhibitors is highly desirable. The efforts oriented towards carrying out efficient in situ saccharification and requiring low energy inputs aim at development of compatible IL/DES and hydrolytic enzymes. Further, the screening of IL/DES-stable lactic acid producers is another challenging aspect which needs to be probed so that one-pot processing is achieved. *L. brevis*, stable in 1-ethyl-3-methylimidazoliumacetate [EMIM][Ac], is an ionic liquid that is able to produce lactic acid from [EMIM][Ac] pretreated lignocellulosic substrates in one pot (Grewal and Khare 2018). The screening for novel lactic acid producers capable of direct utilization of complex polymers, i.e. cellulose and xylan, is another major constraint, as currently there are no known wild-type producers. Recently lactic acid-producing strains are being engineered to express hydrolytic enzymes, viz. xylanase and endoglucanase, so that the bacteria can directly utilize such complex polymers.

1.4.2 Mixed Sugar Utilization and Carbon Catabolite Repression

The inability of lactic acid producers to ferment both hexose and pentose sugars released in hydrolysate after biomass saccharification restraints optimal productivity of biomass-based lactic acid production. The use of promising microorganisms with capability of consumption of mixed sugars is further limited by their sugar utilization pattern as the presence of glucose generally suppresses ingestion of other sugars. Thus, selection of wild-type or engineered lactic acid producers with carbon catabolite repression (CCR)-negative phenotypes is very critical for optimising the fermentation of lignocellulosic substrates. *L. brevis* has been reported as promising CCR-negative strain for use in lignocellulosic biorefineries (Kim et al. 2010; Grewal and Khare 2018). The development of mixed cultures, screening of novel CCR-negative microorganisms, and construction of recombinant strains expressing both hexose and pentose sugar assimilating enzymes are the strategies which need to be worked for agro-waste-based lactic acid production.

1.4.3 Formation of Nondesirable By-products Due to Heterofermentation

The fermentation of sugars via phosphoketolase (PK) pathway leads to generation of other products, viz. acetic acid, ethanol, and carbon dioxide, in addition to lactic acid. Thus, microorganisms metabolizing sugars through heterofermentative pathway result in low yield of lactic acid. Thus, for achieving biobased lactic acid production in high yields, the microbial strains and cultivation conditions should be channelled towards Embden-Meyerhof-Parnas (EMP) pathway, i.e. homofermentative mode. The selection of native homofermentative lactic acid producers and development of genetically modified or metabolically engineered strains need to be actively pursued so that other released by-products do not decrease the yield and increase the purification cost of biobased lactic acid production (Arsköld et al. 2008).

1.4.4 Optical Purity and Stereospecificity of Lactic Acid

The high enantiomerical purities obtained by microbial fermentation are the major advantage as compared to chemical synthesis for lactic acid production. However, the choice of fermenting microorganism and fermentation conditions needs to be critically evaluated for enantiomerically pure production of lactic acid. Both D- and L-isomeric forms of lactic acid affect thermal and mechanical properties of the biopolymer produced, and thus, both are appropriately required for different applications (Tashiro et al. 2013; Ma et al. 2016; Okano et al. 2017). The specificity of

lactate dehydrogenase of selected lactic acid producer, feedstock used, fermentation mode, temperature, aeration, and pH determines the optical purity of produced lactic acid and thus needs to be stringently regulated. Most of the lactic acid producers are known to produce L-form, and thus, screening for D-form-producing strains is gaining attention. *Lactobacillus* sp. and *Sporolactobacillus* sp. in their native forms have been shown to produce D-lactic acid of high purity from lignocellulosic hydrolysates. However, for biotechnological production at industrial scale, genetically modified strains by random mutagenesis or knocking out L-lactate dehydrogenase genes are being developed.

1.4.5 Selection of Extremophilic Strains for Lactic Acid Production

Extremophilic microorganisms are an attractive alternate to mesophilic organisms for use in lignocellulosic biorefinery due to their ability to withstand harsh conditions. The low pH due to high lactic acid concentration leads to inhibition of mesophilic producers, and thus addition of large amounts of neutralizing agents, viz. gypsum, calcium, and carbonate, is required. This leads to complicated downstream processing and increased costs. The selection of potent acid-tolerant and thermophilic lactic acid producers is another challenging aspect as most of the lactic acid bacteria are mesophilic (growing at 37 °C). Further, the uses of acid-tolerant and thermophilic strains also diminish the contamination risks and increase fermentation efficiency. Thermophilic *Bacillus coagulans* LA204 produced high-titre lactic acid (97.59 g/L) from alkali-pretreated corn stover under non-sterile conditions (Hu et al. 2015). Singhvi et al. (2015) developed an acid-tolerant FM1 strain by protoplast fusion between *Acetobacter pasteurianus* and *L. delbrueckii*, which reduced the requirement of calcium carbonate by 50%. The specificity of lactate dehydrogenase of selected lactic acid producer and the fermentation conditions determine the optical purity of lactic acid produced. The feedstock used, fermentation mode, temperature, aeration, and pH need to be stringently regulated to obtain high purity lactic acid.

1.5 Metabolic Engineering of LAB

In the current developments of scientific technologies like genomics, proteomics, and metabolomics, molecular biology interactions and genetic engineering along with different bioinformatics software have allowed to accomplish metabolic engineering more methodically and advantageous approach for the industrial solicitation of lactic acid bacteria. The metabolic pathways of lactic acid bacteria are incompetent in terms of their energy gain (i.e. one or two ATP per sugar molecule), which is alleviated by high conversion rates. Consequently, the overall biomass gain, defined as the yield on sugar as a carbon source, is low. Also, the central carbon metabolism

and biosynthetic pathways required for cell growth are largely uncoupled in lactic acid bacteria, due to their adaptation to nutrient-rich niches. Such niches allow these bacteria to take up many cellular building blocks, rather than synthesize them from the carbon source (Gaspar et al. 2013; Sauer et al. 2017). This in turn leads to the fact that the carbon source is only used for (inefficient) energy gain. Thus, large amounts of sugar are fermented, and not much sugar is 'lost' for biomass production. This amalgamation leads to high yields of their natural fermentation products, which makes them industrially fascinating, and some applications have already been suggested. Basically, lactic acid bacteria are classified into three groups: obligate homolactic fermenters (lactic acid as the single fermentation product), obligate heterolactic fermenters (which produce lactic acid, ethanol/acetate, and CO_2), and facultative heterolactic organisms (Gänzle 2015).

Lc. lactis shows homolactic pathway with rapidly metabolized sugars with more than 90% of the metabolized sugar being converted to lactic acid. Deviation from the homolactic pathway is witnessed under aerobic conditions or during the metabolism of galactose or maltose (Neves et al. 2002a). Lactic acid is used as a preservation (acidifier) and flavour-enhancing agent by the food industry, as an emulsifying and moisturizing agent by the cosmetics industry, in the synthesis of optically pure pharmaceuticals and as intermediate in pharmaceutical processes, and also by the tanning industry (van Maris et al. 2004). L-lactic acid is also used industrially as the starting material in the production of valuable synthetic biopolymers. The polylactic acid is expected to replace various polymers conventionally derived from the oil industry in applications ranging from fibres to packaging. Polylactic acid (PLA) is a very promising biodegradable, renewable, and biocompatible polymer (Bai et al. 2004). Apart from its production, its application in different field (not only in commodity applications but also as durables and in biomedicine) is also increasing. The briskly growing demand for PLA has led to a rapid increase in lactic acid demand worldwide (Van Wouwe et al. 2016; Zhu et al. 2016; Zia et al. 2016).

A clear target for metabolic engineering aiming to increase the lactic acid production lies in the area of sugar utilization/broadening the range of carbon sources and the subsequent glycolytic and lactate fluxes. Regulation of glycolysis and the shift between different fermentation modes have been extensively studied with *Lc. lactis* (Garrigues et al. 2001a, b). Andersen et al. (2001) showed the role of phosphofructokinase (PFK) on the glycolytic flux in *Lc. lactis*, where a particular enzyme plays an important role as both the glycolytic and lactate fluxes were decreased proportionally by a twofold reduction of PFK activity. A key role of PFK with regard to the glycolytic flux control was also reported by Neves et al. (2002b). Papagianni et al. (2007) showed that the control of the glycolytic flux resides to a large extent in processes outside the glycolytic pathway itself, like glucose transport and the ATP consuming reactions, and allosteric properties of key enzymes like the PFK have a significant influence on the control. In continuation of the abovementioned work, Papagianni and Avramidis (2011) raised *Lc. lactis* strains with altered PFK activity, by cloning the pfkA gene from *Aspergillus niger* and studied the effects of increased PFK activity levels on the glycolytic capacity of *Lc. lactis* and lactic acid production. The results confirmed the direct effect of PFK activity on the glycolytic flux in *Lc. lactis* and lactate formation.

Lactate dehydrogenase (LDH) is the preceding enzyme in the pathway converting sugar to lactate in *Lc. lactis*. Disruption of the respective gene (ldh) leads to diversion of the majority of pyruvate towards mixed-acid products (Papagianni and Avramidis 2011). Several researchers studied about the extent of the control of LDH on metabolic fluxes in wild-type *Lc. lactis* cells through construction of a series of mutant strains with different LDH activities (Koebmann et al. 2002). Estimation of flux control coefficients reveals that LDH did not exert any control on the glycolytic flux and also not on the flux catalysed by the enzyme itself, i.e. on lactate production.

LAB can produce lactic acid in its L- or D-isomer form. L-lactic acid is mainly preferred for food and pharmaceutical applications and as starting material in the production of biopolymers, while D-lactic acid causes acidosis in humans. Therefore, metabolic engineering studies have concentrated on the production of pure L-lactic acid by homofermentative LAB (Upadhyaya et al. 2014; Wang et al. 2015; Rodrigues et al. 2017). *L. helveticus* has been the first LAB to be used and combined with ldhD inactivation by chromosomal integration, which produces higher amount of pure L-lactic acid comparable to that obtained by the wild-type strain (Bhowmik and Steele 1994). With a similar approach, pure enantiomers of lactic acid have also been obtained by inactivation of the specific LDH in other LAB. Kylä-Nikkilä et al. (2000)also constructed two stable ldhD-negative strains of *L. helveticus*: one carrying an additional copy of the ldhL gene under the control of the ldhD promoter and the other deleted the ldhD promoter region. This resulted in improved L-lactic acid production by more than 20% under low pH conditions. Similarly, inactivation of ldhD in *L. johnsonii* resulted in the production of pure L-lactate (Lapierre et al. 1999). Davidson et al. (1995) were working with *Lc. lactis* with increased copy number of the LAS (lactic acid synthesis) operon genes, which includes phosphofructokinase (pfk), pyruvate kinase (pyk), and the ldhL genes but observed very small increase in L-lactic acid production. The construction of an L-lactate overproducing *Lc. lactis* strain by UV mutagenization was reported by Bai et al. (2004). The production of pure D-lactic acid was achieved by the deletion of the ldhL gene in *L. plantarum* but with the overexpression of the ldhL gene in the same organism had no effect on the production of L- and D-lactic acid (Ferain et al. 1994). The ldhL gene from *L. plantarum* was inserted into *L. mesenteroides* through the plasmid vectors pLeuCM and pLeuCM42. The expression level of ldhL in *L. mesenteroides* was increased through pyruvate kinase promoter, which was fused to ldhL and cloned. As a result, L-LDH activity and L-lactate productivity were both significantly increased during fermentation along with decrease in the D-/L-lactate ratio (Jin et al. 2016).

The renewable resources mainly include lignocellulosic and starchy agro-wastes for the production of lactic acid, and other commodity chemicals have attracted several researchers in recent years. For producing value-added chemicals by utilising agroindustrial residues, a major drawback is to develop genetically engineered organisms, which can utilize both hexoses and pentoses from the hydrolysate (Tanaka et al. 2002; Yoshida et al. 2011; Mazzoli et al. 2014). These sugars are the most abundant renewable assets on the planet earth and are being considered as the fuel of the future (Abdel-Rahman et al. 2011; Boguta et al. 2014). In this

perspective, the significance of the metabolic engineering for the utilization of pentose sugar is completely justified (Okano et al. 2009; Yoshida et al. 2011). Yoshida et al. (2011) inserted the xylAB operon in the *L. plantarum* genome from *L. pentosus*. It was observed that the engineered *L. plantarum* strain was able to ferment both pentose and hexose sugar (mixture of pentose and hexose in 1:3 ratio) without carbon catabolite repression effects and produced D-lactic acid with a yield of 0.78 g g^{-1} of mixed sugar. Another successful strategy for fermenting arabinoses and producing lactic acid of 0.82 g g^{-1} of consumed arabinose was obtained in *L. plantarum* (Okano et al. 2009).

1.5.1 Metabolic Engineering of LAB for Improved Cellular Traits Against Different Stress

The ability of microorganisms in industrial processes depends upon their capability to cope up with the different stresses. In an industrial process, among variety of stresses, acid stress is the most important one. The growth of LAB is accompanied by lactic acid production leading to acidification of the medium. Lactic acid is present in medium as undissociated form, which simply diffuses inside the cytoplasm eventually leading to arrest of cell growth and subsequently cell death (Serrazanetti et al. 2009). Intracellular lactic acid affects the integrity of purine bases by changing the intracellular pH, and disrupting the cytoplasmic anion pool results in denaturing of essential enzymes inside the cells. Thus, improving the acid resistance is important for the industrial application of LAB. Several researchers have proposed different mechanisms to elaborate the acid stress resistance of lactic acid bacteria (Hartke et al. 1996; Rallu et al. 2000; Serrazanetti et al. 2009; Wu et al. 2014; Papadimitriou et al. 2016).

As an outcome, it was found that several genes and pathways present are indispensable for survival and adaptation of LAB in a low pH environment (Rollan et al. 2003; Budin-Verneuil et al. 2005; Guzzo et al. 2009; Bachmann et al. 2017). In acid-resistant mutant of LAB, it was found that generally relA gene is affected, which synthesizes guanine nucleotides. The *Lc. lactis* mutant with relA gene showed increased resistance against acid shock with the decrease in the production of lactic acid and low glycolytic flux (Rallu et al. 2000; Mercade et al. 2006). Antioxidants are mainly protecting cells from oxidative damage, and glutathione is well known for its antioxidant property and for improving bacterial stress resistance (Masip et al. 2006). Based on these leads, Zhang et al. (2007) engineered a *Lc. lactis* strain by cloning and overexpressing the glutathione genes from *E. coli* resulting in an improved resistance under acidic condition. This engineered *Lc. lactis* survived for hours under mild acid (pH 4.0) shock but for a shorter time span under severe acid stress (pH 2.5). It was hypothesised that glutathione-induced acid tolerance could be due to stabilization of GAPDH activity via S-thiolation and/or an increased intracellular pH (Zhang et al. 2007). Genetically engineered LAB strains with improved high acid resistance were also reported by using nonspecific approaches like genome

shuffling and adaptive evolution (Patnaik et al. 2002; Yu et al. 2008; Zhang et al. 2012).

For raising *Lactobacilli* cells with moderate acid tolerance, they must be cultured at low pH for several generations or in the presence of a mutagen compound (NTG). The acid-resistant cells were picked and shuffled by pool-wise recursive protoplast fusion. This study showed that *L. plantarum* can survive at substantially lower pH and at same time can produce higher lactic acid titre at pH 4.0 than the wild type (Patnaik et al. 2002). In an adaptive evolution experiment, *L. casei* was sequentially transferred into fresh medium during their mid-exponential phase. After 7–8 generation, *L. casei* mutant (lb-2) showed approximately 300-fold higher survival rate as compared with the wild strain along with increase in biomass (Zhang et al. 2012). Ye et al. (2013) used error-prone whole genome amplification to amplify nonspecific DNA fragments and moved it into the wild-type *L. pentosus* by electroporation. This resulted in enhanced acid tolerance (pH 3.8) of *L. pentosus* mutants as compared with the wild-type strain which did not survived.

1.6 Conclusion

Emphasis on lactic acid production based on agroresidues by microorganisms has been discussed. The industrial feasibility for the large-scale lactic acid production by microbial fermentation relies on several constrains like tolerance to inhibitors formed during hydrolysis of lignocellulosic material and mixed sugar utilization as glucose and xylose are the two abundant sugars in lignocellulosic hydrolysate. The industry requires microorganisms that can sustain low pH, high alcohol concentration, and different stress like acid, osmotic pressure, heat, etc. More or less, all of these requirements are fulfilled by lactic acid bacteria (LAB), which make them an appropriate cell factory for industrial use. The phenotypic variations are always present between different species of LAB which was exploited as important source for innovation in food technology but not yet utilized in chemical industry. The biodiversity of LAB strains should be screened and characterized for different commodity chemicals. The metabolic engineering of LAB needs a major attention and specially to improve its transformation capability. Our coming future should be completely based on renewable sources where LAB will play a major role in producing commodity chemicals.

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Chapter 2
Solid-State Fermentation: Use of Agroindustrial Residues

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Abstract Solid-state fermentation is a process carried out with microorganisms that grow on solid and moist substrates that act as sources of nutrients and support microbial growth. It is used for the production of products for the food, pharmaceutical, textile, biochemistry, and bioenergy industries. Organic residues from agricultural, industrial processing, and domestic food residues are the most suitable substrates to be used due to their abundance and composition. In addition, by using these organic wastes as substrates, environmental pollution problems can be minimized. SSF is influenced by a large number of factors, each of which is critical to the technical and economic viability of the process development. This includes the selection of microorganisms and substrates, optimal process parameters, and the steps for the separation and purification of the products. In this chapter, the main variables that influence the development of the SSF process for the production of products will be presented, such as the choice of microorganisms, the fermentation conditions, and the most used types of bioreactors. Some applications of SSF for the production of enzymes such as cellulases, amylases, and pectinases will also be presented, in addition to nutritional enrichment for animal feed.

Keywords Microorganisms · Amylases · Enzymes · Cellulases · Pectinases

2.1 Introduction

Solid-state fermentation (SSF) is a process performed with microorganisms that grow on solid and moist substrates that act as sources of nutrients and support microbial growth (Yazid et al. 2017). Its historical importance to mankind dates back thousands of years, mainly for food processing, both in western countries (bread and cheese) and in eastern countries (Koji). It is used for the production of important biomolecules and products for many industries, including food, pharmaceuticals, textiles, biochemistry, and bioenergy, among others (Soccol et al. 2017).

Recently, it is gaining a lot of attention due to the increasing use of different types of organic waste to obtain products like enzymes. The search for sustainable and ecological processes to transform traditional chemical processes also highlights the potential of the SSF. Thus, the bioconversion of organic waste into valuable biological products could replace nonrenewable materials and transform chemical processes into cleaner practices in the industrial sector (Thomas et al. 2013; Yazid et al. 2017).

The main counterpart of SSF is submerged fermentation (SmF), a process in which microorganisms grow in a liquid medium, with a high content of free water. The biological processes performed at SmF have notable advantages concerning instrumentation and control (monitoring of pH, dissolved oxygen, temperature, the concentration of water-soluble molecules), separation of biomass after fermentation, mixing, aeration, and scaling (Farinas 2015).

On the contrary, SSF resembles the natural habitat of most microorganisms, mainly fungi, and it is less susceptible to bacterial contamination. With regard to products, it allows greater enzymatic productivity for many enzymes, and it is less susceptible to substrate inhibition and, therefore, allows a greater final concentration of the products. It has several environmental advantages, as it allows the use of organic solid waste as a substrate and/or source of energy in its natural form and facilitates the management of solid waste, in addition to less effluent production (Soccol et al. 2017; Arora et al. 2018).

The SSF opened a new paradigm for the bioconversion of solid organic waste through the production of biologically active metabolites, both on a laboratory and industrial scale. The application of SSF in the production of different bioproducts has been widely reported, including enzymes, organic acids, biofertilizers, biopesticides, biopolymers, biosurfactants, bioethanol, aromatic compounds, animal feed, pigments, vitamins, and antibiotics (Thomas et al. 2013; Yazid et al. 2017).

The SSF is governed by a large number of factors, each of which is critical to the technical and economic feasibility of developing the process, and must be assessed together. This included the selection of microorganisms and substrates, optimal parameters of physical-chemical and biological processes, and also the purification of the desired products, which are a challenge for SSF. In general, fungi and yeast cultures were considered the most suitable microorganisms for SSF. This has been based on the theoretical concept of water activity, as fungi and yeasts have lower requirements for water activity, generally around 0.5–0.6 (Thomas et al. 2013).

Bacterial cultures have a greater need for water activity, around 0.8–0.9; however, some species of bacteria, for example, *Bacillus subtilis*, *Bacillus thuringiensis*, and *Lactobacillus* sp., were used to successfully produce solid-state enzymes. Actinomycetes such as *Streptomyces* sp. are also indicated for SSF processes, as they present characteristics such as abundant solid waste colonization, production of a wide range of degrading enzymes, and high resistance to extreme conditions (Thomas et al. 2013; Soccol et al. 2017).

The selection of suitable substrates also plays a key role in the efficient and economical production of the desired product. In selecting suitable substrates for the SSF process, it is important to guarantee the availability and cost of the substrates. They can provide adequate nutrients and physical support for the development of microorganisms in the SSF. Organic residues from agricultural and industrial processing and domestic food residues are the most suitable substrates to be used due to their abundance at a low or no cost and their chemical composition. However, in some cases, an additional supplement must be added to the organic waste. In other cases, chemical or mechanical pretreatment is necessary due to the inaccessibility of certain nutrients to microorganisms (Yazid et al. 2017).

Residues that can be used as substrates for the SSF include cane bagasse, cassava bagasse, cereal bran such as wheat bran, rice bran, oat bran and soybean meal, pulp and coffee husks, fruit skins and pulps, corncobs, straw, and husks of different origins. These materials are basically composed of cellulose, hemicellulose, lignin, starch, pectin, and other fibers (Farinas 2015; Soccol et al. 2017).

In this chapter, the main variables that influence the development of SSF for the production of bioproducts will be presented, such as the selection of microorganisms, the fermentation conditions, and the types of bioreactors most used. Some applications for the production of enzymes such as cellulases, amylases, and pectinases and nutritional enrichment will also be discussed.

2.2 Microorganisms

The interest in the production of enzymes, antibiotics, organic acids, biological control agents, and second-generation ethanol, through SSF, has been increasingly highlighted as being an alternative to SmF (Singhania et al. 2009; Lizardi-Jiménez and Hernández-Martínez 2017).

Since SSF occurs in a wet solid matrix, it is important to consider the choice of the microorganism and substrate that will be involved in the process (Soccol et al. 2017). Bacteria, yeasts, and filamentous fungi can be used in the production of biomolecules by SSF. However, filamentous fungi demonstrate an advantage in this type of fermentation, since wet solid substrates resemble the natural environments in which they grow and develop (Hölker et al. 2004; Yu et al. 2008; Zu et al. 2010; Farinas 2015).

In the SSF process, fungi grow by forming a network of mycelia on the substrate. Mycelia grow inside the substrate matrix (penetrating hyphae) and on the substrate surface (wet mycelium layer) and appear on the air-liquid interface (aerial hyphae). In this type of fermentation, there is the consumption of O_2 and the production of CO_2, H_2O, other biochemical, and heat. Thus, gradients develop within the biofilm, which, for example, forces O_2 to diffuse from the gas phase to the innermost regions of the biofilm and CO_2 to diffuse from these regions to the gas phase. The development of heat leads to a rapid rise in temperature, which can be a serious problem during SSF. Heat is removed from the medium by conduction and evaporation. The bioproducts of interest, such as enzymes, which are released inside the solid matrix and in liquid-filled pores, during fermentation, can be absorbed by the solid, requiring its extraction at the end of the SSF process (Hölker and Lenz 2005).

After choosing the type of microorganism, the preparation of the inoculum is another important point in SSF. The inoculum can be defined as a preparation of viable microorganisms that are added to the fermentation medium, aiming at microbial growth and development. The age and physiological state of the inoculum, in addition to the medium used, are of great importance in fermentation processes (Manan and Webb 2017). If the inoculum is not physiologically viable, there will be a considerable decrease in the production of secondary metabolites (Crafack et al. 2014).

Among the methods of preparation of fungal inoculum, commonly applied in SSF, we can highlight the suspension of spores, mycelium discs, mycelium suspension, and pre-inoculated substrates (Yoon et al. 2014).

The spore suspension can be obtained after washing the surface of the fungi grown in a Petri dish, with distilled water or sterile saline solution. Spores in the suspended liquid can be counted with the help of a Neubauer chamber and microscope, with the inoculum density being adjusted by adding water or saline solution to the suspension to achieve the desired spore concentration as an inoculum, often from 10^6 cm^{-3} for SSF (Hong et al. 2011; Yoon et al. 2014).

To obtain the mycelium disk-shaped inoculum, a cut of the agar is made, in the region of the periphery of the fungal mycelium, in active growth. Then, the disc can be used directly for inoculation in the specific fermentation medium (Yoon et al. 2014; Hong et al. 2011).

The inoculation of a mycelium suspension consists of transferring mycelium discs, with fungi growing actively in a Petri dish, to a liquid medium, for 5–7 days. Then, the homogenized mycelium can be used for inoculation in vials containing the specific substrate for SSF (Shrestha et al. 2008; Elisashvili et al. 2009).

The inoculum from pre-inoculated substrates can be done, for example, from the inoculation of mycelium discs on substrates such as sterile wheat grains. After about 6–20 days of growth, depending on the amount of mycelium and substrate used, the grains with mycelium can be used as an inoculum (Velázquez-Cedeño and Savoie 2002; Reddy et al. 2003). Some studies point out the importance of supplementing sterile wheat grains, before adding mycelia, with calcium carbonate and calcium sulfate, aiming at adjusting the pH to facilitate fungal growth (Reddy et al. 2003; Yoon et al. 2014).

The evaluation of fungal biomass in the SSF is fundamental to characterize its growth, kinetics, and productivity, during fermentation processes. However, this evaluation by direct methods is difficult, as the fungi are closely linked to the substrate, forming a complex separation matrix (Steudler et al. 2015). Despite the difficulties encountered in this determination, indirect methods have been cited for biomass measurements, which are based on the measurement of some metabolic activity (O_2 and CO_2) or some specific biomass component (ergosterol, glycosamine, number of fungal cores, nucleic acids, protein content, genomic DNA). For Steudler and Bley (2015), the most appropriate cellular quantifications for control and modeling of processes are the ergosterol content, number of nuclei, and respiration (O_2 and CO_2).

Ergosterol is a lipid present in the cell membrane of fungi and can be used as an indicator of the biomass of these microorganisms (Mille-Lindblom et al. 2004). Its main advantage, in comparison with other biomarkers, such as chitin and ATP, is its specific association with fungi, since only reduced amounts of this lipid can be found in algae and protozoa. Ravelet et al. (2001) evaluated the fungal biomass in sediments through the quantification of ergosterol. The method was based on saponification and liquid-liquid extraction of ergosterol by ethyl acetate. Quantification and detection were performed isocratically by liquid chromatography.

The quantification of fungal biomass by the number of nuclei, using flow cytometry, was described by Steudler et al. (2015) as a practical, low-cost method. They used an appropriate buffer solution that promoted cell lysis and released intact

nuclei. The nuclei were stained and quantified according to their fluorescence by flow cytometry.

O_2 consumption and CO_2 release result from respiration, a metabolic process by which aerobic microorganisms extract most of their energy for growth. These metabolic activities are therefore associated with growth and can be used to estimate biomass synthesis (Raimbault 1998). In this perspective, Manan and Webb (2020) pointed out in their study that CO_2 can be used to describe fungal growth in SSF in an excellent way.

One of the advantages that SSF has is the possibility of using mixed cultures of microorganisms and, thus, exploring metabolic synergisms between various fungi. In their natural habitats, such as soils or plant materials, fungi grow in symbiotic associations, promoting the biodegradation or mineralization of these complex solid substrates. This requires the participation of a wide spectrum of different molecules produced by different fungi (Hölker et al. 2004).

A study on a mixed fermentation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 as an economical method to produce cellulases for the hydrolysis of corn residues, carried out by Kumar et al. (2016), demonstrated that the production of cellulases was more effective and economical when the fungi were grown concomitantly, instead of *A. nidulans* AKB-25 alone. For Gutierrez-Correa et al. (1999), the mixed culture between *Trichoderma reesei* LM-UC4 and *Aspergillus niger* ATCC 10864 at SSF, with pretreated sugarcane bagasse for the production of cellulolytic enzymes, was beneficial for an economical production of cellulases from agricultural residues, nutritionally poor, without the need for supplementation with high-cost organic substances.

After selecting the microorganism, the type of culture, and the form of inoculation, it is important to adjust the operational parameters such as substrate type, pH, temperature, medium humidity, and SSF duration, which can directly interfere with the productivity of biomolecules or compounds of interest.

2.3 Fermentation Conditions

The SSF process is affected by several parameters, which are related to the metabolic aspects of the microorganisms used and the composition of the fermentation medium. Therefore, one must determine which values are most suitable for humidity, pH, temperature, microorganism, amount of inoculum, type of substrate, and porosity of the fermentation medium, in order to obtain maximum production of enzymes (Farinas 2015).

The humidity of the fermentation medium will depend on the requirements of the microorganism and the nature and composition of the substrates. It generally ranges from 18% to 85%. It must have a value that minimally meets the metabolic requirements of the microorganism. High humidity will hinder the transfer of oxygen into the particles, reduce the surface area of the fermentation medium, and

increase the risk of contamination. Little moisture will affect the microorganism's metabolism, reducing the transfer of nutrients and metabolites (Pinto et al. 2006).

However, the water content in an SSF process is not one of the controlled parameters, but the water activity, since it determines the amount of water actually available for use by the microorganism. It is defined as the ratio between the vapor pressure of water contained in the sample (P) and the vapor pressure of pure water (Po), evaluated at the same temperature (Santiago et al. 2014). The minimum water activity value required in the process varies from one microorganism to another, and for filamentous fungi, this value is around 0.7.

The temperature value used in the process influences the development of microorganisms, as they have an optimal temperature value for their development, which varies from one species to another. The accumulation of metabolic heat can be harmful, as it raises the temperature of the process, impairing the development of microorganisms. In addition, high-temperature values can promote the denaturation of the enzymes produced. Since in the SSF there is an absence of free water, the removal of metabolic heat is impaired, due to the limited thermal conductivity of the solid substrate and the low thermal capacity of the air (Raghavarao et al. 2003; Farinas 2015).

According to Pinto et al. (2006) for filamentous fungi, temperature directly influences spore germination, growth, and product formation. The pH of the fermentation medium directly affects microbial metabolism, as it influences the biochemical reactions involved in the processes of catabolism and cellular anabolism. There is a minimum, optimal, and maximum pH value for each type of microorganism. Filamentous fungi thrive best at acid pH, between 3.5 and 5.0 (Santiago et al. 2014).

Due to the dependence on the presence of ionic species to maintain the conformations that allow binding to the substrate, the enzymatic activity is strongly influenced by pH. However, monitoring and controlling this parameter in SSF is not simple, limiting the work in the literature to describing the influence of the initial pH of the enzyme production medium (Farinas 2015).

Regarding substrates for SSF, these must contain carbon sources and nutrients suitable for the development of the microorganism. Also, it must contain in its composition the inducing source of the product to be produced, especially when it is desired to produce enzymes. Agroindustrial residues are widely recommended as substrates for SSF, as they are widely available and have the necessary macro- and micronutrients for the development of the microorganisms used (Soccol et al. 2017).

The determination of the suitable substrate depends on the product to be produced and the microorganism to be used. It also depends on its availability and ease of processing, in addition to its composition. The medium that allows the best development of the microorganism does not always favor the greatest amount of product (Santiago et al. 2014).

Various agroindustrial residues can be used as solid substrates for SSF, such as sugarcane bagasse, orange bagasse, cereal bran, cassava, and fruit peels, among others. The structures of these materials, such as cellulose, hemicellulose, starch, pectin, and fibers, determine the properties of the substrate and act as sources of

carbon and energy for the development of microorganisms. Eventually, supplementation is necessary (Farinas 2015).

Agroindustrial waste needs physical treatment before being used in the composition of the medium. Such processing varies from one type of material to another and often consists of drying the materials, followed by crushing and screening.

The choice of the microorganism is one of the most important criteria for obtaining enzymes by SSF. Microorganisms that have been isolated, selected, or mutated should be used to expand their ability to adapt to the fermentation medium and promote an increased concentration of the enzyme activity produced. For SSF, fungi that excrete enzymatic complexes instead of accumulating the enzymes of interest inside the cells are more suitable, as they facilitate downstream processes for recovering the enzymes produced (Soccol et al. 2017).

The inoculum concentration also affects the results of an SSF process. If it is a low quantity, there may be the formation of little biomass and facilitate contamination by undesirable agents. On the other hand, large amounts of inoculum can promote rapid depletion of available nutrients, impairing cell development, and, consequently, product formation (Santiago et al. 2014). Values between 10^5 and 10^7 spores per gram of fermentation medium are generally used.

For the formation of the fermentation medium, it is also important to pay attention to the size and shape of the particles of the substrate, since these parameters directly affect the porosity value of the formed bed. Very small particles can promote agglomeration, reducing the surface area available for microbial respiration and, consequently, its development. Large particles, in turn, can favor the transport of oxygen and removal of the metabolite heat but reduce the surface area available for microbial growth (Soccol et al. 2017).

2.4 Bioreactors for Solid-State Fermentation

In SSF, the bioreactor provides the environment for the growth and activity of microorganisms that lead to biological reactions. During the processing period, it must be able to prevent the release of microorganisms and fermentation medium into the environment as well as prevent the entry of foreign substances into the medium (Thomas et al. 2013; Manan and Webb 2017).

The ideal bioreactor should have several characteristics; in particular, the construction material must be nontoxic and able to withstand pressure, usually pressurized steam for sterilization. It should not be affected by chemical corrosion. There must be proper arrangements for aeration and agitation with sample removal, loading, and unloading compartments, and also a cooling mechanism to control the energy generated by metabolism in the form of heat. In addition, bioreactor systems should be able to operate under aseptic conditions (Arora et al. 2018).

The bioreactor model is a vitally important factor that determines the efficiency of the process. Aspects such as the careful selection of the reactor, consideration of the model, type of reactor, and scaling of the reactors are extremely important. Some

important considerations in the development of the bioreactor model are as follows (Thomas et al. 2013; Manan and Webb 2017):

- It is necessary to mix and how it can be done
- The degree of aeration required
- The rate of heat that needs to be removed
- The parameters that need to be measured and controlled
- The type of substrate, its properties, and the handling of solids during preparation of the medium, inoculation, and the taking of samples from the process
- Sterilization and contamination prevention
- The mode of operation
- The criteria used in scaling up
- Capital and operating costs

Some design considerations will be discussed, although definitive rules for the design of bioreactors have not been developed.

The SSF process can be divided into three groups according to the mixing regime used: static, periodically agitated, and continuous agitation. Agitation facilitates the maintenance of homogeneous conditions in the bioreactor, specifically with respect to temperature and aeration (Manan and Webb 2017).

The lack of agitation has a profound influence on the amount of substrate that can be used. In static SSF, it is observed that the temperature gradient increases with increasing bed height. If forced aeration is not used, oxygen in the space between the particles can drop to the limit level, and the concentration of carbon dioxide rises to an inhibitory level (Thomas et al. 2013; Arora et al. 2018).

In periodically agitated SSF, agitation serves to fill the spaces between particles with fresh air. The required mixing frequency depends on the void fraction of the substrate. The void fraction depends on the substrate properties such as particle size and shape.

However, mixing the solid substrate can also cause fermentation damage, including an adverse effect on the porosity of the substrate, disruption of the microorganism's attachment to the substrate, and damage to the fungus mycelium due to the forces caused by abrasion between the particles. Filamentous fungi are particularly susceptible to these forces. Aerial hyphae are crushed on the substrate surface, resulting in inhibition of sporulation (Santos 2007).

Stirring stimulates initial growth to the point that the released metabolic heat cannot be removed easily, leading to overheating of the substrate. The balance between the advantages and disadvantages of the mixing effects is different for different processes considering the substrate, the microorganism, and the bioreactor in the SSF. In any case, agitation should only be provided if necessary, and periodic agitation is usually sufficient.

A large number of SSF processes involve aerobic microorganisms; the transfer of oxygen to the microorganism on the surface of solid particles is of paramount importance. Aeration also promotes the removal of metabolic heat, gases, and other volatile metabolites from spaces between particles (Manan and Webb 2017; Sala et al. 2019).

The transfer of oxygen in the spaces between particles will be limited in the static SSF without forced aeration. The spaces between particles can be filled either by forced aeration or by gentle mixing of the substrate, which allows gas exchange at the top of the bioreactor. Of these two methods, forced aeration is preferable in cases where the microorganism is susceptible to damage from the mixture. The depth of the stagnant gas layer on the particle surface is a function of the speed with which the gas passes through the substrate mass and can be minimized by the high speed of forced aeration. However, high aeration speed is not economical, and they tend to remove water from the substrate (Arora et al. 2018).

The transfer of oxygen in the spaces between particles is influenced by the porosity of the substrate mass. The growth of the microorganism in the spaces between particles, the agglomeration of the substrate, high moisture content, and the use of small particles or the presence of fines between larger particles can form a barrier to the efficiency of oxygen transfer. The high percentage of moisture results in low porosity of the substrate, which prevents oxygen penetration, while the low moisture content can lead to low accessibility of nutrients, resulting in impaired microbial growth (Singhania et al. 2009).

The transfer of oxygen within the particles is limited by molecular diffusion. Diffusivity depends on the substrate and is not affected by the aeration method. Furthermore, oxygen must pass through microorganisms actively breathing to the surface, so very little oxygen will reach the particle itself. The growth of the microorganism is then restricted to the surface of the substrate particles by the availability of oxygen.

Where forced aeration is used, it is quite common for airflow to be upward through the substrate supported by a perforated base. The perforated base must have a large number of small holes instead of a few wide holes; this is to prevent air from traveling in "preferred paths."

The SSF process is characterized by the generation of a large amount of heat. The rate at which heat must be removed depends on the metabolic activity of the microorganism and the amount of substrate in the bioreactor. The solid nature of the substrate and its low humidity lead to very low heat transfer.

Heat generation due to the metabolic activities of microorganisms, which is desirable, for example, in composting, is generally fatal for biotechnological processes because most of the enzymes produced during fermentation can be denatured by heat at the end of the process (Singhania et al. 2009).

In static SSF, the low heat transfer rate causes a large rise in the substrate temperature. This is a problem generally seen on a large scale. Several heat removal strategies can be synthesized:

- Forced aeration with moist air removes heat by conduction
- Forced aeration with dry air removes heat by evaporation
- Cool the outer surface of the bioreactor, with a water jacket
- Circulate chilled water through an internal heat exchanger
- Place the bioreactor in a room with controlled temperature or in a bath with water (small scale)

Such methods can be used in combination. Removing heat by evaporation is the most efficient method of removing the heat generated (Singhania et al. 2009). However, moisture loss poses a serious problem where the moisture content is already low. However, evaporative cooling can be accompanied by moisture replacement.

During fermentation, it is necessary to prevent or minimize the entry of contaminants or to prevent the escape of organisms from the process. The substrate is steam cooked for two purposes: to sterilize and to modify physical properties and increase susceptibility to microbial attack.

The prevention of contamination during the fermentation period can be easily achieved in closed bioreactors. The air used in forced aeration can be sterilized by filtration. In open bioreactors like the tray ones, you have to rely on using a dense and vigorous inoculum and selecting conditions of acidic pH and low humidity. Rooms can be painted with antimicrobial paints. The substrates must be covered with paper or fabric to prevent contamination of the open trays with air. It is also essential to prevent condensates inside the room that may fall on the open tray. Retention of the process microorganism may be necessary if it is a health risk to process workers. Spore retention is usually desirable since it can trigger allergic reactions. Retention is easier to do in closed bioreactors than in open ones.

2.4.1 Main Types of Bioreactors

The bioreactors commonly used for SSF can be classified by the type of aeration used in forced and non-forced, and by the mixing system employed, with or without mixing. The main types of bioreactors used in the SSF will be presented below (Farinas 2015; Arora et al. 2018).

2.4.1.1 Tray Bioreactor

SSF in trays has been used for the production of fermented foods, such as tempeh, miso, koji, and soy sauce (Durand 2003; Arora et al. 2018) in some Asian countries. The trays, with or without perforations, are filled with the substrate and stacked one above the other in rooms with controlled temperature and humidity. Expansion is generally achieved by increasing the surface area and/or increasing the number of trays. The main parameters that must be controlled are the height of the operational bed, the nature of the medium, and the initial moisture content of the substrate and its effect on productivity. Tray bioreactors have been used in the study on a laboratory, pilot plant, and industrial scale (Farinas 2015; Arora et al. 2018).

Tray-type bioreactors were used for the production of cellulolytic enzymes by SSF, in shallow ($50 \times 40 \times 5$ cm^3) and deep ($4 \times 2 \times 1.5$ m^3) trays, using corncob residue as a substrate for the fermentation of *T. reesei* ZU-02. The results showed

that the deep tray bioreactor with forced aeration provided high cellulase productivity, compared to shallow tray bioreactor (Farinas 2015).

The tray bioreactor was used in the production of cellulolytic enzymes using a co-fermentation technique involving *T. reesei* and *A. oryzae*. Soy bran and wheat bran (4:1) were used as substrates (1 cm high), and optimum conditions of temperature (30 °C), humidity (70%), and pH (5.0) were also obtained. The importance of an appropriate C:N ratio in the substrate was emphasized, and they concluded that the process led to high cellulase activity, but also resulted in the balanced production of glycanase (endo and exo) and β-glycosidase, recommended for biofuel production. High levels of β-glucosidase were obtained (91.8 IU $g.s^{-1}$) when A. niger was grown in plastic trays (40 × 25 × 12 cm), and operational conditions were optimized using the response surface methodology (Arora et al. 2018).

Although the tray bioreactor constitutes an important portion of commercial SSF processes in the industry, it still has some disadvantages. Heat transfer occurs mainly by conduction, and due to the low thermal conductivity of the substrate, heat dissipation is generally not efficient, imposing limitations on the height of the bed. As a result, control of the optimum temperature and moisture content in the reactor bed is limited to the bed height. Moreover, it requires a large operational area, and the process is laborious. Most of the time, the substrate requires separate sterilization. It is difficult to apply this technology to sterile processes, except only if large aseptic rooms are built and procedures and equipment are provided to employees, which can be prohibitive and expensive (Durand 2003; Arora et al. 2018).

2.4.1.2 Column Bioreactor

Packaged bed bioreactors or column bioreactors consist of columns in which the solid substrate is supported on a perforated base, from which air is forced through the substrate bed. This type of bioreactor is suitable for processes in which the mixture is undesirable due to adverse effects on microbial growth or on the physical structure of the final product. Column bioreactors can be equipped with a water jacket for temperature control during the process (Farinas 2015).

Forced aeration through a static bed assists in the replacement of O_2 and moisture and reduces the accumulation of heat and CO_2. This offers better control and facilitates a higher substrate load than tray bioreactors. It is important to check parameters such as thermal conductivity, density, specific heat, and particle size of the substrate and empty fraction, as well as kinetic parameters such as specific growth rate, generated metabolic heat, and maximum concentration of cell biomass, which can significantly affect the yield and metabolite productivity (Arora et al. 2018).

Column bioreactors were used for the production of enzymes. The production of endoglycanase by SSF in plastic bags and column bioreactor, using the fungus *Myceliophthora* spp. in a medium composed of wheat bran and sugarcane bagasse, showed that the endoglycanase activities obtained were similar for both systems. The use of residues for the production of endoglycanase by *A. niger* cultivated for

7 days in a column bioreactor was also evaluated. The conditions of incubation temperature, initial moisture content of the substrate, and airflow were evaluated. The incubation temperature and the interaction between the initial moisture content and the aeration rate influenced the production of cellulase (Farinas 2015).

The effect of forced aeration was studied on the production of glucoamylase and protease during the fermentation of *Aspergillus awamori* in a 0.5 and 1 L column bioreactor. The use of 1.5 vvm (volume of air per volume of medium per minute) was considered excellent, and a model describing its effect on enzyme production was developed, successfully predicting the adverse effect of the aeration rate below and above 1.5 vvm. Similar observations were made during the production of cellulases from olive oil and *Aspergillus uvarum* processing residues in a glass column. The high aeration rate decreased the cellulase production, attributed to the low water activity in the bed (aW) and the high shear stress in *A. uvarum* (Arora et al. 2018).

The operation of the SSF in column bioreactors has improved over the tray bioreactor, where it is possible to work with higher amounts of substrate, in addition to greater control of process variables. However, column bioreactors, even on a laboratory scale, are faced with problems of heat accumulation, substrate compaction, preferential air paths, bed drying, and process heterogeneity, imposing limitations on the heights of the operational bed. In addition, operations such as sterilization, inoculation, product removal, and post-fermentation treatment seem cumbersome. Zymotis has been the most promising design among the existing packaged bed bioreactors, with its ability to minimize temperature gradients. However, further improvements in design and operation strategies would be needed to fully realize the industrial potential of SSF technology (Durand 2003; Arora et al. 2018).

2.4.1.3 Rotary Drum Bioreactor

The rotating drum bioreactor consists of a drum-shaped container that can be mounted on a roller, rotating device. Air is normally blown through the upper space above a bed of substrate particles, and the bioreactor can be rotated intermittently or continuously. The mixing of the substrate bed is generally facilitated by the drum rotation action; however, agitated drum bioreactors can also be used with blades, where the blades mounted on a central axis perform the mixing with the remaining static drum. The drum may come with internal deflectors of different sizes and shapes to facilitate mixing (Durand 2003; Farinas 2015; Arora et al. 2018).

The rotating drum with paddles provides more efficient oxygen transfer and reduces the clumping of substrate particles during microbial growth. The agglomeration of substrate particles during the growth of the mycelium can lead to increased difficulty in regulating the temperature of the solid medium. In addition, the oxygen transfer within these medium balls, agglomerated by the fungus hyphae and also very often by the viscosity of the substrate used, may be very low or not at all (Durand 2003).

Bioreactors in this category include gentle agitation, and forced aeration can be added to improve heat and mass transfer and microbial growth. The mixture improves convective transport, as it increases the surface area of the substrate exposed to humid air and/or cooling fluid. However, the challenge is to maximize productivity with minimal mixing events, as these can potentially damage fungal mycelia and also consume a lot of energy (Arora et al. 2018).

A twofold increase in cellulase production in a 50 L drum bioreactor has been reported, compared to studies in flasks using 4 kg of substrate. The increase was attributed to better aeration and mixing. The effect of agitation on the production of hydrolases in a 0.25 L laboratory rotary drum bioreactor was studied, where *A. awamori* was grown in grape marc and orange peel (1:1). The maximum activities for xylanases, exo-polygalacturonase, and CMC were obtained with a high rate of aeration (120 and 200 $mL.min^{-1}$) and a very low agitation rate (1 $min.day^{-1}$). Constant agitation without aeration caused substrate agglomeration, resulting in low growth. The intermittent rotation was considered favorable to the production of citric acid during the fermentation of apple bagasse by *A. niger*; however, the continuous mixing resulted in a 34% reduction in the production of citric acid. The mixture resulted in a significant reduction in endoglycanase activity during the fermentation of wheat bran by *A. niger*. Cellulase activity decreased by 17% with increasing mixing frequency in a bioreactor using *A. niger*. Even if the organism is unable to tolerate mixing, it may still be necessary to mix the bed aseptically during inoculation and sampling to ensure uniform distribution. There is no general mixing strategy for a wide spectrum of bioprocesses; the choice for mixing must be a function of the morphology of the microorganisms, the chemical and physical nature of the substrate, the configuration of the bioreactor, and the mixing regime employed (Arora et al. 2018).

The potentiality of using SSF processes has highlighted the need to work on the operational problems that are involved in conducting them, especially with regard to the use of bioreactors, aiming at scaling up and large-scale production of many compounds of industrial interest.

The limitations inherent to the SSF acquire greater proportions when the scale of production is increased, consequently generating a greater impact of the problems on the process. Among the limitations of SSF, in large-scale operations, the heterogeneity of the process stands out, which makes it difficult to control variables such as temperature, humidity, agitation, aeration, and concentration of nutrients and products. In order to minimize this effect, one must mainly work on the correct choice of the bioreactor and adapt its operational variables to the product's characteristics.

In this text, the main models of bioreactors used were presented; other models based on the models presented were developed. There has been a substantial improvement in the fundamental understanding of aspects of biochemical engineering, particularly in mathematical modeling and in the design of bioreactors, which has helped in the development of various projects for bioreactors. This also helped to better understand the effects of heat and mass transfer, leading to better design of processes and product developments.

All types of bioreactors suffer from some type of engineering limitation that makes scaling difficult. Research focused on the development of instrumentation for SSF bioreactors is therefore necessary to achieve significant advances in the application of this technology. Another possibility is SSF's future research and development, which should focus on high-value products that may be economically viable on a relatively small scale.

2.5 Applications

2.5.1 Production of Cellulases

Cellulases are a group of enzymes capable of hydrolyzing cellulose to glucose and are produced by microorganisms, such as bacteria and fungi, the latter gaining more attention. Fungi have the advantage of adapting more quickly and easily in the environment where they are grown and also end up releasing the enzymes that they produce directly in this environment (Guimarães et al. 2006). Among the various species of fungi, *T. reesei*, *A. niger*, and *Humicola insolens* (Bhattacharya et al. 2015; Payne et al. 2015) stand out as good producers of cellulase enzymes, being used by several authors in their research throughout the years.

Cellulase enzymes secreted by different microorganisms are formed by three main components that act together to release glucose monomers: endoglycanases (EC 3.2.1.4), exoglycanases (EC 3.2.1.91 and EC 3.2.1.74), and β-glycosidases (EC 3.2.1.21). Endoglycanases initiate cellulose hydrolysis and act randomly between cellulose fibers, breaking internal bonds and generating long-chain polysaccharides, with reducing and non-reducing regions. Exoglycanases, in turn, act unidirectionally in these polysaccharides, cleaving them and releasing disaccharide units called cellobiosis. There are two types of exoglycanases: those that act on the reducing ends (cellobiohydrolases) and non-reducing ones (cellodextrinases). Finally, β-glycosidases (or cellobiases) cleave cellobiose, releasing glucose and completing hydrolysis (Singhania et al. 2010; Kuhab et al. 2011; Chandel et al. 2012; Juturu and Wu 2014; Payne et al. 2015; Singhania et al. 2017).

Due to their potential to convert cellulose-rich materials, such as agroindustrial waste, into fermentable sugars, these enzymes have been studied since the 1950s and have proved to be very important in several areas of the industry (Bhat 2000). Its applications in the industry are also diverse, and its use can be highlighted in the textile sectors, removing impurities and modifying certain properties of the fibers of fabrics; animal feed, removing substances considered antinutritional present in grains and vegetables; detergents, improving appearance and color and removing more difficult dirt particles; drinks, acting in the extraction and whitening stages of juices, nectars, wines, beers, and purees; and paper and cellulose, pharmaceutical, and also biorefinery, where glucose (released from cellulose hydrolysis) can be used for the production of bioethanol (Karmakar and Ray 2011; Ferreira et al. 2014; Sharma et al. 2016).

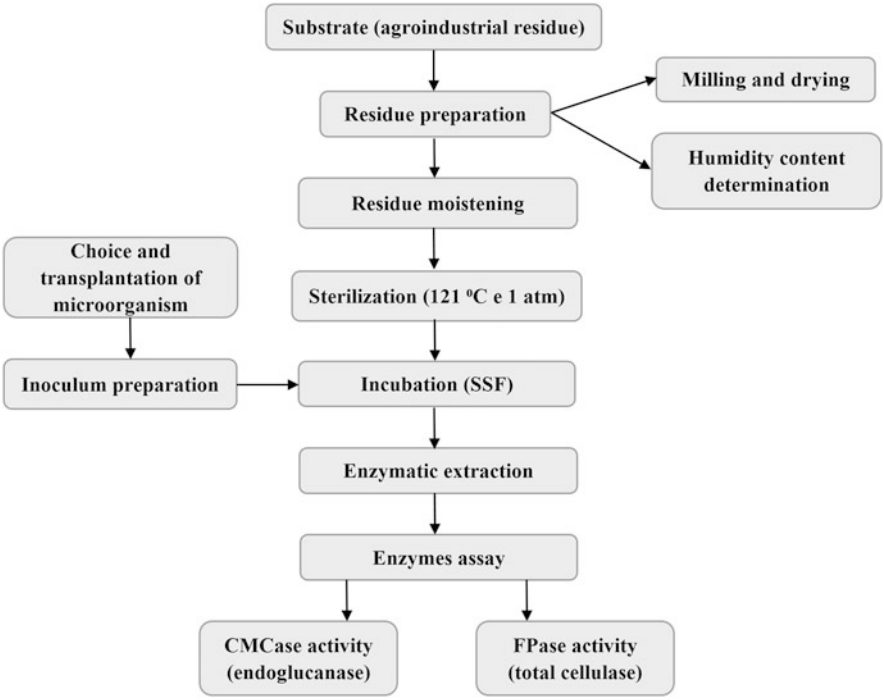

Fig. 2.1 Flowchart of the steps involved in the production and quantification of cellulase enzymes by SSF

Cellulases can be synthesized by both SmF and SSF, using different lignocellulosic biomasses as substrates (Kuhab et al. 2011; Chandel et al. 2012). The research groups that produced these enzymes used agroindustrial residues widely produced in the nearby regions that have expressive levels of cellulose and that can be purchased at low cost or free of charge. The experiments for the production of this enzyme were carried out at the Bioengineering Laboratory (LABIO), belonging to the Chemical Engineering Department, located at the Technology Center of the Federal University of Paraíba, and followed the steps shown in Fig. 2.1, which will be explained in detail later on.

In different researches, residues from wheat (bran), bananas (pseudostem and leaves), sugar cane (straw and bagasse), and corn (straw and cob) were used. Of these, only wheat bran has a well-defined use, being used as animal feed. The remaining residues are generated in surplus and are either burned, so as not to accumulate in the environment, or are left in the field after harvest. Therefore, it is interesting to use them in order to generate value-added products. The waste was obtained from local merchants through donation or purchase. Wheat bran has already been purchased as a dry powder and ready to be used. The rest were sanitized, crushed (conventional or forage blender, depending on the hardness of

the material), dried in an oven, and then stored in containers at room temperature. The moisture determination was carried out with the residues already dry and followed the methodology of the Adolfo Lutz Institute (2008). In the wetting step of the residues, the moisture values found previously were used in order to prepare a solution proposed by Mandels and Weber (1969) or ammonium sulfate solution 1%, which serve as sources of nitrogen and carbon and induce growth of the microorganism and the production of enzymes. The properly moistened residues were then distributed in 1000 mL Erlenmeyer flasks (100 g each) and sterilized in an autoclave for 121 °C and 1 atm.

In all fermentations carried out, two fungi isolated from the soil of a sugar and alcohol industry located in the state of Paraíba (Carvalho-Gonçalves 2017) were used, being called *Penicillium* sp. FSDE15 and *Aspergillus* sp. FSDE16. They were inoculated in a medium composed of potato, dextrose, and agar (BDA) 39 $g.L^{-1}$, incubated for 7 days at 30 °C, and kept refrigerated until later use. The SSF was carried out with fermentation times (7–10 days), spore concentrations (10^6 and 10^7 $spores.g^{-1}$ of substrate), and varied humidity (50, 60, and 70%), using the spores suspended from the fungi that were chilled.

The enzymatic extraction was performed using sodium citrate buffer solution (pH 4.8 and 50 mM) as well as only distilled water in varying solid-liquid proportions (6–30 mL per gram of fermented medium) and later filtered using filter paper qualitative. Previous studies by Carvalho-Gonçalves (2017) showed that there is no significant difference in enzyme activity when using citrate buffer or distilled water. Finally, enzymatic quantification was performed with the enzyme extract supernatant, previously centrifuged for 5 min at 12,000 rpm. The CMCase and FPase activities were determined according to a methodology adapted from Ghose (1987), which is based on the measurement of the concentration of reducing sugars released during the degradation of the CMC substrate and Whatman filter paper no. 1, respectively.

The main results obtained for the production of cellulases will be discussed below. For corn residues (cob and straw) and wheat bran, the total fermentation time was 168 h, the humidity was 60%, and the microorganism used was *Aspergillus* sp. FSDE16. The maximum CMCase activity obtained was 7.96 $U.g^{-1}$ for fermentation carried out with bran and cob in the proportion of 50% and 6.44 $U.g^{-1}$ when straw and bran were used in the same proportion, both at 168 h. In another study, carried out only with wheat bran and under the same conditions of humidity and fermentation time, the greatest production of CMCase was at 120 h, with a value of 6.696 $U.g^{-1}$. These results were greater than those obtained by Bonfim (2014), who carried out an assay with the microorganism *Penicillium* sp. FDSE15 (isolated from the same sugar and alcohol industry), wheat bran as a substrate, and 50% humidity, where the peak of CMCase activity was 6.265 $U.g^{-1}$ at 96 h of fermentation. It is noticed that the activity values were close but that *Aspergillus* sp. FSDE16 showed slightly higher values in their respective peaks of activity.

Analyzing the different conditions of the process, another study was carried out with *Aspergillus* sp. FSDE16 and wheat bran as a substrate, varying the inoculum concentration, humidity, and incubation temperature. The best result was obtained at

168 h, humidity of 50%, and a spore concentration of 10^7 esp.g^{-1} of the substrate, where the value was 12.51 U.g^{-1}.

As for the residues from bananas and sugarcane bagasse, the best results show that the largest CMCase production was 6.81 U.g^{-1} at 144 h, for fermentation with sugarcane bagasse and wheat bran. In descending order, there is 5.35 Ug^{-1} at 168 h, for fermentation carried out with banana pseudostem and wheat bran, and 5.20 U.g^{-1} at 48 h, for fermentation carried out with banana leaf and wheat bran; all fermentations were carried out in the proportion of 50% and with the microorganism *Aspergillus* sp. FSDE16.

Evaluating the results of the experiment carried out with *Penicillium* sp. FSDE15 and with sugarcane straw and wheat bran as substrates, under conditions of 70% humidity, a temperature of 38 °C, and a spore concentration of 10^6 esp.g^{-1} substrates, it was possible to obtain CMCase and FPase activity values equal to 22.97 U.g^{-1} and 1.37 U.g^{-1}, respectively. Rocha (2010), in turn, obtained a maximum CMCase value of 9.00 U.g^{-1} at 120 h of solid state fermentation and 40 °C of temperature, using the fungus *A. niger* ATCC 16404 and rice bran and passion fruit rusk in a 1:1 ratio as substrates.

Another research was carried out to evaluate the influence of supplementation of the medium with different nutrients (1% yeast extract solution (m/m_{H2O}), 1% ammonium sulfate solution (m/m_{H2O}), and Mandels and Weber solution (1969) in the production of cellulases by *Aspergillus* sp. FSDE16 using wheat bran as a substrate, under the conditions of 38 °C, 168 h, and concentration of 10^7 esp.g^{-1} of a substrate. The best result of CMCase activity was obtained for the fermentation carried out with ammonium sulfate, with a value of 6.28 U.g^{-1} in 168 h. Comparing these results with those obtained by Cavalcanti et al. (2018), it is noticed that the use of the fungus of the species *A. niger* together with sugarcane bagasse as a substrate, temperature of 30 °C, humidity of 55%, and nutrient solution formed by $(NH_4)_2SO_4$, 10 g.L^{-1}; KH_2PO_4, 3 g.L^{-1}; $MgSO_4.7H_2O$, 0.5 g.L^{-1}; $CaCl_2$, 0.5 g.L^{-1}; $FeSO_4.7H_2O$, 0.005 g.L^{-1}; $MnSO_4.H_2O$, 0.0016 g.L^{-1}; and $ZnSO_4.7H_2O$, 0.0014 g.L^{-1}, provided a peak CMCase activity of, approximately, 2.40 U.g^{-1} in 72 h. This shows that it is interesting to use ammonium sulfate as a nitrogen source for the production of cellulases since its use provided good results, and it also presents itself as a more economically viable source.

2.5.2 Production of Amylases

Starch is the second most abundant biopolymer in nature. It is present in several types of plants and is considered an important source of human and animal food. It consists of two molecules in its structure: amylose and amylopectin. Amylose is a straight-chain molecule composed of glucose units joined by α-1,4 glycosidic bonds, while amylopectin is a branched-chain molecule composed of α-1,4 glycosidic bonds between glucose monomers and α-bonds 1.6 at the branch points (Sindhu, Binod and Pandey 2017).

Amylases are a group of enzymes that hydrolyze the glycosidic bonds present in starch. They are divided into three subgroups: α-amylase, β-amylase, and glucoamylase. A-amylase (EC 3.2.1.1) is an endoenzyme capable of hydrolyzing α-1,4 glycosidic bonds in polysaccharides; however, it is not capable of hydrolyzing α-1,6 bonds (Selvam et al. 2016). B-amylase (EC 3.2.1.2) is an exoenzyme that hydrolyzes the α-1,4 bonds of the polysaccharides from the non-reducing end forming successive maltose units. Up to this point, hydrolysis is not complete; dextrins are still present because β-amylase is not able to hydrolyze α-1,6 bonds at branch points (Liu and Kokare 2017). Glucoamylase (EC 3.2.1.3) breaks the α-1,6 glycosidic bonds, in addition to catalyzing the hydrolysis of successive α-1,4 at the non-reducing ends of glucans, producing glucose, thus leading to complete hydrolysis of starch (Taniguchi and Honnda 2009).

These enzymes come from various sources such as plants, animals, bacteria, and fungi. Generally, microbial sources are the most used, most of them fungal because they have extracellular means of production and high acceptance status being considered safe (Ayansina et al. 2017).

Since the first reports of industrial bacterial amylase in the 1920s, a large number of microbial amylases from different sources have been widely studied and applied extensively in various industrial sectors, such as the food, textile, detergent, biofuel, and paper industries. Among the enzymes available on the market today, most are produced by filamentous fungi (McKelvey and Murphy 2017). Several species and strains of filamentous fungi have been studied for their potential to produce enzymes such as *Aspergillus* sp., *Trichoderma* sp., *Rhizopus*, *Paecilomyces variotii*, and *Penicillium* sp. for its ability to produce several enzymes and, among them, amylase. The production of fungal enzymes is carried out mainly by SSF and SmF (Viniegra-González et al. 2003; Mrudula and Murugammal 2011). Studies show that SSF produces better results when using filamentous fungi (Mrudula and Murugammal 2011). That is because filamentous fungi are better for growing on solid materials with low water activity (Rawat 2015; Walker and White 2017). In addition to fungal species/strains and fermentation methods, enzyme production can also be affected by culture conditions, such as the nature of the substrates, temperature, oxygen, and water activity, among others.

The production of enzymes by SSF developed by the LABIO group, in general, follows the development of the steps shown in the flowchart of Fig. 2.1, differentiating in the stages of extraction and analysis of enzymatic activity, where for each type of enzyme to be analyzed different methodologies are used. The tests are conducted so as to make the production of the enzyme of interest feasible. In this way, the chosen substrates, usually from agro-industrial residues, are rich in starch, when it comes to the production of amylases. Agribusiness residues used in LABIO's research and Distilled Yeast Products Laboratory, belonging to the Chemical Engineering Department and located in the Technology Center of the Federal University of Paraíba, such as wheat bran, malt bagasse, cassava peel, and crude were obtained from local producers and processed according to their nature. In the case of malt bagasse, residues obtained from the processing of beer and residues from cassava peel and crude, obtained from the production of cassava flour, for

having high humidity and to avoid possible contamination, they were subjected to drying in an oven at 60 °C until a constant weight was obtained; afterwards, they were crushed and stored at room temperature in an airtight container. Wheat bran, on the other hand, was obtained in a form suitable for use, being stored only in a hermetically sealed container at room temperature.

The substrates, after due processing, were characterized in order to know their physical-chemical composition and adapt it later to the fermentation process. Such determinations follow the standard methodology of the Adolf Lutz Institute (2008). After this process, the residues, which already had known humidity, were subjected to a wetting step, where the humidity adjustment was carried out in the range of 60–70% to enable the growth of the fungi used. The moistened substrates were sterilized in an autoclave at 121 °C and 1 atm in glass flasks that function as small bioreactors and were added with inoculum volume in concentrations between 10^5 and 10^7 of esp.g^{-1} of fermentation medium.

The microorganisms used in the research in question are filamentous fungi of the genera *Aspergillus* sp. FSDE16, *Penicillium* sp. FSDE15, and *Trichoderma* sp. *Trichoderma* sp. was obtained from the insulation of a cork stopper by the Industrial Microbiology Laboratory of the Federal University of Paraíba. Fermentations were carried out at times between 120 and 360 h.

The enzymatic extraction was performed using a citrate-phosphate buffer solution, pH 4.8 according to the methodology described by Israel (2005) for the process carried out using the microorganism *Trichoderma* sp. As for the studies carried out with *Penicillium* sp. FSDE15, enzymatic extraction was performed with distilled water as a solvent. The enzymatic quantification was performed later with the supernatant of the obtained crude enzymatic extract, where the methodology described with adaptations by the Manual of Protocols and Methods of Analysis in Agro-food Biotechnology and Human Health Laboratories was followed, as well as the methodology proposed by Aiyer (2004), with adaptations.

In studies with malt bagasse (90%) and crude (10%) as substrates, *Penicillium* sp. FSDE15 as the microorganism, medium humidity of 65% and total fermentation time of 168 h, it was possible to reach maximum amylase activity in 120 h, with a value of 5.30 U.gds^{-1}. For the test carried out with 20% of crude and the same previous conditions, the best result of enzymatic activity obtained was 5.01 U.gds^{-1} in 72 h of fermentation. In another study also carried out with FSDE15, the residue used as a substrate was wheat bran and humidity adjusted to 70%, and the peak enzyme production of 22 U.gds^{-1} in 120 h was obtained in this test. As for the study carried out using the microorganism *Trichoderma* sp., the malt bagasse as a substrate, 70% humidity, and 360 h fermentation time, the amylase activity was maximum in 216 h with a value of 29.77 U.gds^{-1}. In their studies, Santos et al. (2017), when evaluating the production of amylases by *Penicillium* spp. LEMI A11 in a medium containing banana stem as a substrate with 70% humidity, obtained a result of enzymatic activity of 0.24 U.gds^{-1} in 96 h of fermentation.

A study was also carried out to assess the influence of the presence of salts $(NH_4)_2(SO)_4$ and KH_2PO_4 in the humidification solution of the fermentation medium. This study was carried out with *Aspergillus* sp. FSDE16 in medium

containing malt bagasse as a substrate, humidity of 70%, and fermentation time of 120 h. For the test containing fermentation medium moistened only with distilled water, the result obtained from amylase activity was 10.01 U.gds^{-1}, while for the test with medium containing salt solution, the value obtained from amylase activity was 26.19 U.gds^{-1}. In another study carried out with FSDE16, in a medium containing wheat bran and cassava peel as substrates, humidity of 70%, and fermentation time of 120 h, the activity of amylases showed the result of 63.71 U.gds^{-1}. According to Pruckler et al. (2014), wheat bran is very rich in proteins and carbohydrates, which makes it a great source of carbon and nitrogen for several living beings.

In general, the results obtained show that the microorganisms, as well as the carbon and nitrogen sources evaluated, are promising for the production of amylases by SSF.

2.5.3 Production of Pectinases

Pectinolytic enzymes or pectinases form a heterogeneous group of enzymes that hydrolyze pectic substances (Jayani et al. 2005). They are capable of smoothing glycosidic bonds, degrading or modifying pectin, one of the abundant components of fruits. Pectin functions as a crosslinking polysaccharide on the primary cell wall and medium lamella of fruits and vegetables joining the cellulose and hemicellulose fibers; the use of pectinases improves the access of cellulases in their substrates (Rabello et al. 2017).

They are used in the fruit juice industries to reduce viscosity, to improve and increase the efficiency of filtration and clarification and preliminary treatment of grapes in wine industries;, and to improve the extraction of vegetable oils and the treatment and degumming of natural fibers for the textile and paper industry. Recently, it has been suggested that pectinases could be used to hydrolyze pectin in biorefineries in agricultural residues rich in pectin, such as citrus pulp and beet pulp (Uenojo and Pastore 2007; Garg et al. 2016; Pitol et al. 2016; Rabello et al. 2017). Pectinases have received worldwide attention as an ecological biocatalyst that comprises a 25% share of the global food and beverage enzyme market (Amin et al. 2019).

Pectinases are classified based on the mode of action on polygalacturonase (PG, EC 3.2.1.15), pectinesterase (PE, EC 3.1.1.11), pectin lyase (PL, EC 4.2.2.10), and pectate lyase (PGL, EC 4.2.2.2). These enzymes act on the structures of polygalactopyranose with activities and specificities that partially depend on the degree of methylation. Based on their cleavage specificities, pectinases can be grouped into cleaved smooth regions or branched regions of pectin. Depending on the pattern of action, that is, random or terminal, these enzymes are categorized as endo- or exoenzymes, respectively (Rabello et al. 2017; Amin et al. 2019).

They can be produced, in different combinations, by plants and by microorganisms such as fungi, yeasts, and bacteria (Silva et al. 2005; Santos 2007). Generally, pectinolytic enzymes derived from fungi are acidic in nature, while alkaline enzymes

are secreted mainly by bacterial strains. The microorganism most used in commercial preparations is filamentous fungi, particularly *A. niger*. The main justification is the fact that it is recognized as safe (GRAS), as well as the nontoxicity of its metabolites. Many other species of *Aspergillus* have also been reported in the literature for the biosynthesis of acidic pectinases. In addition, several other acidic pectinases are produced mainly by fungi and yeasts of the genus *Penicillium* sp., *Rhizomucor* sp., *Rhizopus* sp., *Trichoderma* sp., *Aureobasidium* sp., *Thermotoga* sp., *Saccharomyces* sp., *Candida* sp., *Pichia* sp., *Schizosaccharomyces* sp., and *Kluyveromyces* sp., while *Bacillus* sp., *Klebsiella* sp., *Cryptococcus* sp., and *Pseudomonas* sp., have been documented for their ability to produce alkaline pectinases. Pectinase-producing strains with unique characteristics can be isolated from natural materials (i.e., decomposition of plants, soils) as well as artificial habitats (food industry waste). Various types of pectinases produced by fungi, bacteria, and yeast cultures are available on a commercial scale (Amin et al. 2019).

Polygalacturonase is the enzyme with the main hydrolytic function. For most industrial uses, polygalacturonases produced by fungi prove to be useful due to their high activity and optimal activity in a low pH range, serving for most applications in fruit and vegetable processes (Zheng and Shetty 2000; Santos 2007).

The production techniques most frequently used for the cultivation of microorganisms on suitable substrates to produce pectinases are submerged and solid-state fermentation. Most enzyme manufacturing industries generally carry out SmF to produce enzymes. On an industrial scale, 90% of enzymes are produced using the SmF technique. However, there has been an increasing trend in the SSF technique for enzyme production (Amin et al. 2019). SSF allows the production of more concentrated crude enzymes and, consequently, with lower extraction and purification costs. Typical substrates are agro-industrial residues, such as citrus peel, beet bagasse, and wheat bran (Silva et al. 2005).

The production of pectinases by microorganisms is influenced by fermentation conditions, in particular, by the culture medium, type and concentration of the carbon source, pH, and temperature of the fermentation, in addition to other factors (Bravo et al. 2000). An important aspect of SSF is the adequate recovery of the produced metabolites. The extraction efficiency is a critical factor that determines the economic viability of SSF for enzyme production. Temperature and type of solvent are known as important parameters in the extraction of solutes from solids. Additionally, when dealing with enzymes, it is necessary to take into account the thermal stability of the enzyme, which is a function of the exposure time (Castilho et al. 2000).

Santos (2007) studied the production of pectinases through SSF, using the dry cashew stalk and the microorganism *A. niger* CCT 0916 as a substrate. It used the methodology of factorial experimental planning and surface analysis to verify the influence of moisture initial medium (40, 50, and 60%), supplementation of the medium with a nitrogen source (0; 0.5; 1.0), and phosphorus source (0; 0.3; 0.6). Ammonia sulfate was used as the nitrogen source, and monobasic potassium phosphate was used as the phosphorus source. As a fermentation medium, two residues of the cashew peduncle were used: residue without washing and washing residue.

These residues differ due to the treatment given before drying. Unwashing was obtained by drying the residue after extracting the juice, while washing was obtained by washing with water five times after extracting the juice, in the proportion of 1 kg of bagasse to 2 L of water. The physical-chemical characterization of the residues showed different compositions, mainly concerning the levels of reducing sugars and pectin. For the washed cashew stalk, the levels of reducing sugar and pectin were 0.21% and 13.41%, respectively, whereas for the non-washed cashew stalk, these values were 35.45% and 7.31%, respectively.

Fermentations were carried out in a 250 mL conical flask containing 20 g of the medium. To ensure uniformity of the sampling, the medium was prepared and then distributed to the Erlenmeyer. The vials were covered with a cotton plug wrapped with gauze and autoclaved at 0.5 atm for 5 min. In the cold environment, 10^7 esp. g^{-1}were inoculated and incubated at 30 °C. The samples were taken in regular periods during the process for the extraction of the enzyme complex and subsequent performance of activity measures. For each sampling, an Erlenmeyer was removed from the greenhouse, all being in the same initial conditions as the process. The enzyme complex was extracted by adding 2.5 $mL.g^{-1}$ of the fermented medium in 200 mM acetate buffer pH 4.5. After homogenization, the samples were left for 1 h in a water bath at 30 °C and then were filtered. The filtrate was stored in a freezer for further analysis of enzymatic activity.

The measurement of the polygalacturonase activity of the enzyme extract was based on the increase of the reducing groups formed by the action of the enzyme, according to Couri and Farias (1995). In test tubes containing 4 mL of 0.25% w/v polygalacturonic acid solution prepared in 200 mM acetate buffer, pH 4.5, previously acclimated to 35 °C, 0.25 mL of enzymatic extract was added, continuing the reaction enzymatic for 30 min at 35 °C. After the reaction, 0.25 mL of the reaction mixture was transferred to test tubes containing 1 mL of the DNS reagent; after homogenization, 0.75 mL of distilled water was added, and the procedure for analysis of the reducing groups by the DNS method was followed. The tests were done in duplicate; in the blank tests (also in duplicate), the enzyme was added to the polygalacturonic acid and immediately transferred to the tubes containing the DNS. The standard curve was obtained with a solution of galacturonic acid in the range of 0 to 1 $mg.mL^{-1}$. One unit of activity corresponds to the amount of enzyme that releases 1 μmol of galacturonic acid per min of reaction, under the reaction conditions. The results were expressed in units of activity per g of moist fermented medium ($U.g^{-1}$).

The best condition found was for cashew bagasse without washing with 40% humidity, 1% nitrogen, and without adding phosphorus source, thus reaching 15.55 $U.g^{-1}$polygalacturonase activity at 30 h of fermentation. For the washed cashew stalk residue, with a fermentation time of 22 h, under the same conditions, polygalacturonase activity of 10.1 $U.g^{-1}$was obtained.

Souza et al. (2010) used the dry residue of the passion fruit peel, and albedo (*Passiflora edulis*) with *A. niger* CCT 0916 obtained polygalacturonase activity of 20.9 Ug^{-1}, in 66 h of process, with 40% of initial moisture and 1% of the concentration of the nitrogen source. The polygalacturonase from the crude

enzymatic extract showed good thermal stability up to temperatures of 50 °C. This enzyme remained stable at pH between 3.5 and 5.5 and was not detected at pH values above 6.5. Santiago et al. (2014) using guava peel and the same microorganism obtained activity of 12.64 $U.g^{-1}$ at 30 h of fermentation with initial humidity of the culture medium of 50% and nitrogen concentration at the source of 1.0%.

Pitol et al. (2016) studied the increase in the scale of pectinase production in compacted bed bioreactors, from 12 to 30 kg of dry substrate. When the compaction occurred, bed temperatures of up to 47 °C were recorded, and the pectinase activity in different regions, from bed at 26 h, ranged from 11 to 28.103 Ug^{-1}. When compaction was avoided, the maximum bed temperature was 32 °C, and pectinase activity at 26 h varied from 17 to 20.103 $U.g^{-1}$. The best result was obtained with a bed height of 40 cm containing 27 kg of wheat bran and 3 kg of sugarcane bagasse, with a change in the temperature of the saturated inlet air between 24 and 32 °C, and the surface airspeed is kept constant at 0.1 $m.s^{-1}$.

Pectinases are important and potentially useful biocatalysts in several industrial sectors, due to their ability to catalyze reactions under environmental conditions. The studies carried out show that it is possible to obtain the pectinase enzymes using agro-industrial residues.

2.5.4 Nutritional Enrichment

The need for advancement in food production has led to an increase in the generation of agro-industrial waste, which, when improperly disposed of, can represent risks to health and the environment. However, special attention has been given to minimizing or reusing these residues as a way to remedy environmental pollution and waste (Roberto et al. 1996; Wanderley et al. 2011). In particular, residues from the food industry involve appreciable amounts of bark, fiber, bagasse, and seeds that can be used as a source of organic matter, enzymes, and essential oils to promote nutritional enrichment for animal and human food.

Several agro-industrial residues have been nutritionally enriched through biotransformation promoted by microorganisms, mainly filamentous fungi that use them as substrates for the production of different groups of enzymes and proteins (Panesar et al. 2016).

Fruit processing, for the extraction of juices and pulp, is the sector of the food industry that generates a high amount of waste that is not usable for human consumption. Fruit residues are known to be sources of compounds with high nutritional quality (Soares et al. 2020), as well as residues from breweries, malt bagasse, trub, and yeast (Saraiva et al. 2018). This characteristic makes them potential substrates for fermentation with microorganisms capable of producing biocomposites with protein content, promoting nutritional enrichment of these residues by SSF. Research has focused on the use of residues from the food industry for protein enrichment (Moreira et al. 2012; Panda et al. 2016; Santana-Neto et al. 2017; Sousa et al. 2020).

Nutritional enrichment through SSF using different agro-industrial residues was also of interest to the research group at the Bioprocess Engineering Laboratory (LEBp) and the Molecular Genetics and Plant Biotechnology Laboratory (LGMBiotec) at the Federal University of Biotechnology (CBiotec) from Paraíba (UFPB) that used alternative substrates such as mesquite fiber, wet malt residue, and acerola residues for the development of *Beauveria bassiana* and enzymatic production. The *B. bassiana* strain used was provided by the URM library of the Federal University of Pernambuco—UFPE. The residues used in the research were mesquite fiber from the semi-arid region of Rio Grande do Norte, acerola residues donated by a fruit pulp industry in the city of João Pessoa (PB), and malt residues donated by the Chemistry Laboratory Applied Organic Chemistry of CBiotec-UFPB.

Here we highlight the novelty in the use of the residues mentioned in the promotion of nutritional enrichment by *B. bassiana*, a species recognized for being an entomopathogenic fungus, with no reports in the literature on enzymatic production by SSF. In all experiments, the residues were used in natural form, with no nutrient supplementation, only moisture adjustment (70%), and distributed in 250 mL Erlenmeyer flasks (30 g each) and sterilized at 121 °C and 1 atm. The *B. bassiana* strain was maintained on the Agar-Sabouraud-Dextrose medium (pH 5.6) at 30 °C for 15 days and inoculated at a concentration of 1×10^6 spores. g^{-1} for the enzymatic production process at 30 °C for 10 fermentation days (Sturmer et al. 2004).

During the 10 days of the process, there were macroscopic changes in the substrates, such as mycelial development and growth of the fungus represented by a white powdery layer on the entire surface. After the observation of visible fungal growth, enzymatic extraction was performed using sterile distilled water in the proportion of each gram of substrate, 10 mL of solution, and filtered on qualitative paper (Whatman no. 1). The net fraction of the extraction of each of the substrates was considered as crude enzymatic extract and analyzed for pH, proteolytic enzymes (Giongo 2006), total proteins (Bradford method), and protein increase (Araújo 2004) promoted by the enzymatic production process.

At the end of the process, a pH variation (4.93–6.60) was observed in the crude enzymatic extract obtained from each residue, leading to believe that there was a production of metabolites that led to an increase in pH throughout fermentation, which may be associated with enzyme production. The proteolytic activity was analyzed to characterize the enzymatic complex produced by *B. bassiana*, since proteases are normally produced by this strain and are strictly linked to the insect infection process due to its entomopathogenic action. The proteases allow the fungus germ tube to penetrate the insect's cuticle during its bioinsecticidal activity (Hepburn 1985). Thus, the analyzed residues (mesquite fiber, wet malt residue, and acerola residues) showed proteolytic activity, with mesquite fiber being the highest activity (0.514 ± 0.009 U.mL^{-1}) when compared to other residues. A study by Ito et al. (2007) on extracellular proteases by *B. bassiana* reached an activity of 0.489 U. mL^{-1}, a result similar to that obtained in this research, even using substrates in natura and without previous treatment, therefore, a very satisfactory result.

Regarding the amount of total soluble protein observed in the residues, an increase in protein content (AP) was found during the proposed enzymatic production. All residues analyzed showed a significant increase in the number of total proteins. The mesquite fiber in natura had a protein concentration of 0.107 ± 0.33 mg.mL^{-1} and, at the end of the process, reached the amount of 10.821 ± 0.13 mg.mL^{-1}, that is, a protein increase of 100 times; for malt residues, the increase was more significant, reaching a value of 335.15 times (38.32 ± 0.06 mg.mL^{-1}) when compared to its initial value (0.114 ± 0.31 mg.mL^{-1}), and finally, for the acerola residues, it was 240.16 times; the amount of protein in natura was 0.110 ± 0.33 mg.mL^{-1} and after protein enrichment 29.57 ± 0.25 mg.mL^{-1}. Thus, it was widely shown that the metabolism of these residues by *B. bassiana*, under the conditions studied, promoted intense production of enzymes and, as a consequence, a considerable increase in proteins in the residues. The potential for nutritional enrichment was evidenced in the results obtained by the action of the *B. bassiana* strain and in the process conditions, adding greater nutritional value to agro-industrial waste when compared to the same in natura that are normally wasted (Raimbault 1998; Pandey 2003). Several studies have shown protein enrichment from bioprocesses using bacteria, yeasts, and filamentous fungi, but there are no reports in the literature on this nutritional enrichment promoted by an entomopathogenic fungus of the species *B. bassiana*.

The protein increase shown in the results gives the potential application of these residues in animal feed, as a way to improve the digestibility and nutritional quality of ruminants and birds, for example. Therefore, agro-industrial residues present in their composition sufficient nutrients for the development of microorganisms in order to add value to the product generated. It is then suggested that there is a real possibility of reducing treatment costs before disposal and a more noble application of waste.

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Chapter 3
Microemulsified Systems and Their Environmental Advantages for the Oil Industry

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Abstract Microemulsified systems are being used more frequently in several industrial processes, such as in the oil industry. Many microemulsified systems present formulations based on natural components, which turn them less harmful to the environment, with biodegradability and value for money being considered critical factors. Several phases can exist in equilibrium in microemulsified systems; however, the main objective is to identify compositions that form a single phase when mixed. Surfactants have as their main characteristic the double polarity: a hydrophilic region with an affinity for the aqueous phase and a hydrophobic region with an affinity for the organic phase. Depending on the surfactant characteristics, the microemulsions formed can be oil-in-water (O/W) or water-in-oil (W/O). The oil and natural gas industry search for environmentally-friendly products; thereby, preflush fluids and drilling fluids are developed based on biodegradable components to reduce environmental impacts, e.g., vegetable oils in the formulation of these fluids have shown incredible significance. Drilling fluids play an important role because they offer a favorable environment for effective and efficient drilling, thus improving well productivity. Preflush fluids are cleaning fluids used during the cementing operations for removing the filter cake formed in the wellbore walls by the drilling fluid and inverting the rock formation wettability. The correct fluid choice is essential in the drilling and cementing operations because it directly influences the operation's quality and costs.

Keywords Surfactant · Microemulsion · Vegetable oil · Drilling fluid · Preflush fluid

3.1 Introduction

Microemulsified systems are being used more frequently in several industrial processes, such as in the oil industry (Santanna et al. 2009; Quintero et al. 2011; Bera and Mandal 2015; Santos et al. 2017; Shafiee Najafi et al. 2017; Curbelo et al. 2018; Ferreira et al. 2018; Carvalho et al. 2019; Hernandez et al. 2019; Pal et al. 2019; Zhou et al. 2020), food industry (Zhong et al. 2009; Ma and Zhong 2015; Abbasi and Radi 2016; Uchiyama et al. 2019; Mendonça et al. 2020), and cosmetics and pharmaceutical industry (Huang et al. 2003; Naoui et al. 2011; Debbih et al. 2017; Solans and García-Celma 2017; Volpe et al. 2018; Das et al. 2020), due to their solubility in polar and nonpolar substances, thermodynamic stability, ability to reduce interfacial tension, and others.

Microemulsions, also known as Winsor IV systems (Winsor 1948), have the advantage of being able to solubilize components soluble in water and oil, under certain conditions of temperature and pressure. Microemulsions differ from emulsions not only in terms of their physical appearance, which is homogeneous from a macroscopic point of view, but also because of their thermodynamically stable and easy-to-prepare characteristics, since they are formed spontaneously through the solubilization of both polar and non-polar compounds by the action of tensor molecules in the form of micelles, when little or no energy is supplied to the system (Schulman et al. 1959; Danielsson and Lindman 1981; Fanun 2008; Zhong et al. 2009; Goshen and Magdassi 2012; Ma and Zhong 2015).

To promote the microemulsion formation, it is necessary that the surfactants are in high concentrations, generally above the critical micellar concentration (CMC). In this case, the higher the concentration of surfactants, the greater the number of micelles present, and the amount of surfactants in the form of monomers is practically constant (Binks 1993; Rosen and Kunjappu 2012). Thus, the more the surfactants present, the greater the solubilization of one component in the other, which consequently forms a single phase (Holmberg et al. 2002; Daltin 2011; Burguera and Burguera 2012; Bera and Mandal 2015).

On the other hand, emulsions are formed when two immiscible liquids are brought into contact with or without surfactants. However, unlike microemulsions, surfactants are in low concentrations, commonly below the CMC (Bus et al. 1990; Miqan et al. 2013; Alizadeh and Suleymani 2020). In this case, only the dispersion from one phase to the other occurs, due to the mechanical energy that is supplied to the system. As the amount of surfactants is low, no micelle is formed, and after a certain time, the two distinct phases will become separate by coalescence. Although they are kinetically stable, the emulsions are thermodynamically unstable (Israelachvili 1994; Fanun 2008; Daltin 2011; Ali et al. 2015; Rocha e Silva et al. 2017; Awad et al. 2018).

Micellar aggregates are structural organizations of surfactant molecules that can take different forms and directly depend on the molecular characteristic and concentration at which the surfactant is in the solution. Therefore, the CMC is considered the most important physical-chemical characteristic of a surfactant, and knowledge of it is important to target its industrial application more effectively (Winsor 1968; Holmberg et al. 2002; Daltin 2011; Bera and Mandal 2015; Alizadeh and Suleymani 2020).

Many microemulsified systems are being formulated based on natural components in order to be less harmful to the environment, with factors such as biodegradability and low cost being considered the most important. Among the natural components, plant-based oils are receiving greater prominence and are increasingly being used to replace those that cause damage both to the environment and human health (Zhong et al. 2009; Schwarz et al. 2012; Araújo et al. 2018; Cortés et al. 2018; Curbelo et al. 2018; Amiri-Rigi and Abbasi 2019; Asgari et al. 2019; Curbelo et al. 2019; Garnica et al. 2020; Mitsou et al. 2020; Monton et al. 2020).

3.2 Microemulsions

In microemulsified systems, several phases can exist in equilibrium; however, the main objective is to identify the aqueous phase, oil phase, surfactant, and sometimes cosurfactant (usually short-chain alcohols) compositions that form a single phase when mixed.

Surfactants, also known as amphiphilic compounds, have as their main characteristic the double polarity, that is, a hydrophilic (polar) region with affinity for the aqueous phase and a hydrophobic (apolar) region with affinity for the organic phase (Winsor 1968; Holmberg et al. 2002; Daltin 2011; Rosen and Kunjappu 2012).

Depending on the surfactant characteristics, the microemulsions formed can be oil-in-water (O/W), when drops of oil are dispersed in the aqueous phase, or water-in-oil, when water droplets are dispersed in the oil phase (W/O) (Schulman et al. 1959; Daltin 2011; Burguera and Burguera 2012; Bera and Mandal 2015).

3.2.1 From Phase Diagrams to Microemulsion

In order to obtain the microemulsions, several experiments are carried out to verify the behavior of the present phases when 3 or 4 components are mixed, depending on the mass fraction of these components in the system, and to identify them through ternary phase diagrams (3 components) or pseudo ternary phase diagrams (4 components) (Acharya and Hartley 2012; Moghimipour et al. 2012; Ma and Zhong 2015; Mitsou et al. 2019; Zhao et al. 2020).

The construction of the phase diagram (Fig. 3.1) is usually done by plotting the mass fractions of 3 or 4 components (aqueous phase, oil phase, surfactant, and, in some cases, cosurfactant), which correspond to a point in the region within the diagram.

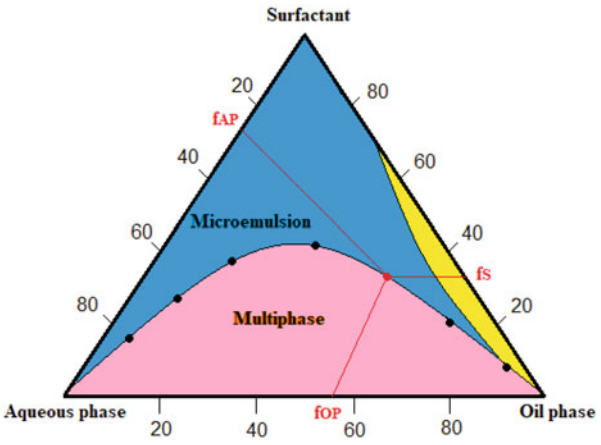

Fig. 3.1 Ternary phase diagram

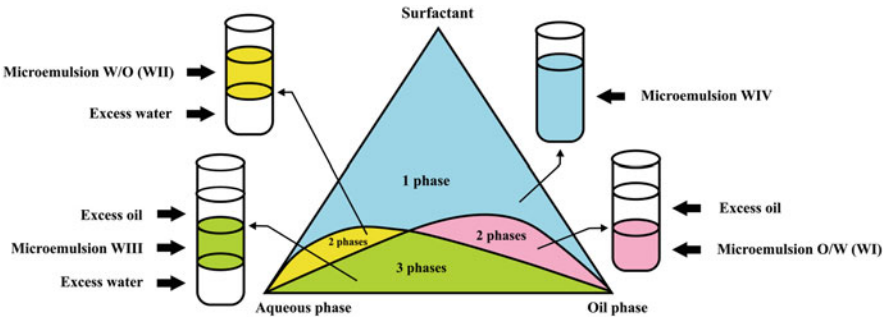

Fig. 3.2 Ternary phase diagram with Winsor regions

For a ternary phase diagram, the mass fractions f_S, f_{AP}, and f_{OP} of each point in the diagram (Fig. 3.1) are calculated by mass balance from the quantities of each component obtained experimentally through the titration methodology. Initially, the mass ratio of two components is fixed, and a titration with the third component starts until the transition from turbidity to transparency, and with this, a monophasic ternary mixture (blue region) appears, meaning that the microemulsion region (Winsor IV or WIV) was achieved.

The region below the solubility curve (pink region) corresponds to a mixture of three components with two or three phases. A mixture with two phases in this region can be an oil-in-water (O/W) microemulsion in equilibrium with the excess oil phase (Winsor I or WI), or a water-in-oil (W/O) microemulsion in equilibrium with the excess aqueous phase (Winsor II or WII). The presence of 3 phases corresponds to a microemulsion in equilibrium with the excess aqueous and oil phases (Winsor III or WIII). The yellow region, shown in the figure for information purposes only, corresponds to a partial miscibility between the surfactant and the oil phase, and the mass ratio between the three components has not yet been sufficient for the microemulsion formation. Figure 3.2 shows a typical phase diagram with the presence of the Winsor regions.

3.3 Petroleum Industry Applications

In the search for a sustainable and economic perspective for the oil and natural gas industry, it is necessary to mitigate the effects on the environment by reducing the levels of toxicity of the components that are used, mainly in the upstream stage. To reduce environmental impacts, efforts have been made to use and/or develop biodegradable products. In the following, specific characteristics of materials used in the drilling of the well (drilling fluid) and cementation (preflush fluid) stages are presented.

3.3.1 *Preflush Fluid*

Preflush fluids are cleaning fluids used during the cementing operations, with the purpose of removing the filter cake formed in the well walls by the drilling fluid and, when necessary, inverting the rock formation wettability. This fluid is responsible for mitigating risks of formation damage (Brege et al. 2012; Lucas et al. 2019), improving the adhesion of cement in the casing-formation system, and providing less interventions in operations and better well productivity.

One of the main challenges of cementing oil wells is the incompatibility of drilling fluid with cement slurry. Due to its physical-chemical characteristics, the contact between the fluid and the cement slurry can provide a significant increase in viscosity, causing an inefficient removal of the drilling fluid and, consequently, a change in the properties of the slurry (Bishop et al. 2008; Li et al. 2016, 2018). Therefore, in order to minimize these problems, intermediate fluids called preflush fluids and spacers are used (Harder et al. 1993).

The formation's wettability is considered an important factor for cementing as it affects the relative oil-water permeability, the movement of fluids, the effective cleaning, and the mobilization of solids (Brege et al. 2012). Wettability is defined as the preference for a fluid to spread or adhere to a solid surface rather than another, which may be water-wetting or oil-wetting, considering the balance of surface and interfacial forces (Anderson 1986; Abdallah et al. 2007). However, inadequate considerations regarding wettability can cause damages to the formation (Alotaibi et al. 2010).

The surface tension determines the behavior of the spherical drops on a surface. When the surface tension is lower, the liquid spreads easily over the surface, acquiring a shape called bead. This shape presents a certain contact angle with the solid surface (Daltin 2011). Therefore, contact angle measurements are used to assess the ability of a preflush fluid to change the surface wettability from oil-wetting to water-wetting (Brege et al. 2012; Wanderley Neto et al. 2019).

Some basic criteria must be considered in the preflush fluid choice and applicability such as the following:

- Present compatibility with the fluids used in drilling and completion
- Non-interfere with cement slurry viscosity and pumping time
- Non-interfere with the mechanical properties of the cement slurry, within the established limits
- Presentation of low fluid loss to the formation
- Allowance of turbulent regimes at low pumping rates, thus facilitating the drilling fluid removal

Numerous applications of microemulsions are reported in the literature, demonstrating the efficiency of these fluids. Some examples are shown below.

Quintero et al. (2015) found that microemulsions, in the presence of suitable surfactants, provide the preflush fluids with the ability to withstand variations in temperature, brine salinity, contamination of drilling fluids, and crude oils, without

decreasing the well cleaning capacity. In addition, the presence of the surfactant in the microemulsion reduces the interfacial tension between the preflush fluid and the non-aqueous fluid and then solubilizing the oil and reversing the surface wettability. Hence, these properties provide the filter cake removal with efficiency and minimal energy and at the same time improve the condition of the reservoir's rock surface.

Quintero et al. (2015) developed a preflush fluid based on a microemulsified system to promote the drilling fluid removal in deep-water wells consequently guaranteeing their cleanliness. The laboratory test results before the field application showed that the cleaner fluid could solubilize the drilling fluids, reversing the rock wettability from oil-wetting to water-wetting and having ultra-low interfacial tension. Based on the key performance indicators, such as brine completion turbidity, volume used, and cleaning efficiency, the displacement and removal of drilling fluids using the cleaner fluid showed excellent performance during circulation in the well, especially in deep-water wells. In addition, they showed that microemulsions can be applied over a wide range of temperature and brine density.

Pernites et al. (2015) developed a spacer capable of removing aqueous and non-aqueous drilling fluids and allowing inversion of the rock's wettability, ideal for cement adhesion. The rheological study of the fluid was determined at different densities (10–16 ppg) and test temperatures (68 °F, 125 °F, 190 °F) to demonstrate the factors that influence the effective fluid flow in the annular space that surrounds the well. It was observed that the density of the fluid increased with the addition of barite, showing that the fluid does not require an additional viscosifier. The compatibility study showed positive results with the water-based fluid and with the cement slurry with no phase separation, gelation, coagulation, or hardening, even after 2 h of testing.

Curbelo et al. (2018) developed preflush fluids based on microemulsified systems using vegetable oil, glycerol solution, and two non-ionic surfactants (T80 and NP150). Both fluids showed excellent drilling fluid removal performance, wettability inversion, and thermal stability at high temperatures. Furthermore, they proved to be highly compatible with the olefin-based drilling fluid and/or cement slurry, and when contaminated with the slurry, they showed satisfactory compressive strength. Therefore, the study showed that the characterization and preparation of microemulsified systems based on vegetable oil for application in well cleaning provide a clean technology, strongly aligned with the current environmental challenges faced in the oil industry.

Another study developed by Curbelo et al. (2019) was the development of microemulsified systems based on vegetable oil to remove the filter cake formed by the non-aqueous drilling fluid. However, they used a 2% wt KCl brine and a nonionic surfactant NP40. The results showed that the preflush fluids were able to remove over 75% of the non-aqueous drilling fluid.

Wanderley Neto et al. (2019) proposed the application of an oil-in-water (O/W) microemulsion as a preflush fluid. They evaluated different compositions in the Winsor IV region, with a maximum surfactant fraction above 40 wt % and concluded that all systems had an efficient fluid removal. However, to evaluate the performance of the microemulsion (O/W), they chose a point with 86 wt % of the aqueous phase

and 2.4 wt % of the oil phase for inversion, compatibility, and compressive strength tests. The results showed 100% of efficiency in the fluid removal as well as compatibility and resistance suitable for operation. Moreover, through the contact angle, they verified that the wettability was improved only after 24 h of the treatment.

Da Silva et al. (2020) formulated new microemulsion systems containing produced water to remove filter cake from the olefin-based drilling fluid. The produced water was synthesized under the condition of 1497 ppm (KCl—Synth), 1506 ppm ($MgCl_2$—Scientific Exodus), 4229 ppm (NaCl—Synth) and 4875 ppm ($CaCl_2$—Scientific Exodus) mixed with 400 ppm of oil. Three microemulsified systems were evaluated using aviation kerosene as an oil phase (OP), produced water as an aqueous phase (AP) and non-ionic surfactants (S) (NSL60, NSL90 and NSL30), and with 1-butanol as a cosurfactant (C). The mass ratio of the cosurfactant/surfactant used was equal to 0.5. In view of comparing the efficiency of the microemulsion system with different surfactants, a point in the same mass composition (67.2% AP, 1% OP and 31.8% C/S) was chosen for each surfactant. There was a 100% filter cake removal for microemulsions with NSL60 and NSL90 surfactants and good thermal stability for using as a cleaning fluid.

3.3.2 Characterization of Preflush Fluids

The analyses commonly performed to characterize preflush fluids are removal test, wettability inversion test, compatibility with cement slurry, and resistance to compression. In addition to these, for microemulsion-based preflush fluids, thermogravimetric and thermal stability analyses are also recommended (Curbelo et al. 2019).

3.3.2.1 Removal Test

This test's main objective is to verify the drilling fluid removal from the borehole walls by the preflush fluid. Experimentally, following the recommendation of the M12 test (determination of the preflush fluid efficiency for cementing oil wells) from Procelab (Campos 2014), 200 mL of the microemulsion (preflush fluid) is heated up to 190.4 °F under agitation, thus simulating the well temperature. At the same time, the non-aqueous drilling fluid is homogenized in a Hamilton Beach mixer for 15 min.

To start the test, about 4 mL of the drilling fluid is distributed in a beaker until it fully covers the viewing window (Fig. 3.3 a), forming a uniform film of drilling fluid considered to be the filter cake (Fig. 3.3 b). Then, on the opposite side of the viewing window, the microemulsion is carefully poured (Fig. 3.3 c) to keep the film intact. The removal is performed in a Fann 35 A viscometer (Fig. 3.3 d) for up to 10 min,

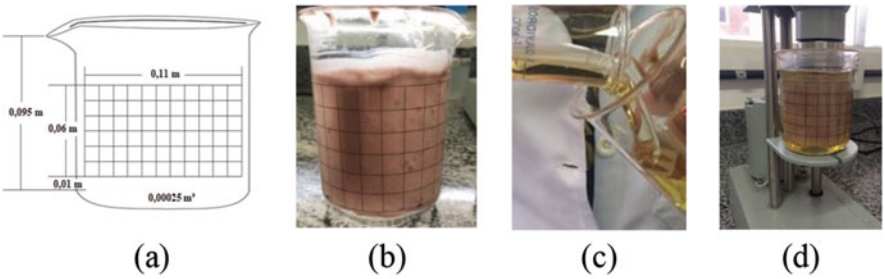

Fig. 3.3 Removal test: viewing window (**a**), filter cake drilling fluid (**b**), microemulsion used for the test (**c**), and Fann 35 A viscometer (**d**)

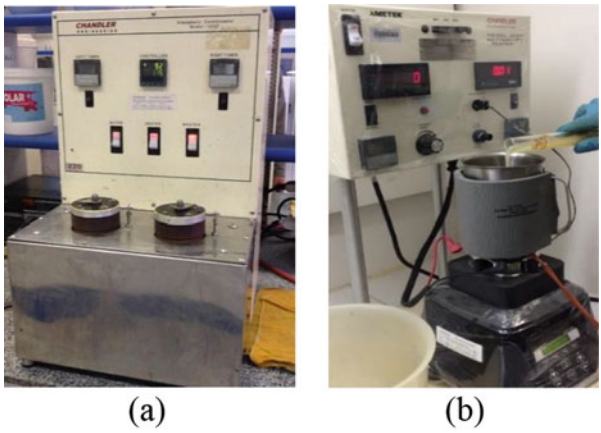

Fig. 3.4 (**a**) Atmospheric consistometer, (**b**) wettability test apparatus

being completed beforehand if all drilling fluid has been removed from the viewing window.

The removal percentage is calculated by the clean area in relation to the total area of the viewing window, thereby obtaining the preflush removal efficiency Eq. (3.1).

$$\text{Removal Efficiency } (\%) = \frac{\text{Removal Area}}{66\ \text{cm}^2} \times 100 \tag{3.1}$$

3.3.2.2 Wettability Inversion Test

The preflush and drilling fluids are separately preconditioned in an atmospheric consistometer (Fig. 3.4 a) until reaching the temperature of 190.4 °F. Subsequently, using the wettability test (Fig. 3.4 b), the inversion is measured through the electrical conductivity provided by the titration of the drilling fluid with the preflush fluid. The

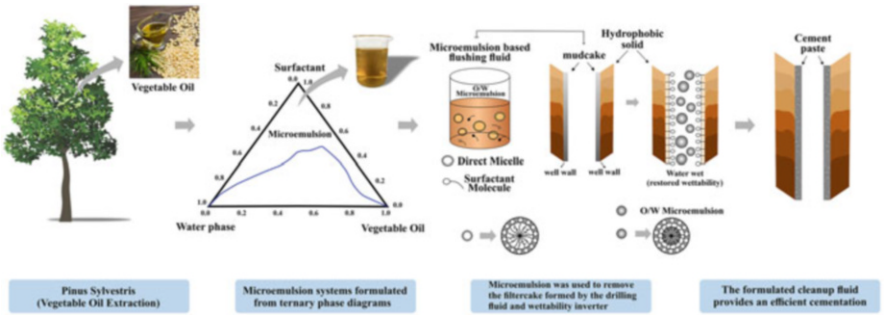

Fig. 3.5 Scheme for removing the filter cake and reversing the wettability of the wellbore walls

titration is carried out until the electrical conductivity meter has a stable value greater than 1 (initial calibration value).

Figure 3.5 shows the scheme for removing the filter cake and wettability inversion in the wellbore walls by microemulsion.

3.3.2.3 Compatibility Test

This test is performed to check the compatibility between the fluids that are used in the cementing operations. Compatibility occurs when mixtures of drilling fluid/preflush fluid/cement slurries, in different proportions, do not show chemical reactions or undesirable physical changes.

Compatibility tests are performed between the drilling fluid/preflush fluid, cement slurry/preflush fluid, and drilling fluid/preflush fluid/cement slurry, following the API RP 10B-2 (2013) testing recommendations.

The ratios (v/v) recommended by API are 25/50/25, for the mud/cleanup fluid/cement mixture, and 75/25, 50/50, 25/75, and 5/95, for the cleanup fluid to cement. The mixtures are taken to the Fann 35 viscometer where the deflection angle readings are performed at rotation speeds of 300, 200, 100, 60, 30, 20, 10, 6, and 3 rpm.

The incompatibility between drilling fluid/cleanup fluid/cement slurry is determined when large differences in the rheology readings of the mixture are observed, which stands for gelation, sedimentation, or fluid destabilization.

3.3.2.4 Compressive Strength

The compressive strength test aims to estimate the resistance that the hardened cement slurry will exhibit in the oil well. It is a nondestructive method for determining the relative strength development of a cement sample under conditions of temperature and pressure and performed by the Ultrasonic Cement Analyzer (UCA), Model 4262, Chandler Engineering (Fig. 3.6).

This test is performed with cement slurry (CS) contaminated with drilling fluid (DF), in a volumetric proportion of 90 (CS)/10 (DF), according to section 13.5 of

Fig. 3.6 Ultrasonic cement analyzer

Fig. 3.7 Morphology of a STD slurry (**a**) and the cement contaminated by compatible microemulsion (**b**) and incompatible with microemulsion (**c**)

API RP 10B-2, 2013. Continuous monitoring of the compressive strength development, usually over a 24-hour period, is carried out. Compressive strength versus time graphs are obtained and evaluated, in which the resistances of the contaminated cement slurries are expected to be lower, but close to that of the pure slurry.

Strength indications are determined by measuring the change in speed of an ultrasonic signal transmitted through the cement sample as it cures. The characteristics of the binder phase, which is the hardening of the cement paste, provide indications of the time that a paste has consistency and allows it to be moved under usage conditions, which are evaluated by the initial and final setting time, defining respectively the handling limit and the beginning of the mechanical resistance development.

Figure 3.7 exemplifies a standard cement plug without contamination (Fig. 3.7 a), compatible with microemulsified preflush fluid contamination (Fig. 3.7 b), and incompatible with microemulsified preflush fluid contamination (Fig. 3.7 c).

3.3.3 Drilling Fluid

Drilling fluids play an important role in the search for oil and gas. They offer advantageous environments for conducting an effective and efficient drilling, thus improving well productivity. They need to be formulated in order to ensure that problems, such as lost circulation, inefficiency in cleaning the well, potential to harm the environment, and others, can be avoided.

The performance of a drilling fluid can be verified by evaluating key parameters of the operation, costs related to the process, and, mainly, its environmental impact. The reduction of environmental effects caused by certain drilling fluids has a direct relationship with the chosen additives and with the base on which the fluid is formulated.

Oil-based drilling fluids have an efficient performance, mainly in the inhibition of swelling of hydratable formations; however, they do not present correct environmental adequacy, being quite toxic to the environment (Addy et al. 1984; Daan et al. 1992). On the other hand, water-based drilling fluids are characterized by being environmentally acceptable, but when encountering shales, they will cause a large cation exchange with the clay minerals present in the microstructure of this formation, which characterizes the swelling phenomenon.

The use of microemulsified systems is a good alternative for obtaining drilling fluids and has been studied by some researchers, such as Hayes et al. (1977), Davies et al. (1997), Quintero et al. (2009), Numkam and Akbari (2019), and Okoro et al. (2019), in which they presented stability advantages over a wide range of saline conditions, corrosion resistance, good lubricity characteristics, gel strength, less permeable and thin filter cake formation, low interfacial tension, and high detergency. These are important characteristics for the performance of the drilling fluid functions under different operating conditions, in order to guarantee an efficient and safe drilling process. Depending on the type of microemulsion obtained, it is possible to formulate synthetic oil-based drilling fluids (microemulsion W/O) and water-based fluids (microemulsion O/W) (Hayes et al. 1977; Davies et al. 1997; Agarwal et al. 2013; Jha et al. 2015; Dias et al. 2015; Garnica et al. 2018; Sousa et al. 2020; Garnica et al. 2020; Leal et al. 2020).

3.3.4 Characterization of Drilling Fluids

An appropriate fluid for drilling operations must have some specific characteristics, such as being chemically stable and the ability to stabilize the well walls, keep solids in suspension when at rest, facilitate the separation of drill cuttings at surface conditions, be inert in relation to the producing rocks, accept any physical and chemical treatment, be pumpable, present a low degree of corrosion, facilitate the geological interpretations of the perforated rock, and present compatible cost with the operation (Thomas 2001).

The drilling fluids must have the following functions: the ability to clean the bottom well of the generated drill cuttings and transport them to the surface; exert hydrostatic pressure on the formations, in order to avoid the influx of undesirable fluids (kick) and stabilize the well walls; and cool and lubricate the drill string and drill bit.

The main physical and chemical characteristics of the drilling fluids to be studied are specific mass, rheological parameters, filtration tests to control fluid loss, aging test, solids content, pH, and lubricity (Caenn and Chillingar 1996; Ratkievicius et al. 2016; Santos et al. 2018; Stephanou 2018).

These properties are important, since the rheological properties studies can assist in the calculation of pressure losses in the pipeline and in determining the transport speed of the cuttings and the gel forces for indicating the degree of gelation of a drilling fluid and providing information on the resistance of a fluid to restart its flow after a period of rest from drilling activities.

In addition, pH is able to provide information about the fluid's ability to promote equipment corrosion, the solids content that in high quantities influences other properties such as specific mass, gel forces, and viscosity, and can even increase the likelihood of equipment wear and fracture of rock formations.

3.3.4.1 Rheological Parameters.

The rheological parameters are determined on the Fann 35 A viscometer (Fig. 3.8), which are apparent viscosity (AV), plastic viscosity (PV), initial gel (IG), final gel (FG), gel strength, and yield point (YP).

Table 3.1 presents how the rheological properties of the drilling fluid are determined.

Fig. 3.8 Fann 35A rotational viscometer

Table 3.1 Rheological properties obtained in a Fann 35-A viscometer

Property	Description	API units	Conversion to SI units
Apparent viscosity (AV)	$L_{600}/2$	cP	1 cP = 1 mPa s
Plastic viscosity (PV)	L_{600}–L_{300}	cP	
Initial gel (IG)	L_3 after 10 s	Lbf/100 ft^2	1 Lbf/100 ft^2 = 0.48 pa
Final gel (FG)	L_3 after 10 min	Lbf/100 ft^2	
Gel strength	IG-FG	Lbf/100 ft^2	
Yield point (YP)	L_{300} – PV	Lbf/100 ft^2	

L_x = reading at x rpm

Fig. 3.9 Fann series 300 Filter Press (**a**) Fann HPHT Filter Press (**b**)

3.3.4.2 Filtration Test

The static filtration test can be performed both on API Fann 300 series filtration cells, using Fann filter paper (Part. Number. N88000100) as the filter medium, with an area exposed to 2.3 × 10^{-3} m^2 filtration (Fig. 3.9 a), under 100 psi, at room temperature, and for 30 min, and in HPHT (high pressure high temperature) filtration cells in the Fann Press Filter (Fig. 3.9 b).

3.3.4.3 Dynamic-Aging Test

The dynamic-aging test is carried out in a Fann Roller Oven (Fig. 3.10), in a special cell, usually at 150.8 °F for 16 h, according to API 13B (2012). Subsequently, the rheological test is redone to check if the fluid has lost its rheological properties with the effect of time, agitation, and temperature increase.

Fig. 3.10 Fann roller oven

Fig. 3.11 Oil and water retort kit (10 mL)

3.3.4.4 Solids Content

The solids content is determined in an oil and water retort kit (Fig. 3.11). The equipment consists of a fluid chamber with capacity of 10 mL or 50 mL, an upper boiling chamber, a distillation tube, and a condenser. The distillation takes place in approximately 35 min and reaches a temperature of 932 °F, with which the distillate is collected in a graduated collector.

3.3.4.5 Lubricity

The lubricity coefficient is obtained in a lubricity tester equipment (Fig. 3.12). Initially, the fluid is stirred for 5 min on the Hamilton Beach shaker at a speed of 17,000 rpm. Subsequently, it is transferred to the equipment container, with an initial zero torque and a speed of 60 rpm. A force of 150 in/lb is slowly applied over 5 min,

Fig. 3.12 Lubricity tester (Ofite)

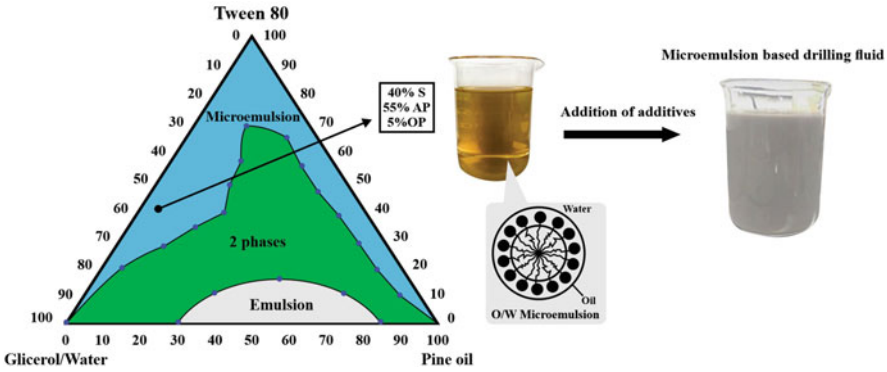

Fig. 3.13 Microemulsified drilling fluid

reading the torque exerted by the fluid, and then the lubricity coefficient is calculated (Amorim et al. 2011).

Figure 3.13 shows a water-based microemulsified drilling fluid. The microemulsified system consisted of the surfactant Tween 80 (S), pine oil (OP), and glycerin and water (AP) in the mass ratio 1:1.

3.4 Conclusion

Based on the latest research, it is possible to note the concern in the development of drilling fluids as well as preflush fluids based on biodegradable components, in order to cause less environmental impacts. The use of vegetable oils in the formulation of these fluids is incredibly significant. The cost of drilling oil wells depends not only on the composition and properties of these fluids but also on the efficiency of their operation throughout the operation. The correct choice of fluid to act in the drilling

process as well as the cleaning fluid to remove the formed filter cake and invert the rock formation's wettability is of extreme importance in the cementing operation, since these factors provide a good adhesion of the cement slurries to the rock formation, directly influencing the operation's quality and costs.

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Chapter 4
Microbial Exopolysaccharides as Biosurfactants in Environmental and Industrial Applications

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Abstract Microbial exopolysaccharides (EPS), a carbohydrate biopolymer containing several distinct monosaccharides and non-carbohydrate substituents (molecular mass from 10 to 1000 kDa), constitute mainly sugar residues. The EPS is both homopolymer and heteropolymer supported their shape and size depend on degree of polymerization, derived from various microorganisms. Biosurfactants can be distinctive as the surface-bioactive biomolecules produced by microorganisms with broad array of applications. It has the viscoelastic quality maintained in the temperature range of 5–60 °C. Up to date, microbial EPS (biosurfactants) have found many applications including environmental remediation, pharmaceuticals, cosmetics, and food industry. First and foremost, the biosurfactants achieved a prospective interest for environmental applications of inorganic and organic contaminants, mainly in hydrocarbons, heavy metal removal from water and soil, enhanced oil recovery, and pharmaceutical products. The modified EPS act as suitable agent for biodegradation of textile dyes. It has potential and promising application in textiles, paper and pulp, coal, ceramic processing, health care and cosmetics, food industries, detergents, pesticide and herbicide formulation, uranium ore-processing and mechanical dewatering of peat, etc. The EPS and their derivatives which include alginate, dextran, gellan, pullulan, and xanthan are nontoxic, biodegradable drug carriers which help in drug delivery system. These molecules also exhibit immunomodulator, antibacterial, antioxidant, antibiofilm, antiviral, and antitumor activity and expand to scaffold engineering. The distinctive rheological properties of those molecules are helpful for jelly formation in food industry. It also acts as crystallization inhibitor which helps in ice-cream manufacturing, and their stable emulsion property maintains well-built emulsification indexes with soya bean oil and hydrocarbons. Thus, the biosurfactants act as multifunctional biomolecules with wide applications in various fields.

Keywords Microbial EPS · Biosurfactants · Bioremediation · Agriculture · Pharmaceutical · Cosmetics and food industries · Promising applications

4.1 Introduction

Biosurfactants can be distinctive as the surface-bioactive biomolecules produced by microorganisms with broad array of applications. Biosurfactants are the amphiphilic compounds which are produced from the living surfaces like microbial cell surface, and sometimes biosurfactants are extracted from the extracellular hydrophobic and hydrophilic moieties which confer the ability to accumulate between the fluid phases thus reducing surface and interfacial tension at the surface and interface, respectively (Cunha et al. 2004). Surfactants are found in soaps and detergents as the active ingredients have the ability to concentrate at the air-water interface and mainly to separate oily materials from a particular media because of the fact that they have the ability to increase aqueous solubility of non-aqueous phase liquids (NAPLS) by reducing the interfacial tension at air-water and water-oil interfaces (Yin et al. 2009). Biosurfactants are structurally diverse group of surface-active substance which is produced by microorganisms. All the biosurfactants are amphiphiles which consist of two parts: a polar (hydrophilic) and a nonpolar (hydrophobic). A hydrophilic group consists of mono- and oligopeptides. A hydrophobic group consists of saturated, unsaturated, and hydrophilic-lipophilic balance (HLB) that specifies portion of hydrophilic and hydrophobic constituents in surface-active substances (Lang 2002). In view of the fact that of the amphiphilic structure in the biosurfactants, it increases the surface area of hydrophobic water-insoluble substances and increases the water bioavailability of such substances which change the properties of bacterial cell surface. Surface activity in the surfactants makes it as excellent emulsifiers, foaming and dispersing agents (Desai and Banat 1997).

Due to the potential advantage of biosurfactants that are most commonly used in many industries like agriculture, food production, chemistry, cosmetics, and pharmaceutics industries (Muthusamy et al. 2008). The biosurfactants comprise of emulsification, de-emulsification, dispersion, foaming, wetting, and coating characteristics which allow them to be productive in biological remediation technologies for organic and metal contaminants. It increases the bioavailability of hydrocarbon which results in the enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria which are present in the polluted soil (Banat et al. 2010). The heavy-metal polluted soil biosurfactants form the composites with metal in soil interface and followed by desorption of metal and removal from soil interface which guides to enhance of metal ion concentration and the bioavailability in the soil solution (Soberón-Chávez and Maier 2011). The recently produced surfactants are derived chemically from petroleum. The synthetic tension active agents are generally toxic and are difficult to break down by the action of microorganisms. In the current years, this problem made the scientific community to seek the surfactants which are more environmentally friendly like as those achieved through microbial production which are known as biosurfactants (Vijayakumar and Saravanan 2015). The concerns regarding the environment on the part of consumers and environmental control legislation led to the development of natural surfactants as alternative for the existing products (Silva et al. 2014a, b). Biosurfactants attracted many industries by their

advantages like structural diversity, low toxicity, greater biodegradability, and ability to function in wide ranges of pH, temperature, salinity, greater selectivity, and lower CMC, and the production involves renewable sources which are industrial wastes and industrial by-products (Cerqueira et al. 2011).

Surfactants are the part of most versatile group potentially used in various industries. In recent times, commercial surfactants are synthesized from petrochemicals, animal fats, plants, and microorganisms, but from the studies, it shows that the majority of production markets are relied on petrochemicals. The development of recent strategies to replace petroleum, coal, and natural gas-based products with the renewable, biodegradable, and sustainable green energies became the challenges for human and environmental protection agencies. In general, petroleum is being considered as a unique source of energy since they are used in thousands of daily consumed products which are sourced from petroleum (Rosa et al. 2015; Pacwa-Plociniczak et al. 2011; Makkar et al. 2011). It is claimed that the increase in the production of petroleum gives two phenomena: first, it increases the level of environmental pollution that affects public health and, second, it declines petroleum production that impacts the economy level of oil-producing countries. From the two phenomena, the demand for the green alternative products will lead to the increase in canter for the potential needs of industrial and domestic applications. The studies show that the natural surfactants are employed to replace synthetic surface-active material from petroleum feedstock, because they are cost-effective as compared to the synthetic origin compounds (Cerqueira et al. 2011).

In the recent days, biosurfactants are becoming an attractive alternative in the market as the manufactures are producing eco-friendly biosurfactants from the different natural and sustainable sources. Surfactants are extracted from the organic compounds (Silva et al. 2014a, b). Natural surfactants (saponins) are considered in the set of secondary metabolites which are richly found in large number of plants, and in some marine organisms in general, they can be found in diverse parts of plants including seeds, leaves, roots, and flowers and in fruits. The saponins are foaming agents from plants and are soluble in water with higher molecular weights (Vijayakumar and Saravanan 2015). Surfactants will increase the solubility of hydrophilic molecules by reducing the surface and interfacial tension at the oil and water interface. Critical micelle concentration (CMC) is the concentration of surfactant at which organized molecular assemblies which are known as micelles are formed and which correspond to the point where the tension active agent attains the lowest stable surface tension (Pacwa-Plociniczak et al. 2011; Makkar et al. 2011).

4.2 Types of Biosurfactants

Many of the biosurfactants may be either anionic or neutral which contain amine group is known as cationic. Hydrophobic moiety has long-chain fatty acids, while hydrophilic moiety may be carbohydrate, cyclic peptide, amino acid, phosphate

carboxyl acid, or alcohol. The biosurfactant molar mass generally ranges from 500 to 1500 Da (Bognolo 1999). Biosurfactants are categorized generally by their origin and chemical composition (Banat et al. 2010; Vijayakumar and Saravanan 2015; Rahman et al. 2003). The major types or classifications of biosurfactants are as follows:

- Glycolipids
- Fatty acids, phospholipids, and neutral lipids
- Polymeric biosurfactants
- Particulate biosurfactants

4.2.1 Glycolipids

The most commonly known glycolipids are rhamnolipids, sophorolipids, and trehalolipids (Chrzanowski et al. 2012a). Rhamnolipids are found as the exoproducts of the pathogen *P. aeruginosa* which are the combination of α-L-rhamnopyranosyl-α-L-rhamnopyranosy-ß-hydroxydecanoyl-ßhydroxydecanoate (Rha-Rha-C10-C10) and α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-ß-hydroxydecanoate (Rha-Rha-C10) and as well as their mono-rhamnolipid congeners (Rha-C10-C10 and Rha-C10) (Abdel-Mawgoud et al. 2010). The rhamnolipid congeners and homologues are discovered by the sensitive analytical techniques.

The rhamnolipid congeners and homologues are produced at different concentration by the species of pseudomonas and bacteria which belongs to other families and classes (Chrzanowski et al. 2012b). Rhamnolipids reduce the toxicity to specific compounds by increasing hydrocarbon solubilization and thereby facilitating biodegradation (Marecik et al. 2012; Chrzanowski et al. 2009, 2011). Sophorolipids are produced by yeasts which are belonging to the genus candida (Cortés-Sánchez et al. 2013; Daverey and Pakshirajan 2009). These glycolipids have a dimeric carbohydrate sophorose which are linked to a long-chain hydroxyl fatty acid through glycosidic bond. Sophorolipids and lactone derived from microbes form a sophrolipid which are preferable in many applications (Gautam and Tyagi 2006).

Mannosylerythritol lipids (MEL) are yeast glycolipids which are one of the most promising biosurfactants called pseudozyma and which are produced from vegetable oils (Lang 2002). Trehalolipids are produced from the species of mycobacterium, *Nocardia* and *Corynebacterium.* Trehalolipids which are produced from another species *Arthrobacter* and *Rhodococcus erythropolis* will able to lower surface and interfacial tension (Vijayakumar and Saravanan 2015).

4.2.2 Fatty Acids, Phospholipids, and Neutral Lipids

Bacteria and yeasts of different groups produce large amount of fatty acids and phospholipid surfactants during the growth on n-alkanes. Phosphatidyl ethanolamine-rich vesicles are produced from the species *Acinetobacter* and form optically clear microemulsions of alkanes in water.

These biosurfactants are used for medical applications. The phospholipid protein complex deficiency is the ultimate reason for respiratory failure in children born prematurely. The isolation and cloning of genes which are involved in the production of surfactants are used in the process of fermentative processes (Gautam and Tyagi 2006).

4.2.3 Polymeric Biosurfactants

The polymeric biosurfactants are emulsan, lipomannan, alasan, liposan, and other polysaccharide protein complexes. Emulsifier for hydrocarbons in water is emulsan, and liposan is an extracellular water-soluble emulsifier which is synthesized by C. lipolytica (Hatha et al. 2007). Studies show the application of liposan as an emulsifier in food and in cosmetic industries (Chakrabarti 2012).

4.2.4 Particulate Biosurfactants

Particulate biosurfactants partition extracellular membrane vesicles to form a microemulsion which exerts an influence on alkane uptake in the microbial cells (Vijayakumar and Saravanan 2015 and Chakrabarti 2012). The following are the chemical structures of some common biosurfactant used in various applications as shown in Fig. 4.1.

4.3 Biosurfactant Producing Major Microorganisms

The important biosurfactants producing microorganism used for various applications are shown in Table 4.1. Microorganisms use a set of carbon sources and energy for their growth. The combination of carbon sources with the insoluble substrates leads to the facilitation of the intercellular diffusion and causes the production of different substances (Deleu and Paquot 2004; Marchant et al. 2014; Chakraborty and Das 2014). Microorganisms like yeasts, bacteria, and fungi are capable of production of biosurfactants with different molecular structures and surface activities (Campos et al. 2013). In current years, there has been an increase in the scientific interest on the topic of isolation of microorganisms which produce tensioactive molecules with

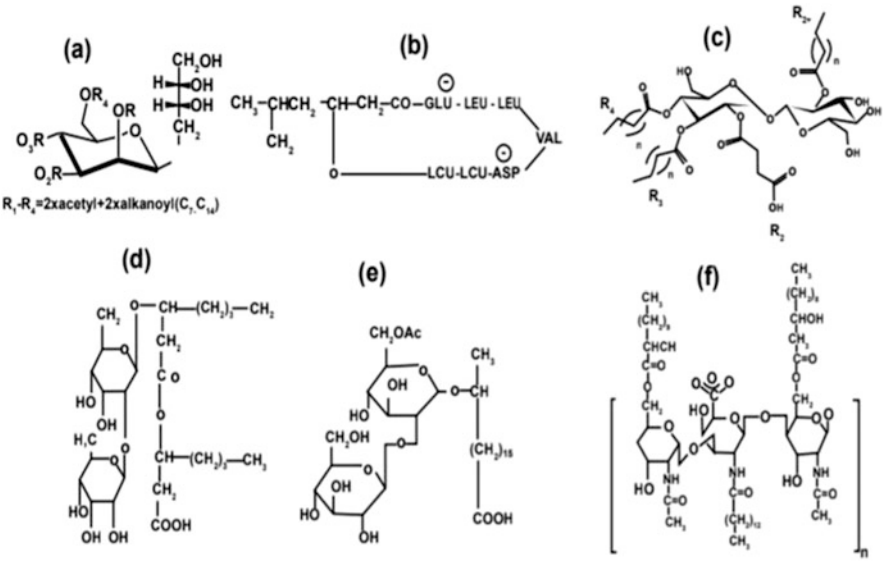

Fig. 4.1 Chemical structure of some common biosurfactants (**a**) mannosylerythritol lipid (**b**) surfactin (**c**) trehalose lipid (**d**) sophorolipid (**e**) rhamnolipid (**f**) emulsan

the good surfactant characteristics like low CMC and less toxicity and with high emulsifying activity (Silva et al. 2014a).

The bacteria of genera *Pseudomonas* and *Bacillus* are some of the great biosurfactant producers (Silva et al. 2014b). Most biosurfactants of a bacterial origin are inadequate for their use in the food industry because of their possible pathogenic nature (Shepherd et al. 1995). While *Candida bombicola* and *Candida lipolytica* are the largely studied yeasts cells for the purpose of the production of biosurfactants in environmental applications. The potential bacterial strain of indigenous pure monoculture (*Pseudomonas aeruginosa*) having the degradability of xenobiotic pollutants includes diesel oil, pesticide (methylparathion) and makes it an ideal applicant in bioremediation (Usharani and Lakshmanaperumalsamy 2017).

The main advantage of using yeasts such as *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, which are generally regarded as safe (GRAS) status. The organisms which have the GRAS status do not offer the risks of toxicity or pathogenicity that allows their use in the industries like food and pharmaceutical (Campos et al. 2013).

4.4 Biosurfactant Properties and Its Advantages

Biosurfactants are amphiphilic compounds that contain both hydrophilic and hydrophobic portions referred to as head and tail. In aqueous, the term hydrophobic groups are used as lyophobic and hydrophilic groups as lyophilic (Fracchia et al. 2012).

Table 4.1 Important biosurfactants producing microorganism and its applications

Biosurfactants		Microorganisms involved in the production	Applications in Environment and Industries
Major group	Major class		
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa* *Pseudomonas putida* *Pseudomonas* sp. *Renibacterium salmoninarum* *P. chlororaphis* *Bacillus subtilis*	Bioremediation, biodegradation, dispersion of hydrocarbons, and vegetable oils, metal, hyrocarbons and pesticides removal. Bioremediation Biocontrolagent Antifungal agent
	Sophorolipids	*Torulopsis bombicola* *Tourlopsis petrophilum* *Tourlopsis apicola* *Candida bombicola* *C. apicola*	Recovery of hydrocarbons, removal of heavy metals, enhanced oil recovery. Emulsifier, microbially enhanced oil recovery, alkane dissimilation
	Trehalolipids	*Mycobacterium tuberculosis* *Rhodococcus erythropolis* *Arthrobacter* sp. *Nocardia* sp. *Corynebacterium* sp. *Tsukamurella* sp. and *Arthrobacter* sp.	Enhancement of bioavailability of hydrocarbons, bioremediation Antimicrobial agent
	Mannosylerythritol lipids	*Pseudozyma (Candida) Antarctica* *Kurtzmannomyces* sp.	Lipase enzymes, food, detergents and pharmaceuticals, biological amphilies biosurfactants; Neuroreceptor antagonist, antimicrobial agent; biomedical applications
	Lipomannan	*Mycobacterium chelonae*,	Immunological studies
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery
	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions, heavy metal sequestrants, dispersion of hydrophilic pigments, emulsion-type organo gel and superfine microcapsules preparation,
	Phosphatidyl ethanolamine	*Acinetobacter* sp. *Rhodococcus erythropolis* *Acinetobacter*	Increasing the tolerance of bacteria to heavy metals clear microemulsions of alkanes in water, medical applications

(continued)

Table 4.1 (continued)

Biosurfactants		Microorganisms involved in the production	Applications in Environment and Industries
Major group	Major class		
		sp. *Aspergillus Corynebacterium lepus*	
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of biodegradation of hydrocarbons, chlorinated pesticides, removal of heavy metals, enhancement of phytoextraction; Antimicrobial agent, biomedical application
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery Hemolytic & chelating agent
Polymeric biosurfactants	Alasan	*Acinetobacter radioresistens KA-53*	Stabilization of hydrocarbons in water emulsion
	Biodispersan	*Acinetobacter calcoaceticus A2*	Dispersion of limestone in water
	Emulsan	*Acinetobacter calcoaceticus RAG-1*	Stabilization of hydrocarbons in water emulsion
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbons in water emulsion; emulsifier in the food and cosmetic industries; extracellular water soluble emulsifier
	Mannoprotein	*Saccharomyces cerevisiae*	Stabilization of hydrocarbons in water emulsion

Surfactants are the active compounds which are found in detergents and in soaps which have the property to reduce the surface tension or interfacial tension at the air-water and water-oil interfaces (Satpute et al. 2010). Biosurfactants can be produced in large quantities from the different low-cost materials which have several advantages over the synthetic chemical-derived surfactants that include low toxicity, bioavailability, biodegradability, high foaming, environment-friendly, low cost in terms of availability, pH, and salinity.

In addition, they are safe and are better alternative to chemical surfactants mainly in food, pharmaceutical, cosmetic, and edible oil (Nitschke and Costa 2007; Fracchia et al. 2015; Bhadoriya et al. 2013). The growing distress in the environment about chemically derived surfactants leads to the concentration on natural surfactants because of their harmless characteristics. The most desirable and effective properties of biosurfactants include the reduction of surface tension, solubility enhancement, and low critical micelle concentration (Jha et al. 2016). An effective biosurfactant is able to reduce the surface tension of water and interfacial tension between a polar and non-polar liquid (Mulligan 2005). The CMC is the maximum concentration of

surfactant monomers in the water that can be influenced by pH, ionic strength and the temperature of the solution. Let us discuss some of the properties of biosurfactants (Pacheco et al. 2010).

4.4.1 Surface and Interfacial Activity

The main essential characteristics for a good surfactant are efficiency and effectiveness. The efficiency of the surfactant is measured by the CMC, and the effectiveness of the surfactant is related to surface and interfacial tension. The CMC of the biosurfactant should be between 1 and 2000 mg/L, and interfacial and surface tensions should be 1 and 30 mN/m (Barros et al. 2007). The surfactant can be called as good surfactant only when it is able to reduce water surface tension from 72 to 35 mN/m and interfacial tension from 40 to 1 mN/m (Barros et al. 2007).

4.4.2 Tolerance to Temperature, pH, and Ionic Strength

Most of the surfactant is being used at high temperature, and the value of pH should range from 2 to 12. It can also tolerate a salt concentration up to 10%, while only 2% of NaCl is enough for inactivate synthetic surfactants (Danyelle Khadydja et al. 2016).

4.4.3 Biodegradability

Biosurfactants are nontoxic biomolecules that are biodegradable in nature. They are easily degraded with the help of microorganisms in water and in soil, which is one of the important properties of biosurfactants, making them as adequate for bioremediation and in waste treatment (Danyelle Khadydja et al. 2016).

4.4.4 Low Toxicity

Because of the low toxicity in biosurfactants, they are used in food, cosmetics, and pharmaceutical industries. It also plays a fundamental importance to environmental applications. Biosurfactants can be manufactured from the largely available raw materials as well as industrial waste (Danyelle Khadydja et al. 2016).

4.4.5 Specificity

Biosurfactants are complex molecules with specific functional groups, and because of this, they have a specific action and a particular interest on the detoxification process of different pollutants and the de-emulsification of industrial emulsions and as well as in specific food, pharmaceutical, and cosmetic applications (Danyelle Khadydja et al. 2016).

4.4.6 Biocompatibility and Digestibility

This is one of the important properties of biosurfactants which allows the biosurfactants to be used in different industries mainly in food, pharmaceutical, and cosmetic industries (Danyelle Khadydja et al. 2016).

4.4.7 Emulsion Forming

This is another property of biosurfactants which can be either emulsifiers or de-emulsifiers. Emulsion is a heterogeneous system which consists of immiscible liquid depressed in another liquid in the form of droplets. Emulsions have the minimal stability, but by the addition of biosurfactant, it can lead to an emulsion to remain stable for month or even for years (Velikonja and Kosaric 1993; Cirigliano and Carman 1985; Campos et al. 2013).

4.4.8 Antibiofilm Properties

Biofilms are groups of bacteria with other organic matter that has populated and accumulated on a specified surface (West and Harwell 1992). Bacterial adherence to the surface is the first step in the establishment of biofilm and is affected by various factors, such as the type of microorganism, hydrophobicity and electrical charges of the surface, environmental conditions, and the ability of microorganisms to produce extracellular polymers that assist the cells in anchoring to the surface (Fig. 4.2). Biosurfactant may help to exterminate biofilms depending upon the habitat they are being located in. It works as antibiofilm formation activities or properties. Di-rhamnolipids from *Lysinibacillus* sp. BV152.1 hold mostly di-rhamnolipids, and it inhibits the biofilm formation. Inhibition of biofilm was identical in the presence or absence of the cell adhesion phase, and di-rhamnolipids affected both cell adhesion and biofilm growth.

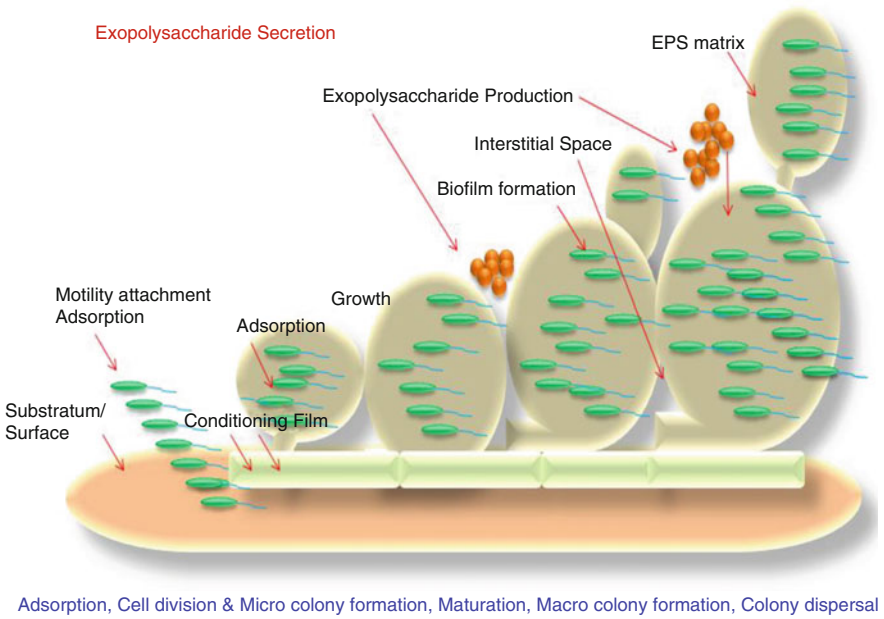

Fig. 4.2 (**a**) Microbial biofilm formation and exopolysaccharide secretion; (**b**) microbial exopolysaccharide secretion

4.5 Factors Affecting Biosurfactants Production

There are some important factors that affect the production of biosurfactants. The composition and emulsifying activity of biosurfactant depends not only on the producer strain but also on the culture conditions like the nature of carbon source,

nitrogen source, and the C: N ratio; nutritional limitations, chemical, and physical parameters like temperature, aeration, divalent cations, and the pH influence not only on the amount of biosurfactant produced but also on the type of polymer produced (Salihu et al. 2009). Some of the factors are discussed further.

4.5.1 Carbon and Nitrogen Sources

Biosurfactant quality and quantity are being affected and influenced by the nature of the carbon substrate (Rahman and Gakpe 2008). Some of the good sources of carbon substrate are diesel, crude oil, glucose, sucrose, and glycerol being used for biosurfactant production (Desai and Banat 1997). One of the important factors for the production of biosurfactant is nitrogen because it is very much essential for the growth of microbial as protein and enzyme syntheses. A variety of nitrogen compounds have been used for the manufacture of biosurfactants like urea peptone, yeast extract, ammonium sulfate, ammonium nitrate, meat extract, and malt extract. The most widely used nitrogen source for the production of biosurfactant is yeast extract due to its usage with respect to concentration of organism and culture medium. Another preferred nitrogen source for the production of biosurfactant is ammonium salts (Adamczak and Bednarski 2000).

4.5.2 Environmental Factors

Environmental factor is one of the important factors in the yield and characteristics of the biosurfactant produced. In order to gain larger quantities of biosurfactants, it is mainly necessary to optimize the bioprocess because the product may be affected by the changes in temperature, pH, aeration, or agitation speed. Many biosurfactants are produced in the temperature range from 25 to 300 °C (Desai and Banat 1997).

4.5.3 Aeration and Agitation

One of the important factors for the production of biosurfactant is aeration and agitation which has the greater influence on production of biosurfactant as both facilitate the oxygen transfer from gas to the aqueous phase. It also linked to the physiological function of microbial emulsifier (Adamczak and Bednarski 2000).

4.5.4 Salt Concentration

Salt concentration is one of the factors that affect the production of biosurfactant. It also had a corresponding effect on the production of biosurfactant as the cellular activities of microorganisms will get affected by salt concentration. The studies state that some biosurfactant products are not affected by concentrations up to 10% (weight/volume) with slight reductions in the CMC detected (Desai and Banat 1997).

4.6 Growth Conditions and Metabolic Pathways

The metabolic pathway of microbial exopolysaccharide production was represented in the following: Figs. 4.2a, b and 4.3. For the process of cell metabolism and for the synthesis of polar moiety of a biosurfactant, microorganisms used hydrophilic substrates, while these hydrophilic substrates are used for the production of the hydrocarbon portion of the biosurfactant (Weber et al. 1992; Desai and Banat 1997). The synthesis of precursors for biosurfactant production involves diverse metabolic pathway and depends mainly on the nature of the main carbon sources employed in the culture medium. While carbohydrates are the only carbon source used for the production of a glycolipid, the flow of carbon is regulated in such a way that both the

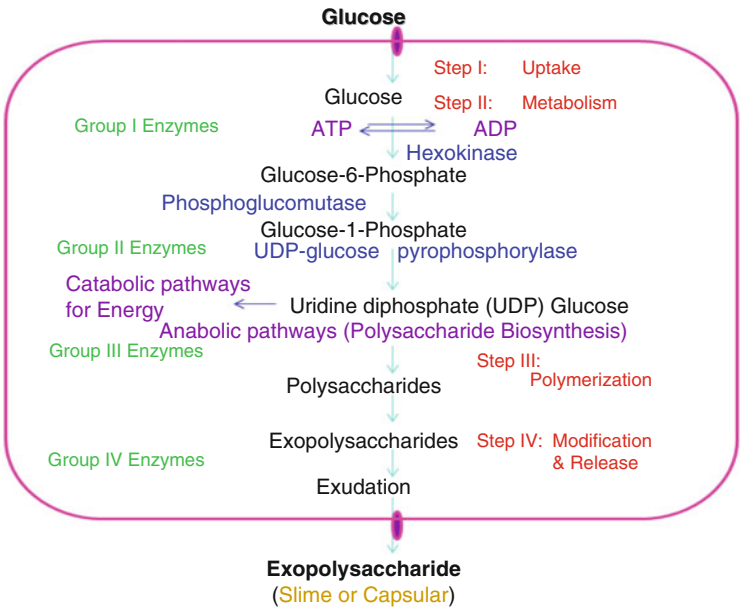

Fig. 4.3 Metabolic pathway of exopolysaccharide production

lipogenic pathways and the formation of hydrophilic moiety through the glycolipid pathway are suppressed by the microbial metabolism (Haritash and Kaushik 2009). Glucose or glycerol which is a hydrophilic substrate is degraded until forming intermediates of the glycolytic pathway such as glucose 6-phosphate which is one of the important precursors of carbohydrates found in the hydrophilic moiety of a biosurfactant. Glucose is oxidized to pyruvate through glycolysis, and pyruvate is then converted into acetyl-CoA for the production of lipids which produces malonyl-CoA when united to oxaloacetate which is followed by the conversion into fatty acid of the precursors for the synthesis of lipids (Hommel and Huse 1993).

The microbial mechanism is directed mainly to the lipolytic pathway and gluconeogenesis which allows its use for the production of fatty acids or sugars when a hydrocarbon is used as a carbon source. The production of sugar is activated by the gluconeogenesis pathway; this pathway consists of the oxidation of fatty acids through ß- oxidation to acetyl-CoA. Starting with the formation of acetyl-CoA, the reaction involved in the synthesis of polysaccharide precursors like glucose 6-phosphate is essentially the inverse of those involved in glycolysis. However, reactions catalyzed by pyruvate kinase and phosphofructokinase-1 are irreversible; thus other enzymes exclusive to the process of gluconeogenesis are required to avoid such reactions (Tokumoto et al. 2009). The biosynthesis of a surfactant takes place through four different routes: (a) carbohydrate and lipid synthesis; (b) synthesis of the carbohydrate half with the synthesis of the lipid half depending on the length of the chain of the carbon substrate in the medium; (c) synthesis of the lipid half with the synthesis of the carbon half depending on the substrate in use, and (d) synthesis of the carbon and lipid halves which are both needy on the substrate (Thaniyavarn et al. 2008). Thus, the length of the n-alkane chain which is used as the carbon source alters the biosynthesis of a surfactant. The previous studies showed that the production of mannosylerythritol lipid (MEL) by the yeast C. Antarctica in the presence of different n-alkanes shows that this species does not grow or produce a biosurfactant in media containing C10 to C18, but anyways production occurred when the species are grown in a medium containing C12 to C18, and octadecane as substrate led to the greatest yield, but in contrast the production is less in the media containing n-alkanes with more than 19 carbons (Kitamoto et al. 2001).

4.6.1 Microbial Biosurfactant Physiological Role

Biosurfactants are formed by a diversity of a microorganism; they are produced either extracellular or attached to parts of cells predominantly during growth on water immiscible substrates (Desai and Banat 1997; Tan 2000). The main physiological role of biosurfactants is to permit microorganisms to grow on water-immiscible substrates by reducing the surface tension at the phase boundary, thus making the substrate more readily available for uptake and metabolism, though the molecular mechanism related to the uptake of their substrates is still not clear and not fully understood (Desai and Banat 1997).

The review suggests that when the surface area turns into limiting, biomass enhances arithmetically rather than exponentially. The fact that emulsification is a natural process brought about by extracellular agents is indirect, and there are certain conceptual difficulties in understanding how emulsification can provide an (evolutionary) benefit for the microorganism producing the emulsifiers (Desai and Banat 1997; Marchant and Banat 2012a). In addition, biosurfactants have been shown to be involved in cell adherence which imparts greatest stability under hostile environment conditions and virulence and in cell desorption when organisms need to find new habits for survival. An additional physiological function of biosurfactants is their antimicrobial activities towards various microorganisms. As a rule, different surfactant inhibits different taxonomy (Desai and Banat 1997).

Microbial growth on water-insoluble carbon sources such as hydrocarbons goes along with metabolic and structural alterations of the cell. The surface-active compounds in the culture medium or attached to the cell boundaries are frequently regarded as a condition for initial interactions of hydrocarbons with the microbial cell. Such compounds are related with growth limitation in the late logarithmic and the stationary growth phase, in which precise enzymes are induced. Adding up of purified biosurfactants to microbial cultures consequences in inhibitory as well as in stimulatory effects on growth (Ron and Rosenberg 2001). Biosurfactants are regarded as prerequisites of hydrocarbon uptake and also as secondary metabolic products. The glycolipid biosurfactant exhibits applicability to the transport, pipelining, processing, and recovery of heavy crude oil.

Biosurfactants are produced by microorganisms either through secretion or adhesion to cells, particularly which cultivated on substrates which are insoluble in water. While the function in microbial cells is not yet fully understood, it has been speculated that biosurfactants are concerned in the emulsification of insoluble substrates (Desai and Banat 1997). The main physiological role of biosurfactants is to allow microorganisms to grow on substrates that are insoluble in water through a reduction in surface tension between phases, making thee substrate more available for uptake and metabolism. The direct uptake of dissolved hydrocarbons in thee aqueous phase, direct contact between cells and large hydrocarbon droplets, and the interaction with emulsified droplets (emulsion) have been described. In addition the emulsification of the carbon source, biosurfactants are also concerned in the adhesion of microbial cells to hydrocarbons.

Microbial cell adsorption to insoluble substrates and the discharge of surfactant compounds permit growth on carbon sources (Desai and Banat 1997). The factor which affects the production of biosurfactant is the growth conditions (Desai and Banat 1997). The genus candida species produce maximum biosurfactant yields in the wide pH range. Moreover, *Pichia anomala* and *Aspergillus ustus* produce the larger amount of biosurfactant yields at pH 5.5 and 7.0, respectively (Thaniyavarn et al. 2008; Kiran et al. 2009).

The various microbial processes are affected by even a small amount of change in the temperature.

4.6.2 *Fermentation Process for Biosurfactant Production*

The microbial biosurfactant production flow chart was shown in Fig. 4.4. The production kinetics has considerable variation among different system.

For convenience, kinetic parameters are grouped as follows (Desai and Banat 1997):

- Growth-associated production
- Production under growth-limiting conditions
- Production with precursor supplementation
- Production by resting or immobilized cells

The parallel relationships which are found between growth, the usage of the substrate and its biosurfactant production, which are all considered in the growth associated production. Production in growth-limiting conditions is characterized by an accentuated increase in concentration of biosurfactants as a result of the limitations of one or more medium components. Production by resting or immobilized cells is a type of biosurfactant production wherein there is no cell multiplication; the cells nonetheless continue to use the carbon source for biosurfactant synthesis. Investigators report that the addition of biosurfactant precursors to the medium leads to quantitative and qualitative changes in the very final product (Desai and Banat 1997). The biotreatment efficiency of *Pseudomonas aeruginosa* dou strain

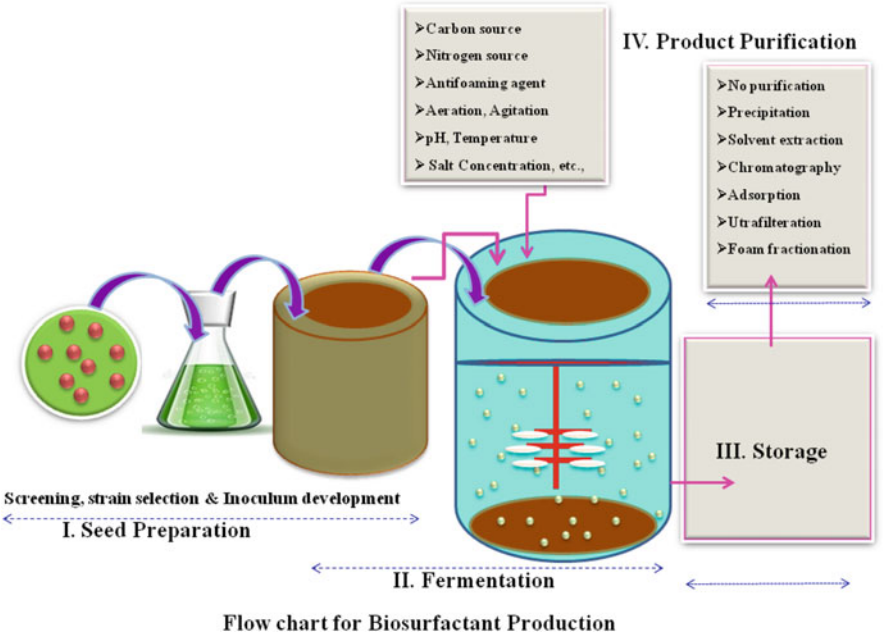

Fig. 4.4 Flow chart for biosurfactant production

was reported to be 83% after seven days of incubation at optimized conditions of pH (7), temperature (35°C) and agitation (150rpm) in modified MS medium at 10% diesel oil (v/v) with optimum concentration of additional nutrients of glucose, peptone, nitrate, phosphate and sodium chloride (Usharani and Lakshmanaperumalsamy 2017).

For instance, the used bacteria were separated from soil contaminated with palm oil from a local area. Half gram of soil obtained from the land contaminated with crude palm oil industry was poured in a small pipe containing 9.9 ml sterilized water for 25 min. The solution was further diluted up to 10^{-8} dilutions and incubated in agar media for 7 days at 37 °C. The agar media contained 0.5% leavened extract, 0.5% NaCl, and 2% bacto agar. The growth and capability of the bacteria for producing biosurfactant were compared with the commercial bacteria for producing biosurfactant compared with the commercial bacteria. The microbes were then selected according to their ability to form an emulsifying zone around the colony and their capability to grow in comparison with those commercial bacteria. The microbes were later incubated in an incubator on agar slants for 24 h at 37 °C. A loop full of cells from the agar slant was then replaced to a 250-ml flask containing 50 ml sterilized propagation medium (PM) to be propagated in a water bath shaker at 140 rpm and 37 °C for 24 h (Hesty and Meilana 2017).

Samples were commonly taken and centrifuged at 13,000 rpm for 10 min to separate the cell mass. Prior to drying at 90 degree C for 24 h, the cells were washed and rinsed with purified water and re-centrifuged. HCL was included to the fermentation broth to obtain pH 2. The acid precipitate was extracted with methanol and then centrifuged at 3000 rpm for 15 min. The solution was stained using a 0.2-mue meter Millipore membrane. The biosurfactant content in the filtrate was additionally analyzed using high-performance liquid chromatography. The temperature of the column was maintained at 40 °C, and acetonitrile solution with 1degree acetic acid was used as the mobile phase at a flow rate of 1.5 mL/min. The glucose concentration was investigated using a biochemistry analyzer. The surface tension was evaluated using a tensiometer (Hesty and Meilana 2017).

4.6.3 Effect of Various Initial Concentrations

The effect of various factors which includes the initial glucose concentration was important on the kinetic profiles of cell mass along with the glucose consumption, biosurfactant and surface tension during the fermentation process using the potential strain. Overall, there was a moderate drop in glucose concentrations for all early concentrations. Glucose used for growth of the strain was more bearable at an initial glucose concentration of 70 g/L. The cell mass decreased by about twice the amount when the glucose concentration because of substrate inhibition. Biosurfactant yield and productivity decreased substantially when the glucose concentration was increased from 50 to 70 g/L; the highest biosurfactant of 50 g/L was produced. This initial glucose concentration resulted in the smallest value of surface tension

obtained in line with the values of surface tension in the literature for *Bacillus* sp. strains. Kinetic models are useful tools that are applied for further process expansion and/or industrial implementation. The extended modified Gompertz kinetic model is generally used for predicting or observing the ethanol production and predicting the cumulative bio-hydrogen production in this study; it was applied for predicting the biosurfactant production. At initial glucose concentrations of 10–70 g/L, *Bacillus* sp. BMN14 produced biosurfactant concentrations of 1–2.46 g/L. The bacteria showed good performance, with a decrease in surface tension up to 27 mN/m. The optimum condition for the production of biosurfactant is C/N ratio of 12.4 and resulted in the highest decrease in surface tension. The cell mass and biosurfactant concentrations could be well observed using the modified Gompertz equation. The modified Gompertz equation very well predicted the surface tension (Hesty and Meilana 2017).

4.6.4 Raw Material for Biosurfactant Production and Its Recovery

The exopolysaccharide of biosurfactant production was shown in Fig. 4.4, and the raw materials commonly used for the production are as follows:

- Animal fat
- Frying oils
- Corn steep liquor
- Molasses
- Whey

According to Barros et al. (2007), the use of agricultural industrial waste is one of the steps toward the implantation of attainable biosurfactant production on an industrial scale, for which the optimization of the different variables involved is required (Makkar and Cameotra 2002; Barros et al. 2007). A number of waste products are employed in biosurfactant production, such as oily effluents, vegetable oils, animal fat, starchy effluents, vegetable cooking oil waste, vegetable waste (whey), molasses, soap stock, corn steep liquor, dairy industry waste, glycerol, and cassava flour waste water. Current culture is characterized by an increase in expenditures, the need to reuse materials, and environmental concerns. Consequently, greater emphasis has been given to reuse, recovery, and recycling. Indeed, the value for environmental preservation has led to the reuse of different industrial and other wastes. Industrial waste had provoked the interest of researches as a low-cost substrate for biosurfactant production. This is mostly valid for the food production industry, the waste products, effluents, and by-products of which can be reused (Banat et al. 2014). The choice of waste products should ensure the proper stability of nutrients to allow microbial growth and consequential biosurfactant production. Industrial waste with a higher content of lipids or carbohydrates is

ideal for use as substrate. Olive oil mill effluent is a concentrated black liquor with a water-soluble portion of ripe water and olives that is used for the removal of olive oil. It has polyphenols that represent a challenge in terms of the environmental disposal. However, it also contains organic acids (5–15 g/L), residual oil (0.3–5 g/L), sugars (20–80 g/L), and nitrogen compounds (12–24 g/L). Mercade et al. successfully employed olive oil mill effluent for the strain of *Pseudomonas* sp. to produce rhamnolipids (Mercade et al. 1993). Edible fats and frying oil are considered rich carbon sources for biosurfactant production. Vegetable oils constitute a lipid carbon source and are mainly comprised of unsaturated or saturated fatty acids with chains of (16–18) carbon atoms. Different oils are sufficient substrates for biosurfactants. Babassu oil (5% v/v) with a carbon source (1% glucose w/v) is a good medium for biosurfactant production. Sunflower and olive oils have proven to be sufficient energy and carbon sources for the production of biosurfactants. *P. aeruginosa* strains produce a biosurfactant on residue from oil plants, canola, soybean, and corn. Sarubbo et al. (1999) found that two strains of *C. lipolytica* (strain No. 1055 and 1120) produce biosurfactants toward the exponential growth phase and onset of the stationary phase Canola oil sodium nitrate, and residue has been reported adequate for microbial growth and the production of up to 8.50 g/L of rhamnolipids. The combination of canola oil and glucose has been used for the fortunate production of a biosurfactant by *C. lipolytica* (Sarubbo et al. 1999). Animal fat and lard can be obtained in large quantities from the meat processing industry and have been used as a source for cooling foods. Recently, such fats have lost a large part of the market to vegetable oils due to the lower degree of harm to health caused by the later (Banat et al. 2014). Animal fat increases the production of sophorolipids by the yeast *C. bombicola* (Deshpande and Daniels 1995). Santos et al. (2013, 2014) pointed out that using animal corn steep liquor and fat, achieved maximum glycolipid production by the yeast *C. lipolytica* UCP 0988 strain. The authors also report that the product has been used in bioremediation as well as recovery and oil mobilization. Molasses is a by-product of beet and sugarcane processing. These inexpensive substrates have magnesium, phosphorus, and calcium (~1%), potassium (1.5–5.0%), protein (2.5%), non-sugar organic matter (9–2%), and dry matter (75%). The pantothenic acid, thiamine, biotin, and inositol content (1–3%) gives molasses its very thick consistency and brown color. The high sugar content (48–56%) makes molasses sufficient for biosurfactant production by various microorganisms (Makkar and Cameotra 1997; Sudhakar-Babu et al. 1996). The dairy industry produces large quantity of whey, such as lactic whey, curd whey, cheese whey, and whey waste, all of which can be used as a substrate for the microbial production of metabolites (Banat et al. 2014; Dubey and Juwarkar 2001, Dubey et al. 2005; Rodrigues et al. 2006). Whey disposal represents a major pollution problem especially in countries that depend on a dairy economy. A large amount of lactose (approximately 75%) is found in lactic whey. Other components, such as organic acids, vitamins, and proteins, are good sources for biosurfactant production and microbial growth (Thompson et al. 2000). Thus, the disposal of this by-product represents a waste of a widely available substrate and a difficult environment (Helmy et al. 2011). The agriculture industry products based on corn, through wet

processing, result in both liquid and solid by-products and, when disposed improperly, become a major source of contamination and harm to the environment. Corn steep liquor is a by-product of the soaking of kernels and washing water as well as fractionating into starch German oil that contains 40% solid matter. This by-product consists of lactic acid (20–26%), proteins (21–45%), approximately 8% ash (containing Ca2+, Mg2+, K+, etc.), approximately 3% carbohydrates, and a fat content (0.9–1.2%) (Cardinal and Hedrick 1948; Akhtar et al. 1997; Helmy et al. 2011). Corn steep liquor, refinery residue, and nut oil are low-cost nutrients for the production of glycolipids by C. sphaerica (UCP 0995). The biosurfactant of this strain is mobilized and removes up to 95% of motor oil on sand, making it useful for bioremediation (Sudhakar-Babu et al. 1996; Luna et al. 2013, 2015).

The production of low-cost biosurfactants is unlikely due to the complex recovery process. In many biotechnological processes, downstream processing accounts 70–80% of production costs. Process development is conducted in order to acquire biosurfactants that can be easily recovered and are inexpensive. For some economic reasons, most biosurfactant production processes need to involve whole cell culture broths or other crude preparations (Helmy et al. 2011; Marchant and Banat 2012a; Marchant et al. 2014). Extraction with ether, acetic acid, hexane, pentane, ethyl acetate, butanol, dichloromethane-methanol, and chloroform-methanol constitutes the most commonly used method in biosurfactant: downstream processing. The disadvantages of using organic solvents for biosurfactant recovery include the increase in production costs due to the price of expensive solvents. The most widely employed products are different ratios of methanol and chloroform, which facilitate the adjustment of the polarity of the extraction agent to the extractable target material. Chloroform is a toxic chloro-organic compound that is harmful to the environment and human health. Thus, there is a need for inexpensive solvents with low toxicity for biosurfactant extraction processes that are suitable for industrial applications (Helmy et al. 2011). Biosurfactant production involves continuous foam formation due to high surface activity. Other product precipitation techniques have also been reported, such as precipitation with ammonium sulfate, adsorption, and centrifugation (Winterburn et al. 2011). Biosurfactant recovery depends mainly on the ionic charge, water solubility, and location (extracellular or cell bond, intracellular). The foam fractionation is a solvent-free process that separates biosurfactant molecules adsorbed to air bubbles in the culture medium. Biosurfactant productions engage continuous foam formation due to the high surface activity. Foam in the broth interferes with mass and heat transfer processes, thereby affecting productivity. Though foam is beneficial to biosurfactant production, as it assists in the continuous removal of product, production and recovery processes can be accomplished in a single stage (Helmy et al. 2011; Marchant and Banat 2012a). Continual foam fractionation in the fermentation process helps to avoid the accumulation of product that could otherwise inhibit biomass growth and product formation and also facilitates extended biosurfactant production in fed-batch or continuous mode operation. Moreover, biosurfactants do not readily undergo denaturation due to their simple structure and small size. More research and development are required to optimize existing recovery processes to make such processes both more competitive and

commercially viable (Winterburn et al. 2011). Ultrafiltration is used in a single step method to purify and concentrate biosurfactants (surfactin and rhamnolipids) from the supernatant fluids of culture. The capability of surfactant molecules at concentrations above the critical micelle allocates these aggregates to be retained by comparatively high molecular weight cut-off membranes. Lower molecular weight impurities such as salts, free amino acids, peptides, and small proteins are effortlessly removed. Various molecular weight cut-off membranes were inspected for the retention of surfactin and rhamnolipids. Amicon XM 50 was the superior membrane for retention of surfactin, and 160-fold purification was continuously achieved. The YM 10 membrane was the most suitable for rhamnolipid recovery. Ultrafiltration can play an important role in biosurfactant purification as large volumes of media can be processed rapidly at highly low cost (Helmy et al. 2011; Cooper and Paddock 1984; Marchant et al. 2014).

4.7 Promising and Emerging Application of Biosurfactants

The exopolysaccharides (EPSs) from microbial sources are biomolecules formed in the extracellular space and possess various biological functionalities which include environmental protection, surface adherence, and cellular interactions. EPSs are biocompatible and eco-friendly nature, consequently making them appropriate for applications in numerous ways and various industrial products. Biosurfactants have a wide range of applications in a variety of industrial applications such as painting, textile, agriculture, waste water, petroleum, food, pharmaceutics, cosmetics, etc., as shown in Fig. 4.5. These compounds are well known as anti-adhesive agent; emulsifying, moisturizing, antimicrobial, wetting, and multifunctional agents; and stabilizing (Fracchia et al. 2014). Primarily, the biosurfactants attained a potential interest for environmental applications of inorganic and organic contaminants, mainly in heavy metal removal from water and soil, enhanced oil recovery, and pharmaceutical products (Al-Wahaibi et al. 2014; Boruah and Gogoi 2013; Vijayakumar and Saravanan 2015).

4.7.1 Environmental Bioremediation and Bioleaching Applications

Most of the studies are focusing on the environmental applications of biosurfactant due to their environment-friendly characteristics, better physiochemical properties, diverse structure, and suitability for many purposes which include removal of heavy metals from contaminated soil and remediation of hydrophobic organic compounds (▪ OCs) from soil (Adamu et al. 2015). Heavy metals are becoming a part of the several environmental problems. The most common metals found in soils are nickel

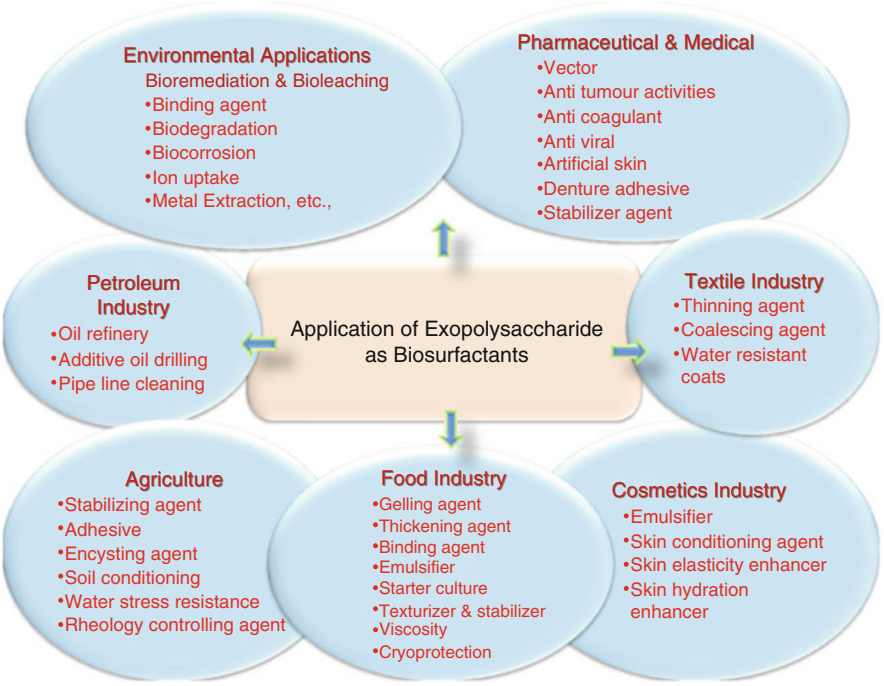

Fig. 4.5 Broad applications of exopolysaccharide

(Ni), copper (Cu), zinc (Zn), chromium (Cr), cadmium (Cd), arsenic (As), mercury (Hg), and lead (Pb) that can create many health issues categorized under inorganic chemical hazards for plants, animals, and humans (Khan et al. 2008; Ali and Al-Qahtani 2012; Liu et al. 2015; Adamu et al. 2015; Hu et al. 2017; Li and Qian 2017). The chemical surfactants had been used to treat heavy metal contaminated solubilize hydrophobic organic compounds and soils but it may cause other environmental issues due to its degradability in the soil. The heavy metal removal from the soil using ionic biosurfactants mainly involved three main steps, that is, the removal of heavy metal from the soil through cleaning with biosurfactant solution (Ali and Al-Qahtani 2012; Khan et al. 2008). The heavy metals adsorbed on the inner surface of soil particles separated through the sorption of biosurfactant molecules at the interfaces between be engaged by the biosurfactants and trapped within the micelle through electrostatic interactions. Finally, the biosurfactant can be retained through the method of membrane separation (Liu et al. 2015; Adamu et al. 2015; Li and Qian 2017). The efficiency of biosurfactants for neglecting heavy metals from sludge, soil and its removal rate for Cd, Cr, Zn and Cu was about 90–100 %. In addition, natural surfactants are found to be efficient in treating contaminated soils with diesel and crude oil (Rosa et al. 2015).

4.7.2 Petroleum Industry and Agriculture Applications

The biosurfactants are valuable in the formulations of emulsifying, demulsifying agents, anti-corrosives, biocides, and other innovative applications in the petroleum industry (De Almeida et al. 2016). It confirmed their value in residual oil recovery by solubilizing trapped oil in rock formations, which is a prerequisite for enhanced oil recovery (EOR). By the same principle, they have also been used in washing contaminated vessels and facilitating pipeline transport of heavy crude oil (Mazaheri-Assadi and Tabatabaee 2010). The anti-corrosion effects on oil-prospecting resources are based on the direction of their polar groups and their antimicrobial activities (Satpute et al. 2016). In the quality improvement of soil, remediation is essential to decrease organic and metal pollutants to an acceptable or tolerable level as seen in the previous section. Plant growth in healthy land desires interaction with soil microbiota in the rhizosphere, and the interactions between these plants and microbes are essential in agriculture activities. The factors that assist these interactions are biofilm formation on the root surface, the release of quorum-sensing molecules, and microorganism motility (Choudhary et al. 2017). The symbiotic relationship manipulates nutrient accessibility and uptake that is important for plant growth promotion (Singh et al. 2018). The plant growth-promoting microorganisms (PGPMS) improve metal phytotoxicity, encourage the growth throughout the induction of defense mechanisms against pathogens, and change metal bioavailability in soil through the process of acidification, chelation, precipitation, complexation, and reduction–oxidation (redox) reactions (Ma et al. 2016). Interactions of pests and pathogens overcome plants in natural ecological settings and human intervention in pest-pathogen control, enrichment of plant–microbe interactions, and soil remediation with an analysis to optimize crop yield and its revenue.

4.7.3 Pharmaceutical Industries, Cosmetics, and Food Industrial Applications

Biosurfactant has attracted interest in pharmaceutical industries and cosmetic because of their potential use of solubilizing, foaming, emulsifying, wetting, detergents, and other many useful properties. Marchant and Banat are two very wide industries; they are one of the vital essential components in producing products such as healthcare products, moisturizers, creams, toothpastes, shower gel, soap, hair conditioners, shampoo, and many other skin care products (Marchant and Banat 2012b). It can replace synthetic surfactants in vast industrial applications as detergents, foaming, emulsifiers, solubilizers, and wetting agents. The change in consumers from natural to synthetic additives and the growing health and environmental concerns have formed a demand for new “natural and green” additives in foods and its industrial applications. Apart from this, biosurfactants have revealed antimicrobial and antibiofilm activities against foodborne pathogens, and therefore, it can be

multipurpose additives or ingredients in food processing (Marcia Nitschke and Sumária Sousa e Silva 2018; Sudhanshu Shekhar et al. 2015). Biosurfactants from microbial sources take part in a functional role in the food industry as a food emulsifier, stabilizer, foaming agent, adhesive, wetting, and antimicrobial agent (Singh and Cameotra 2004, Alizadeh-Sani et al. 2018). Fracchia et al. (2014) carried out a thorough review of biosurfactant applications in the textile industry where they are used as a pretreatment agent, for dye solubility, and to achieve penetration of the fiber. In the paint and papermaking industry, it has wide applications, used as a degreasing, dispersant, defoaming, deresinification, calendaring, coating, and color levelling agent (Kilic 2013) Singh and Cameotra 2004; Pervaiz and Sain 2010). In the commercial laundry detergents, cosmetics, other household and personal care industries, due to the ecological issues, the requirement for green solutions which influence the rising demand for biosurfactants (Global Market Insight 2019). Household and personal care products evidence more than 60% of biosurfactant application followed by industrial cleaners and petroleum biotechnology with the application of the commonest biosurfactant classes which include glycolipids, lipopeptides, and polymeric surfactants (Soonglerdsongpha et al. 2014).

4.8 Advantages of Biosurfactants

Biosurfactants have more advantages when compared to their chemically synthesized variations.

4.8.1 Biodegradability

Biological surfactants are easily degraded by many microorganisms Marchant et al. 2014; Sarubbo et al. 2007; Bednarski et al. 2004; Kitamoto et al. 2001; Desai and Banat 1997; Velikonja and Kosaric 1993).

4.8.2 Low Toxicity

Biosurfactants demonstrate higher toxicity than the chemical derived surfactants. It was also identified that biosurfactants shows higher EC 50 (it decreases 50% of test population) values than synthetic dispersants (Mao et al. 2015; Rosa et al. 2015; Chrzanowski et al. 2011; Cortés-Sánchez et al. 2013; Daverey and Pakshirajan 2009).

4.8.3 Surface and Interface Activity

A good surfactant can minimize surface tension of water from 75 to 35 mN/m and the interfacial tension of hexadecane-water from 40 to 1 mN/M. The surfactin have the ability to the interfacial tension of hexadecane-water to <1 mN/M and minimize the surface tension of water to 25 mN/M (Santos et al. 2016).

4.8.4 Physical Factors

Many biosurfactants are not affected by such environmental factors such as ionic strength tolerances, pH, and temperature (Vijayakumar and Saravanan 2015; Banat et al. 2014; Batista et al. 2010; Tokumoto et al. 2009; Makkar and Cameotra 2002).

4.8.5 Availability of Raw Materials

Biosurfactants are produced from very cheap raw materials which are available in enormous quantities. The source of carbon comes from lipids, carbohydrates, or hydrocarbons, which may be used separately or in combination with each other (Banat et al. 2010; Oliveira et al. 2009; Bednarski et al. 2004; Sarubbo et al. 2006).

The following are the main advantages:

- Nontoxic or low in toxicity
- Biodegradable wastes used as raw materials
- Able to work at critical condition
- Wide applications

The following are the main limitations:

- Low production yield
- High production cost

4.9 Conclusion and Future Perspectives

Microbial exopolysaccharides of biosurfactant are biocompatible and eco-friendly in nature. It could be produced in stirred tank fermenter, and it could be recovered from cell-free culture medium by foam fractionation process. It has good surface activity and low toxicity and is stable under various conditions and has wide applications in various fields. It is prospected to become a substitute chemical surfactant, produced from nonrenewable resources, with option produced from cheap renewable feed stocks and shows potential strategy for the removal of various xenobiotics from the

environment through environmental bioremediation process and its technology. Furthermore, they are attractive since they are less destructive to the environment and yet are robust sufficient for various industrial applications.

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Chapter 5
Biodegradable Polymers for Food Packaging and Active Food Packaging

Priyanka Bagde

Contents

Abstract Owing to consumer health concerns and environmental problems derived from packaging wastes, various research groups have been looking toward green polymers as biodegradable alternatives to synthetic plastic packages. Various biodegradable polymers and composites have studied for their ability to be used as food packaging. To some of these packaging materials, antimicrobial agents have been added to inhibit food spoiling or pathogenic microorganisms which in return also extends the shelf-life of packed food. In this chapter, we will discuss biocomposites used in food packaging, biodegradation of these packaging, and their limitations. We will also see some of the antimicrobial agents used in these food packaging to make it an active food packaging.

Keywords Packaging · Antimicrobial · Biodegradable · Active food packaging · Bacteriocins

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5.1 Introduction

The huge consumption of fossil fuel-derived plastic material has created consistent waste production issues that cause environmental pollution. Tons of plastics are utilized for packaging purposes. Owing to the inappropriate disposal and non-biodegradability of these petroleum plastics, tons of plastics end up in the ocean, and more than 30% of the plastic waste is landfilled. Consequently, the recycling of plastic has become necessary to halt the accumulation of plastic waste in our environment. However, due to contamination of organic substances that is present in food packaging plastics, the recycling system does not work on food packaging systems (Gan and Chow 2018). Hence, food packaging material should not only be maintaining the quality of food but also eco-friendly and biodegradable. For this reason, bio-based polymers, such as proteins, lipids, and polysaccharides, have emerged as an alternative packaging material due to their biodegradability and capability to address the municipal solid waste problem (Sogut and Seydim 2018).

The globalization of food trade has increased demand for fresh produce, minimally processed, and ready-to-eat food products. Most food products are perishable, specially fruits and vegetables. Spoilage can be biological or chemical. Chemical spoilage of food is due to the oxidation process. Biological spoilage is by autodegradation of tissues by enzymes, viral contamination, protozoan and parasitic contamination, microbial contamination, and loss by rodents and insects. The growth of microorganisms is the major problem of food spoilage leading to shortened shelf-life of food (Huis In't Veld 1996). Modern food production processes and cold chains have changed the microbial ecological conditions of food products. Perishable food products requiring cold storage are often vacuum packed or modified-atmosphere packed. These packing methods exploit the growth-inhibiting influence of carbon dioxide on aerobic Gram-negative spoilage bacteria. As lactic acid bacteria (LAB) and some bacteria in the three Enterobacteriaceae family are not susceptible to the influence of carbon dioxide, they are typical spoilage microbes in packed foods. As a part of hurdle technology, active food packaging can be combined with other food preservation techniques or hurdles such as low temperature, acidity, water activity, and modified-atmosphere packaging. Such a multi-preservation proactive approach can increase the shelf-life of foods and reduce the risk of food pathogen growth related to minimally processed, easily prepared, and ready-to-eat "fresh" products. One way of setting up antimicrobial packaging is to adsorb antimicrobial peptides (AMPs) on surfaces (Karam et al. 2013).

The kind of packaging that changes its condition such as to extend the shelf-life or improve safety or sensory properties while not affecting the quality of the food is called active packaging (Khalid et al. 2018). Microbial contamination does not only just reduce the shelf-life of foods but also increases the risk of foodborne illness. Traditional methods used to preserve foods from the effect of microbial growth include thermal processing, drying, freezing, refrigeration, irradiation, modified atmosphere packaging (MAP), and adding antimicrobial agents or salts. However, some of these techniques cannot be applied to some food products, such as fresh

meats and ready-to-eat products like fruits. A promising form of active food packaging is antimicrobial food packaging. The use of films incorporated with antimicrobial agents for packaging could be more efficient, due to slow migration of the agents from the packaging material to the surface of the product. If an antimicrobial agent can be released from the packaging material during an extended period of time, then it can also be used into the transport and storage phase of food distribution (Quintavalla and Vicini 2002).

Several organic and inorganic chemicals have been used as antimicrobial agents in food packaging namely organic acids (da Rocha et al. 2014), bacteriocins (Jin et al. 2009; Yuan et al. 2015), plant extracts (Marcos et al. 2014; Maryam Adilah et al. 2018; Wang and Rhim 2016), natural polymers (Kurek et al. 2014; Pelissari et al. 2009; Schnell et al. 2017), enzymes (Ozer et al. 2016; Yener et al. 2009), nanoclays (Kanmani and Rhim 2014a, 2014b), and metallic oxides (Shankar et al. 2017). As it is confirmed that chemical agents are toxic and susceptible to permeate into the body, the preparation of natural packaging material is very much essential (Lin et al. 2018). The incorporation of antimicrobial agents directly into polymeric packaging is an exciting development, which allows industry to combine the preservative functions of antimicrobials with the protective functions of pre-existing packaging concepts (Scannell et al. 2000).

5.2 Genesis of Packaging Material

Owing to consumer health concerns and environmental problems derived from packaging wastes, various research groups have been looking toward green polymers as biodegradable alternatives to synthetic plastic packages. To acquire this ‘green tag’ by using bioactive preservatives in biodegradable packaging material, researchers have studied incorporation of different bioactive agents in eco-friendly polymers (Imran et al. 2014).

Cellulose-based materials are being widely used as they offer advantages including edibility, biocompatibility, barrier properties, attractive appearance, nontoxicity, non-polluting, and low cost. In formulations of films, different polysaccharide matrices, such as cellulose derivative materials, have been widely used. An example is the polysaccharide HPMC, which is a biodegradable and renewable polymer, as well as an alternative for novel packaging material. Hydroxypropyl methylcellulose (HPMC) is a biopolymer approved by FDA (Food and Drug Administration) and the EU for food uses (21 CFR 172.874). HPMC exhibits good film-forming characteristics, as well as the ability to form thermally induced gelatinous coatings, allowing their use as materials to retard oil absorption in deep frying food products (de Moura et al. 2011).

Lignocellulosics have advantages such as renewability, biodegradability, and low cost over other traditional materials, and hence their use has increased. Biografting provides innovative solutions to increase the performance of lignocellulosics with new properties including strength and stiffness, resistance to moisture, and microbial

attack. Biografting of antibacterial and other organic molecules on lignocellulosic biomass is an environmentally friendly and best approach to incorporate desired functionalities for successful industrial applications. Laccase, lipases, and peroxidases are among the enzymes being investigated for biografting of organic molecules onto lignin for improved properties of lignocellulosic biomass. The lack of availability of suitable antibacterial molecules in large amounts and cost-effectiveness are the major problems for the commercialization of this method (Kalia et al. 2014).

Antimicrobial packaging stops growth of microorganisms in foods and protects the product from external environment, thereby minimizing or totally avoiding addition of preservatives and satisfying the actual demand of consumers for healthier foods, containing less additives. Antimicrobial packaging is a type of active packaging that reduces, inhibits, or retards microorganism growth that can be contaminating the packaged food. Some of the antimicrobial systems include the addition of sachets containing volatile antimicrobial agents into the packages, application of edible coatings with the antimicrobial component, and the incorporation of antimicrobial agents directly to the package material. The incorporation of antimicrobial agents in flexible films is emerging as a promising technology, as the majority of solid or semi-solid foods present high microbial growth in their surface. The antimicrobial component, when establishing contact with food, inhibits the growth of microorganisms present in the surface.

The antimicrobial packaging presents a major advantage over the conventional ones since they avoid addition of preservatives directly to foods, attempting to actual tendency of consumers of searching for healthier foods, minimally processed, and free of additives (Santiago-Silva et al. 2008). Jipa et al. (2012) obtained biodegradable bacterial cellulose (BC)-based films, incorporating sorbic acid (SA) as antimicrobial agents. When BCP concentration was higher, the release of SA was faster. On the other hand, when antimicrobial concentration was increased, the release of SA was significantly slower, due to formed crystal dissolution. Antimicrobial effectiveness was mainly influenced by the amount of SA released and by water solubility of films. These films were effective against *Escherichia coli* K12-MG1655.

5.3 Biocomposites in Food Packaging

Among the various biomaterials present in the market, the one derived from renewable resources, such as starch-based products, are most widespread and economic biomaterials (Avella et al. 2005). Starch is a 2 semicrystalline, widely available, renewable, low cost, and biodegradable agropolymer, consisting of repeating 1, 4-α-D glucopyranosyl units (amylose and amylopectin) and is stored as a reserve in most of the plants (Savadekar and Mhaske 2012). In the presence of a suitable plasticizer, temperature, and shear, the starch granules can be fragmented and melted, and the hydrogen bonds among the starch molecules can be disrupted to

produce thermoplastic starch (TPS), which is capable of being processed and used as replacement of conventional thermoplastics (Castano et al. 2012; Castillo et al. 2013; Rico et al. 2016).

Gliadin films incorporating cinnamaldehyde were highly effective against fungal growth both in vitro and in real food systems. The amount of cinnamaldehyde retained in the film after manufacture, and storage was enough to exert fungistatic and in some cases fungicidal activity against *P. expansum* and *A. niger* in vitro. Active food packaging with gliadin films incorporating 5% cinnamaldehyde increased the shelf-life of both sliced bread and cheese spread (Balaguer et al. 2013).

The agar/CNC bio-nanocomposite film, which is completely biodegradable and biocompatible, has a high potential to be used for food packaging or biomedical applications. Mechanical and water vapor barrier properties of agar film were improved significantly by blending them with the CNC. The tensile modulus and tensile strength of agar film increased by 40% and 3 25%, respectively, in the composite film with 5 wt% of CNC, and the WVP of agar film decreased by 25% after formation of nanocomposite with 3 wt% of CNC (Reddy and Rhim 2014).

Sonia and Dasan (2013) have studied celluloses microfibers (CMF)/poly (ethylene-co-vinyl acetate) (EVA) composites as food packaging material. They were prepared with different weight percentages of CMF. Milk, curd, and orange juice were packed in these composites at normal storage temperatures, i.e., −4, 8, 15, and 25 °C. Barrier property of the composite increased with CMF loading. Biodegradability of composites were studied by inoculating the films with *Aspergillus niger* (*A. niger*) and showed increased biodegradation with CMF.

Nanocomposite films had potential to be used as environment friendly antimicrobial packaging films to improve shelf-life of food (Kanmani and Rhim 2014b). The use of nanomaterials, including metallic as active fillers in polymeric nanocomposites for food packaging, have been extensively investigated. Silver nanoparticles (AgNPs) have been explored as bactericidal agents for technological applications. AgNPs were incorporated into a hydroxypropyl methylcellulose (HPMC) matrix for applications as food packaging materials (Imran et al. 2012). Even if we assume a worst-case scenario where all silver is in its most harmful nanoform, human exposure to silver is likely to be below provisional toxicity limit and current migration limits for conventional migrants. However, it is acknowledged there is still considerable uncertainty about the potential harmful effects of particles at the nanoscale (Cushen et al. 2013).

Ramos et al. (2014) incorporated thymol (the active additive) and modified montmorillonite (D43B) in nano-biocomposite films preparation based on poly (lactic acid) (PLA). Poly (lactic acid) (PLA) is one of the most important bio-based and biodegradable thermoplastic polyesters which is commercially available. PLA is a highly transparent and rigid material with a relatively low crystallization rate, making it a promising candidate for the fabrication of bi-axial-oriented films, thermoformed containers, and stretch-blown bottles.

Composite films of chitosan and gelatin have been reported to have improved mechanical, transport, and physical properties compared with those of single polymer-based films. Chitosan addition increased the antibacterial activity, while it

decreased the antioxidant activities of composite edible films (Jridi et al. 2014). Chitosan–nanocellulose NCP deserved "suitable bio-nanocomposite" title. Tg values of prepared chitosan–nanocellulose bio-composites were higher than those of the most synthetic films. Tm values of the NCPs were in a reasonable range of industrial films; besides, they were particularly attractive for recycling purposes as relating to thermal aspects (Dehnad et al. 2014).

Potato starch based bio-nanocomposite films were made with halloysite nanoclay as the reinforcing materials. These bio-nanocomposites have high potential to be used for food packaging purposes as they have improved barrier and mechanical properties which is due to the addition of halloysite nanoclay (Sadegh-Hassani and Mohammadi Nafchi 2014).

Natural extract obtained from a residual stream generated during the PVPP cleaning process in the brewing industry was incorporated into ethylene vinyl acetate (EVA) films. The films spiked with the highest concentrations of extract provided the best results by retarding both the oxidation of beef samples by around 60% and *S. aureus* growth (Barbosa-Pereira et al. 2014). Antimicrobial nanocomposite films were prepared by incorporating ZnO NPs into the various biopolymers (agar, carrageenan, and CMC) for active packaging. Film possessed a bacteriostatic effect which inhibited growth of various Gram-negative and Gram-positive foodborne pathogens (Kanmani and Rhim 2014a).

Efficient packaging materials with desirable properties (i.e., durability, biodegradability, and mechanical strength) can be formed by using hydrogels. It is a promising and emerging concept, as most of the biopolymer-based hydrogels are supposed to be biodegradable; they can be considered as alternative eco-friendly packaging materials. This article reports about synthetic (polyvinylpyrrolidone (PVP)) and biopolymer (carboxymethyl cellulose (CMC)) based on a novel hydrogel film and its nature of biodegradability under controlled environmental conditions. Variation in mechanical, viscoelastic properties and weight loss of the hydrogel films with time have provided the evidence of their biodegradation (Roy et al. 2012).

5.4 Active Food Packaging

Microorganisms that may be present in the packed food or packaging material are inhibited or retarded by active antimicrobial packaging or active food packaging. An effective antimicrobial packaging or food contact surface should be able to kill or inhibit microorganisms that cause foodborne illnesses (Karam et al. 2013). Controlled release of antimicrobial peptides from active packaging can overcome the quick decline of active compound concentration resulting from dilution into bulk foods and interactions with food components. Diffusion coefficient (D) and partition coefficient (K) values for antimicrobials in packaging films can help in predicting shelf-life of food products.

Bagde and Nadanathangam (2019) incorporated immobilized bacteriocin in corn starch film (57% reduction in bacterial count) which showed enhanced tensile

strength (69%). Bacteriocins (extracted from *P. acidilactici* and *E. faecium*) were immobilized on CNC (crystalline nanocellulose) and were used to reinforce the starch film. The biodegradability of reinforced films was affected due to the use of bacteriocin in fillers. These films stayed fresh for 28 days in an ambient environment, but the films incorporated with bacteriocin alone had fungal degradation in 14 days. This shows that CNC immobilization is required for better stability of bacteriocin during storage.

XRD pattern of NCP in composite films of chitosan and gelatin exhibited that n-cellulose peak disappeared completely after addition to and homogenizing by chitosan matrix, which strengthens the configuring probability of intercalated or exfoliated structures into the NCPs. NCP indicated excellent inhibitory effects against Gram-positive and Gram-negative bacteria, promising for food packaging; application of chitosan–nanocellulose NCP on the ground meat decreased lactic acid bacteria population up to 3.1 logarithmic cycles (compared with nylon packaged sample) at 25 °C during 6 days of storage (Dehnad et al. 2014).

To formulate the next-generation biodegradable films, embedded with either active agent, nano-encapsulated active agent, or both of them, Imran et al. (2012) used fusion of two concepts, i.e., AMP nanoencapsulation and biopolymer immobilizing. Thus, nisin could diffuse/migrate through the liposome (simple model cell membrane) by pore formation as it was demonstrated against bacteria. Nanoencapsulation of nisin in soybean-lecithin was accomplished by an innovative, rapid, efficient and industrially applicable method of microfluidic format without the use of organic solvents. The incorporation of nanostructures in HPMC film-forming solution (FFS) may change the native microstructure (topography and morphology), barrier (O2, H2O), mechanical, color, light transmission, and water sorption properties of formulated packaging films.

5.4.1 Antimicrobial Peptides in Food Packaging

AMPs, also called host defense peptides, are part of the innate immune response and are found among all classes of life. AMPs are small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi (Izadpanah and Gallo 2005). Natural AMPs can be found in prokaryotes (e.g., bacteria) and eukaryotes (e.g., protozoa, fungi, plants, insects, and animals) (Bahar and Ren 2013). The most abundant family of linear AMPs of insect origin are the cecropins including serotoxins and hyphancin produced by moth *Hyalophora cecropia*, *Sarcophaga peregrine*, *Drosophila melanogaster*, etc. (Bulet and Stocklin 2005). In mammals, two broad classes of AMPs have been described—the defensins and cathelicidins (McGwire and Kulkarni 2010). Plant AMPs can be classified into distinct families, viz., thionins, plant defensins, lipid transfer proteins, and hevein- and knottin-type AMPs produced by most of the plants including dicotyledonous species such as *Crambe abyssinica*, *Arabidopsis thaliana*, etc. (Willem et al. 1997).

Nisin, the lantibiotic produced by *Lactococcus lactis* strains, and pediocin, produced by *Pediococcus* spp., are examples of antibiotics produced by bacteria (Papagianni and Anastasiadou 2009).

Bacteriocins are ribosomally synthesized peptides. They bear the characteristics of being nontoxic, heat stable, and non-harmful to humans, with antimicrobial spectrum against food spoilage and pathogenic microorganisms. Bacteriocins are produced by all major groups of bacteria. Bacteriocins, natural metabolites, are mostly heat stable and undergo proteolysis in the gastrointestinal tract. Due to their characteristics, they have gathered the interest of the research and development sector of food industries toward active and intelligent packaging systems from traditional packaging systems, thereby leading to their increased production (Bali et al. 2014).

Pediocins are antimicrobial peptides produced by *Pediococcus* sp., and researches have revealed their ability to inhibit the growth of some pathogenic bacteria. The pediocins are bacteriocins produced for some species of *Pediococcus* genera that exhibit bactericidal effect over some pathogenic and Gram-positive bacteria (Cotter et al. 2005). Their use as natural biopreservatives to overcome the post-processing contamination of meat products (slicing, packaging, peeling, and handling) has been reported, and they are particularly effective against *L. monocytogenes*, pathogenic microorganisms of great importance in food contamination. The films incorporated with pediocin presented high in vitro antimicrobial effect against *L. innocua*, but small effect against *Salmonella* sp. (Paula et al. 2009).

Nisin is a ribosomally synthesized AMP that possesses a relatively broad spectrum of antibacterial activity against Gram-positive foodborne pathogens and spoilage organisms. The Food and Drug Administration (FDA) has recognized nisin (E234) as an approved food additive due to its nontoxicity. The rapid approach using fluorescently labeled nisin can be used to characterize controlled release rates of bioactive peptides from biodegradable films (Imran et al. 2014). Nisin, a bacteriocin produced by strains of *Lactococcus lactis*, has found practical applications in the food industry because of its bactericidal effect against a broad range of Gram-positive bacteria, including many species of *Listeria*, *Staphylococcus*, and spore-forming bacteria like *Bacillus* and *Clostridium* (Karam et al. 2013). Nisin has GRAS status (generally recognized as safe) and is approved for use in the US for pasteurized cheese produced as an anti-botulinal agent, as well as in pasteurized liquid egg ingredients. In other nations, nisin is used on a much wider scale as a food antimicrobial. Nisin and pediocin (a bacteriocin produced by the meat fermentation starter culture bacteria Pediococcus acidilactici) have been demonstrated to be active against *Listeria monocytogenes* and other Gram-positive bacterial pathogens on meat surfaces when applied in a liquid form (Siragusa et al. 1999).

A novel tofu (LAB tofu) was developed by a culture of bacteriocinogenic *W. hellenica* D1501 combined with MTGase. *W. hellenica* D1501 was capable of inhibiting the growth of foodborne spoilage and pathogenic microorganisms in soymilk. Compared with tofu prepared by traditional coagulants, *W. hellenica* D1501 in LAB tofu could effectively prevent the growth of undesired microflora and delay the process of spoilage. During storages at 4 °C for 21 days, 25 °C for

14 days, and 37 °C for 7 days, LAB tofu exhibited a stability of quality properties. In addition, bacteriocins and various antimicrobial volatiles were produced to guarantee the safety of LAB tofu (Chen et al. 2014).

5.4.2 *Bacteriophages in Food Packaging*

Phages can be integrated with edible packaging materials to develop novel active packaging materials for biocontrol application (Vonasek et al. 2013). Phages can interact with their host bacteria and lyse them and then can be used as antibacterial agents. Phages active against *Listeria* or *Escherichia coli* were shown to effectively control the growth of *L. monocytogenes* and *E. coli* O157:H7 in ready-to-eat and raw meat, respectively, under different storage temperatures and packaging conditions (Anany et al. 2011).

Currently, most of the antimicrobial active packaging materials are broad spectrum antimicrobials which do not target bacterial pathogenic species specifically. There is a significant need to develop novel antimicrobial packaging materials that have high specificity to target only pathogenic organisms while maintaining commensal bacteria. The need for specificity in antimicrobial activity is important as pathogens may be a small fraction of the total microbial load present in food systems. Pathogen-specific antimicrobial active packaging materials may improve the antimicrobial efficacy as they act against specific microbes and do not have or have reduced interactions with non-targeted microbes. Further, nonpathogenic microbes are necessary in the production of some dairy and fermented foods. Additionally, the commensal bacteria present may have benefits for human health such as the case with probiotic microbes or even controlling the growth of pathogenic bacteria (Vonasek et al. 2013).

T4 bacteriophage encapsulation was done in edible WPI films. WPI films are able to stabilize phages at ambient and refrigerated conditions without significant loss in phage infectivity over a period of 1 month. Additionally, the WPI films are able to release significant concentration of phages in an aqueous environment within 6 h of incubation. In contrast with the leaf surface, less phage was released to the surface. The release of phages from edible film is mediated by a rapid release of glycerol and swelling of WPI film in aqueous environment. Antimicrobial activity measurements using a growth inhibition assay demonstrate that WPI film encapsulating phages can effectively reduce the rate of growth of *E. coli* (Vonasek et al. 2013).

5.5 Biodegradation of Biodegradable Packaging Films

Biodegradation is a process of breakdown of organic matter by microorganisms, such as bacteria and fungi. The breakdown of materials by microorganisms in the presence of oxygen is aerobic digestion and in the absence of oxygen its anaerobic

digestion. Various factors affect the biodegradation of any material; some of them include type of material, soil condition, microbial flora present in soil, water/moisture, and oxygen content. The more chemical bonds present in the polymer have a significant effect on its biodegradation. Microbes degrade polymers by hydrolysis and oxidation. It is essential that there are hydrolyzable and/or oxidizable (less effective) functional groups along the polymer for biodegradability.

In the process of biodegradation, firstly the long polymer molecules are reduced to shorter and shorter lengths and undergo oxidation (oxygen groups attach themselves to the polymer molecules). This process is triggered by heat (light). Oxidation causes the molecules to become hydrophilic (water attracting) and small enough to be ingestible by microorganisms, setting the stage for biodegradation to begin. Biodegradation occurs in the presence of moisture and microorganisms typically found in the environment. Carbon dioxide, water, and biomass are produced when microorganisms consume the degraded polymer, and they are returned to nature by way of the biocycle.

The speed of biodegradation depends on temperature (50–70 °C), humidity, number, and type of microbes. The degradation is fast only if all three requirements are present. Generally at home or in a supermarket, biodegradation occurs very low in comparison to composting. Polymer-based products are required to biodegrade on a controlled way: natural polymer (like rubber, lignin, humus) and synthetic polymer like polyolefins biodegrade following an oxo-biodegradation mechanism (Arvanitoyannis, 1999) and consequently cannot satisfy the rapid mineralization criteria requested for standard biodegradation. Also, at ambient temperature, oxo-biodegradation is a slower process than hydro-biodegradation as well described by Scott and Wiles (2001). These authors explained that during the oxo-degradation of carboxylic acid, molecules of alcohols, aldehydes, and ketones, biodegradable with low molar mass, are produced by peroxidation, initiated by heat or light, which are the primary cause of the loss of mechanical properties of hydrocarbon polymers. Then, bacteria, fungi, and enzymes start the bioassimilation giving rise to biomass and CO_2 that finally form the humus. Generally, synthetic polymers contain antioxidants and stabilizers added to protect the polymer against mechano-oxidation during the processing operation and to provide the required shelf-life. So, from one hand antioxidants are necessary to improve the performance of these materials, but, on the other hand, for the biodegradation process, it is better not to add these molecules. Hydro-biodegradation is the well-known process that gives bioassimilable products from cellulose, starch, polyesters, etc. Aliphatic polyester is hydrolyzed and bioassimilated rapidly in an aqueous environment in much the same way as starch and cellulose (Scott and Wiles 2001).

Although hydrocarbon polymers make a positive contribution to the environment because they can be mechanically recycled if clean, incinerated with energy recovery, with a calorific value almost identical to the oil from which they come on, they are not compostable. According to the European standard norm UNI EN 13432 (2002), a product to be defined compostable must be biodegradable and disintegrable in brief time, or rather it must be turned from the microorganisms into water, carbonic, and fertile anhydride compost. Finally, to be defined

compostable, the manufactured article must result compatible with a process of composting that means it must not release dangerous substances and must not alter the quality of the produced compost (Scott and Wiles 2001).

5.6 Limitations of Biodegradable Films Used in Packaging

The use of long-lasting polymers as packaging materials is not justified, especially for short application because, due to food contamination, physical recycling of these materials is often impractical. So there is an increasing demand on the use of biodegradable polymers which could be easily renewable (Kale et al. 2006). In real composting conditions, most of the commercialized biopolymer materials do not fully compost though they are biodegradable.

However, some characteristic properties of pure PLA are inadequate for food packaging applications, such as weak thermal stability, low glass transition temperature, low gas barrier properties, and low ductility and toughness. Recently, these poor PLA intrinsic properties have been improved by the reinforcement of the polymer matrix with layered silicates (Ramos et al. 2014). Natural compounds, like cellulose, starch, etc., are not technologically useful for food packaging where water resistance is required as they are hydrophilic polymers, water wettable or swellable, and consequently biodegradable. Between these two extremes are the hydro-biodegradable aliphatic polyesters such as polylactic acid (PLA) and the poly (hydroxyacid) (PHA) (Scott and Wiles, 2001).

5.7 Conclusions

There has been a lot of advancement in food packaging materials for improving or extending the shelf-life of packed food and food products. Various nanobiocomposite films are produced for food packaging. Antimicrobial peptides (AMP), like bacteriocin and nisin, bacteriophages, and other nonmetals, are incorporated in packaging materials as antibacterial agents which retard the growth of food spoiling microbes. Bacteriophages target specific pathogenic bacteria present in food, and hence their use in food packaging materials is significant. Immobilization techniques are the way by which these antimicrobial agents (AMP, bacteriophages, nanoparticles like Ag, cinnamaldehyde) can be incorporated in the packaging materials. Packaging sector can be greatly influenced by nanotechnology. Food packaging can be elevated to new heights by nanoscale innovations in the forms of pathogen detection, active packaging, and barrier formation. Though there is significant research done on biodegradable polymer usage in food packaging, there are still some hurdles which have to be overcome so that they can completely replace and compete with synthetic plastics on a commercial level.

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Part II
Environmental Biotechnology

Chapter 6
3D Printing Technology in the Environment

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Contents

Abstract Environmental pollution has grown at an alarming rate, causing irreversible damages to the planet. Technological advances have contributed to the development of successful strategies for pollution control, through cost-effective remediation processes and devices to monitor such processes. From all different

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types of environmental remediation processes, bioremediation has gained special attention in the last years, considering itself as a leading technology for the treatment of environmental contaminants. However, it still presents several difficulties, mainly when they are large-scale processes. These limitations have driven for new technologies that allow either the environmental remediation as the monitoring of toxic pollutants. Additive manufacturing, also known as 3D printing, is gaining significant attention in the last 10 years, being present in different technological and research fields. Many advantages have been pointed out, such as the rapidity when compared with the traditional subtractive methods, the easiness (price and speed) to produce complex devices, the less waste production, among others. In this chapter, the main aspects concerning 3D printing technology and its application in (bio)remediation are revised, regarding the improvement of the quality of water, soil, and air. Bioremediation strategies with "living ink materials" to the monitoring of toxic pollutants in the environment through printed structures and devices will be analyzed. 3D printing is now an emergent topic that made a paradigm shift in the fabrication of three-dimensional objects, transversal to several areas. Particularly in environmental biotechnology, there is a window of opportunity to explore the full potential of this technology in the development of highly cost-effective strategies for remediation and monitoring of natural samples.

Keywords 3D printing · Environmental biotechnology · Bioremediation · Biomonitoring

6.1 Introduction

The rapid industrialization observed around the world has contributed to the improvement of technological processes. Nonetheless, environmental pollution has grown at an alarming rate, causing irreversible damages to the planet. Global warming, acid rain, air and water pollution, waste disposal, and climate change are some of the main problems that affect every human and animal on Earth. The worldwide concern about this issue has been the subject of several public demonstrations, political summits, sticks foundation nongovernmental organizations, among other actions (Lampert n.d.). The governments and public organizations already recognized these issues and established several laws to minimize the damage. Technological advances have contributed to the development of successful strategies for pollution control, through cost-effective remediation processes and devices to monitor such processes. This can be demonstrated by more than 5000 scientific papers on environmental remediation published since 2010.

The growing demand for effective remediation processes led to the development of various ex situ and in situ physical, chemical, and biological methods (Fig. 6.1). They have been widely used to restore the natural conditions of certain contaminated

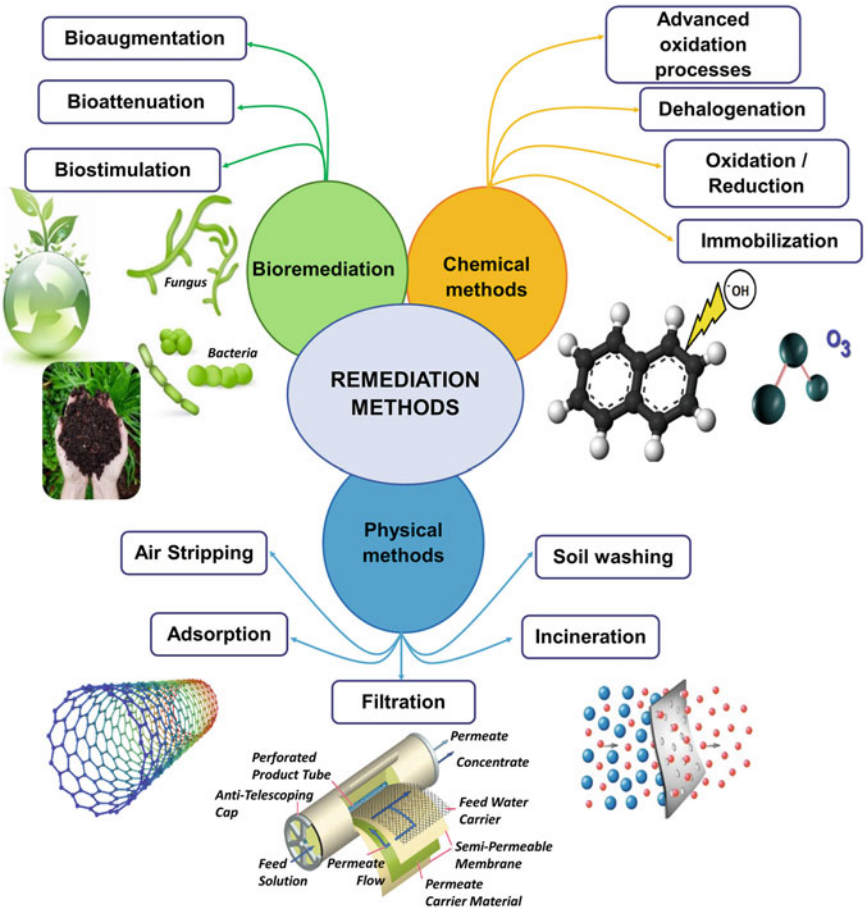

Fig. 6.1 Schematic overview of the environmental remediation methods

environments, such as soils, sediments, surface waters, groundwaters, and air. The physical methods of treatment are considered, among all the methods, the oldest. There are reports of the use of these techniques, from remote times with the ancient Greeks and during the Roman period, where there was technological growth (Angelakis et al. 2018). Physical methods use physical and mechanical barriers to isolate, recover, or separate contaminants. These include filtration, adsorption, coagulation, among others, such as thermal methods (incineration, pyrolysis, thermal desorption, and vitrification). These processes are generally used in the primary treatment of water for human consumption as well as in wastewater, mainly for the removal of turbidity, particles, drugs, heavy metals, and dyes (Andrade et al. 2020; Gerba 2009; Villar da Gama et al. 2018). Chemical treatment methods use chemicals to contain, sequester, precipitate, concentrate, separate, and remove contaminants,

generally, through oxide-reduction mechanisms (Khin et al. 2012). These include advanced oxidation processes, dispersion, emulsification, sonochemical degradation, electrochemical degradation, dehalogenation, among others (Ossai et al. 2020). The application of these methods is much broader compared with physical methods, being effective in the treatment of emerging contaminants (drugs, pesticides, plastics, and natural and artificial hormones) (Adrian and Suflita 1990; Díaz-Rodríguez et al. 2020; Kuhn and Suflita 1989; Rivera-Utrilla et al. 2013), petroleum derivatives (Chen et al. 2016), heavy metals (Basta and McGowen 2004), phenols (Yang et al. 2015), and synthetic dyes (do Nascimento et al. 2020; Martínez-Huitle and Brillas 2009; Robinson et al. 2001). The physical–chemical methods, being widely studied in various pollutant-removal processes, mainly in water treatment plants, have well-established operating mechanisms and parameters. However, they have several disadvantages in their application, such as (1) high-energy costs, (2) high capital for operation and maintenance, (3) generation of toxic waste, (4) addition of toxic chemical agents, (5) training of the operating personnel, (6) poor efficiency in the degradation of organic pollutants, among others.

Biological methods (also known as bioremediation) are based on metabolic activities of certain organisms, such as microorganisms, fungi, plants, or enzymes, that allow the degradation, transformation, or removal of contaminants to harmless metabolic products and restore the natural conditions of a contaminated environment under aerobic (in the presence of oxygen) or anaerobic (in the absence of oxygen) conditions. Bioremediation has gained special attention in the last years, considering itself as a leading technology for the treatment of environmental contaminants (Tan et al. 2016). Before selecting the bioremediation techniques for your application, it is necessary to carefully evaluate some parameters. The nature of the pollutant, the degree of contamination, and certain environmental factors, such as the type of environment, nutrients, oxygen concentration, temperature, pH, among others, are some of the criteria to consider (Azubuike et al. 2016). Generally, these processes have been used successfully to restore environments contaminated with petroleum hydrocarbons, chlorinated solvents, explosives, chlorophenols, pesticides, wood preservatives, and polycyclic aromatic hydrocarbons (PAHs) (Ossai et al. 2020). Bioremediation treatments are considered safe, specific, with fewer energy costs, and more friendly to the environment due to partial or complete bioconversion of organic waste into stable and non-toxic final products (Khan et al. 2013). Another great advantage of these methods is the ability to adapt to the particularities of each effluent and environmental conditions, which draws the attention of the scientific community for the development of new technologies related to this area.

Although bioremediation treatments have the aforementioned advantages, their application still presents several difficulties, mainly when they are large-scale processes (Megharaj et al. 2011; Talley and Sleeper 1997). These limitations can be overcome through a combined approach involving nanotechnology and bioremediation, known as nanobioremediation, in which complex organic pollutants are broken down into simpler and safer compounds through nanoencapsulated biological organisms (Abdel-Aziz et al. 2018; Cecchin et al. 2017; Lee et al. 2007; Mohsenzadeh and Rad 2012). To better understand this process, it must be

remembered that nanoparticles are traditionally produced through physical–chemical methods; however, this situation has been changing due to the need to lower the cost of producing nanoparticles. It is when the biosynthesis of nanoparticles produced initially from microorganisms and later from plant extract arises from the understanding of the mechanisms of metal bioremediation. Metal bioremediation is where microbial enzymes or plant phytochemicals are responsible for the reduction of metallic compounds in their respective nanoparticles, due to their antioxidant or reducing properties, thus allowing the reduction of the costs of synthesis of nanoparticles, known as nanomaterials (Ossai et al. 2020; Yadav et al. 2017). According to Jovanović (2017) , the production of nanomaterials worldwide is underestimated, because, for example, manufacturers in the European Union with <1 ton/year or the United States with <45 tons do not need to report production. Furthermore, monitoring and reporting of the number of nanomaterials in food, food additives, medications, cosmetics, medical devices, or pesticides are not necessary.

New technologies for environmental remediation and monitoring of pollutants have been proposed such as 3D printing technology. It has been adopted in many different areas, allowing the construction of custom-designed 3D objects by additive-manufacturing (AM) processes. 3D printing is now a booming field and is making news everywhere. Not only because of the biggest advantage to construct things inaccessible so far and with reduced waste generation but also by the great opportunity to develop new bioremediation systems and devices capable of monitoring toxic pollutants. For this reason, 3D printing is considered a promising candidate to revolutionize bioremediation technologies.

6.2 3D Printing as a Recent Trend

Additive-manufacturing processes, also known as 3D printing, is a technology with almost forty years old. It uses computer-aided design to build objects layer by layer, while in the opposite, subtractive manufacturing removes the material to create parts. The first published report about AM dates back to 1981 and describes the fabrication of a functional rapid-prototyping system using photopolymers (Kodama 1981). Several outcomes have been described but only since 2011 that 3D printing became a well-recognized technology to produce three-dimensional structures in all manufacturing fields. This trend is demonstrated by the exponential increase rate of published scientific papers that use 3D printing technology (Fig. 6.2a). Many companies are developing and selling 3D printers, which decrease their prices and make them more affordable. Besides, companies are putting hard effort in the improvement of accuracy, selling 3D printers with great resolution. The remarkable advantages on the development cycle of products, such as rapidity and efficiency, combined with the several printable materials available, led to the broad use of 3D printing technology in different areas, which include multidisciplinary material science (e.g., jewellery), electronic engineering (e.g., aircraft and smart robotic

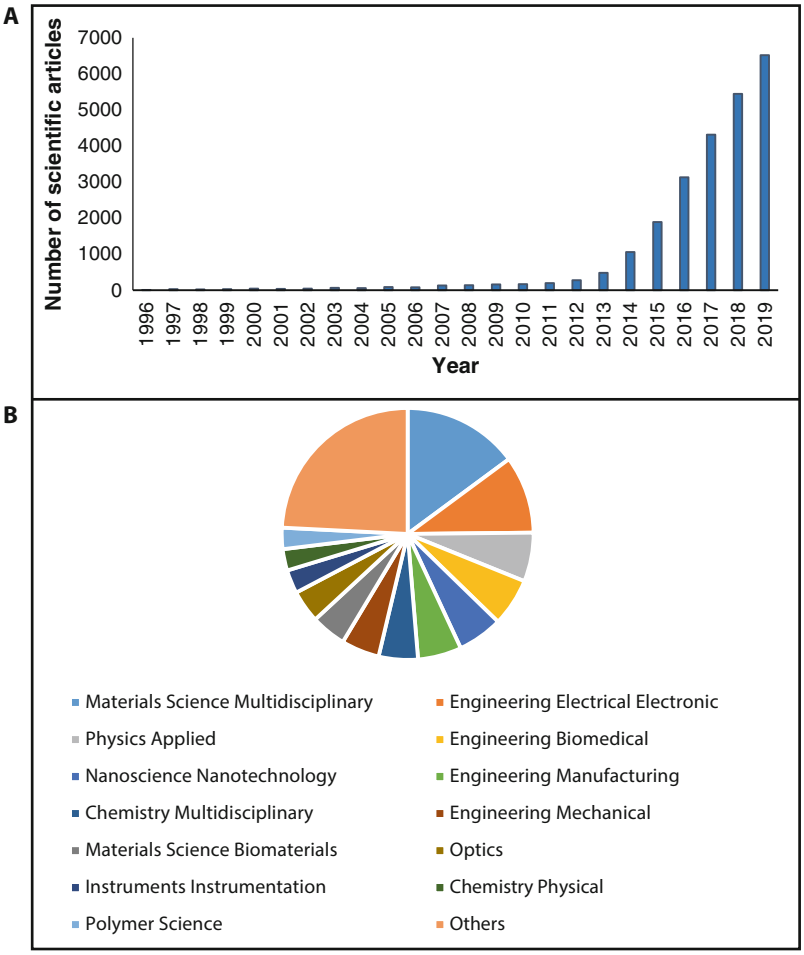

Fig. 6.2 Demonstration of the broad use of 3D printing. (**a**) Evolution of published scientific papers that include 3D printing technology (source: Web of Science database, May 2020). (**b**) Most important fields in which 3D printing has been used in product fabrication (source: Web of Science database, May 2020)

arms), biomedical engineering (e.g., bone replacements), chemistry (e.g., analytical chemistry), among others (Fig. 6.2b).

Different AM technologies have been used, such as powder bed fusion, directed energy deposition, binder deposition, vat photopolymerization, material jetting, and material extrusion (Özel et al. 2016). A brief description of the most used and challenging AM processes is summarized in Table 6.1. Depending on the object to be printed as well as the area where it will be used, a wide range of materials and printers can be used for 3D printing (Table 6.1). Sintered metals, plastics, wax,

Table 6.1 Summary of 3D printing processes and some of their common features

3D printing processes	3D printing technology	Printing process details		Materials	Dimensional accuracy	Lower limit
Material extrusion	FDM (or FFF)	Solid thermoplastic material is pushed through a heated nozzle, melting it in the process		Thermoplastics (PLA, ABS, PP, PEG, TPU)	±0.5%	±0.5%
Vat polymerization	SLA	Point laser	Light source selectively cures a photopolymer resin	Photopolymer resin (standard, castable, transparent, high temperature)	±0.5%	±0.15 mm
	DLP	Voxel approach				
Powder bed fusion	SLS	Thermal energy source will selectively induce fusion		Thermoplastic powder (nylon 6, nylon 11, nylon 12)	±0.3%	±0.3%
Material jetting	MJ, DOD	Droplets of material are selectively deposited and cured on a build plate		Photopolymer resin (standard, castable, transparent, high temperature)	±0.1 mm	–
Binder jetting	BJ	The liquid-binding agent selectively binds regions of a powder bed.		Stainless/bronze, full-color sand, silica (sand casting)	±0.2 mm (metal) ±0.3 mm (sand)	–
Metal powder bed fusion	DMLS, SLM, EBM	Thermal source to induce fusion between metal powder particles one layer at a time		Aluminum, stainless steel, titanium	±0.1 mm	–

FDM fused deposition modelling, also known as fused filament fabrication (FFF), *SLA* stereolithography, *DLP* digital light processing, *SLS* selective laser sintering, *MJ* material jetting, *DOD* drop on demand, *BJ* binder jetting, *DMLS* direct metal laser sintering, *SLM* selective laser melting, *EBM* electron beam melting, *PLA* polylactic acid, *ABS* acrylonitrile butadiene styrene, *PP* polypropylene, *PEG* polyethylene glycol, *TPU* thermoplastic polyurethane

carbon nanomaterials, composites, and more uncommon materials such as stem cells, paper, concrete, food, and yarn are some of the representative examples.

The technological advances of 3D printing have been drawing the attention of scientists and engineers in different areas. Regarding the environmental biotechnology, 3D printing can yield solutions for scientific problems and offer new possibilities for bioremediation processes and biomonitoring of toxic pollutants. As far as we know, in 2017, the first scientific work in which green fuel-driven thumb-sized motors were fabricated by 3D printing for in situ remediation of underwater was published (Yu et al. 2017). The housing structures were printed by stereolithography (SLA), using a photosensitive resin as printable material and further loaded with an engineered *Bacillus subtilis*, for pollutant degradation. The versatility of printable materials and the use of different microorganisms can open new possibilities for highly efficient environmental remediation.

6.3 3D Printing Application in Environmental Biotechnology

3D printing technology has been contributed to the fabrication of structures for physical, chemical, and biological environmental remediation processes. In the first group, the fabrication of membranes based in ceramic for the removal of microbiological particles from water (Hwa et al. 2018) and composite materials to separate oil from water (Al-Shimmery et al. 2019) is one of the representative examples. Other pioneering work of physical remediation described the fabrication of electrospun nanofibers on 3D printed polymers to prepare mechanically stable water filters (Kozior et al. 2019). In the second group, the photocatalytic degradation of amoxicillin in water using 3D-printed chitosan scaffold as support for titanium dioxide (Bergamonti et al. 2019) and the catalytic degradation of other pharmaceuticals and illicit drugs in wastewater using 3D-printed polyvinyl alcohol ferrate (VI) capsules (Czolderova et al. 2018) were reported. In the third group, bioprinting is now a booming field where complex-patterned biomaterials can be fabricated to achieve spatial cellular and chemical composition control. The mixing of bacterial species with various bioinks to produce functional complex materials is the most attractive example.

Biotechnological processes for environmental remediation have been the focus of the biodegradation of organic matter of municipal wastewater and biodegradation/detoxification of hazardous substances in industrial wastewater. Other applications are in the prevention of pollution and restoration of water quality in reservoirs, lakes and rivers, coastal areas, in aquifers of groundwater, and treatment of potable water. The continuous monitoring of the treatment processes and the detection of specific pollutants in the environment are also important applications. However, the efficiency of actual applications depends on the process design and optimization, which should be carried out at the lowest cost as possible to develop cost-effective

solutions. Many failures have been reported due to the ineffective scale-up from lab to field application (Talley and Sleeper 1997), mainly due to the instability and diversity of both microbial properties and conditions in the treatment system.

3D printing has revolutionized the concept of object manufacturing, making an enormous impact on industry and economy. Particularly in the environmental biotechnology, it has rendered practical solutions to scientific problems by offering the fabrication of materials inaccessible so far. The immobilization of living cells and/or microorganism along with bioactive molecules to be distributed into free-formed geometries represents a paradigm shift in biotechnology. This powerful technology shows an enormous potential for applications in bioremediation of wastewater and biosensing of toxic chemicals.

6.3.1 3D Technologies for Bioremediation

Microorganisms have been used for many years to remove organic matter and toxic chemicals from domestic and manufacturing waste discharge. Interestingly, a wide variety of these microorganisms form self-aggregated systems, called "biofilms," which are used to degrade the pollutants in the natural environment and engineered systems, such as the bioreactors. However, dense microbial aggregates may limit the diffusion of nutrients into the biofilm and their metabolites or toxic degradation products out of it. Besides, the lack of manufacturing tools that enable immobilization of bacteria in a biocompatible medium hinders the development of highly efficient and cost-effective technology.

3D printing technology with programmable biochemical machinery of microorganisms can be the solution to create "living materials" with controlled 3D shape, microstructure, and dynamic metabolic response. Ink materials integrate living cells with supporting hydrogels to produce printable ink that can be further transformed into customized 3D geometries. Microorganisms capable of degrading toxins, synthesize vitamins, produce cellulose, and perform photosynthesis can be loaded in these inks (Schaffner et al. 2017). In a recent work, a functional living ink, called "Flink," was described as a biocompatible immobilization medium with suitable viscoelastic properties for 3D printing of various cells through direct ink writing (Schaffner et al. 2017). The medium was a hydrogel ink comprising hyaluronic acid, *k*-carrageenan, and fumed silica, which was mixed with the living organisms. The immobilization of *Pseudomonas putida* in a 3D-printed lattice capable of converting hazardous phenol into biomass is a representative example (Schaffner et al. 2017). This pioneering work represents the high potential for new applications, in which bacteria with different functionalities can be immobilized in limitless complex shapes and material compositions. These shapes can be designed to promote dynamic functionalities, in which the products of bacterial metabolism can diffuse inside the printed structure and work as substrates to other bacteria. Very soon, real 3D-printed bacterial minifactories can be fabricated for bioremediation and environmental clean-up (Fig. 6.3).

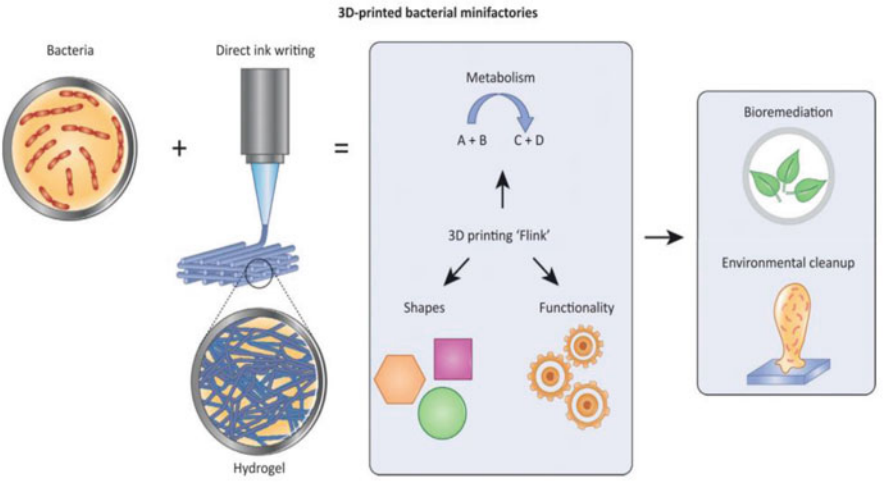

Fig. 6.3 Fabrication of functional living materials using 3D-printed bacterial minifactories. Multifunctional bacteria are embedded in bioink versatile hydrogels and deposited in specific 3D geometries for the creation of living and responsive materials. This strategy promises a future of smart, adaptive, and multifunctional, yet economically and environmentally sustainable, (bio)-materials. Reproduced from Kyle (2018). Copyright © 2018 with permission from Elsevier

Moreover, the ability of microorganisms for bioremediation can be modified and amplified through artificial modifications of their genetic properties. Recombinant DNA techniques or genetic engineering can create a new artificial combination of genes and increase the number of derived genes in the cell. This contributes to the improvement of the metabolic pathways of organisms, expanding the range of the substrate and stabilizing their catabolic activity, which increases the remediation efficiency of the target compounds (Azubuike et al. 2016; Juwarkar et al. 2010). At the same time, some inexpensive commercially available 3D printers can perform multichannel printing, in which multiple types of cells and chemicals can be printed in 3D structures. Therefore, the use of different genetically modified organisms (GMOs) printed with a design allowing the communication between them can create complex logic gates in the final 3D-printed material (Liu et al. 2018). True "living responsive devices" could be fabricated to explore novel functions and revolutionize environmental biotechnology.

Biofilm-based algae cultivation has also received increased attention as a potential platform for bioremediation, particularly of wastewater (Gross et al. 2015). Algae are eukaryotic microorganisms that assimilate light energy and are used in environmental biotechnology for the removal of organic matter and nutrients from water exposed to light (Ivanov and Hung 2010). However, light attenuation due to algal self-shading is a key limiting factor for the photosynthetic efficiency and upscaling of microalgae cultivation. Nonetheless, 3D printing technology already made attractive contributions to overcome this limitation and boost the photon augmentation process.

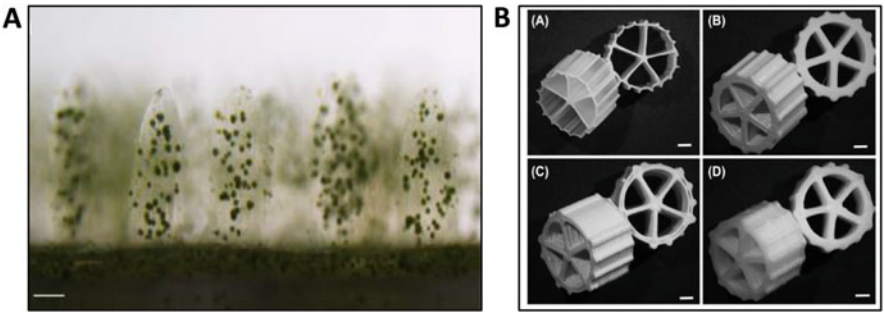

Fig. 6.4 Representative examples of 3D printing applications in algae cultivation. (**a**) Living 3D-printed bionic coral. Horizontal view of 7-day old bioprinted construct, showing aggregates of the green microalga *Marinichlorella kaistiae KAS603*. Scale bar: 0.05 mm. Reproduced from Wangpraseurt et al. (2020). (**b**) Polymeric structures used to settle and cultivate *U. tepida.* (a) Bio-media made of PE purchased off-the-shelf was used as template for 3D-printed structures made of (b) PLA, (c) ABS, and (d) PP. Scale bar: 2 mm. Reproduced from Praeger and de Nys (2018). Copyright © 2018 with permission from Elsevier

To protect or restore the biodiversity and fishery resources in the marine environment, an artificial reef fabricated by a giant 3D printer using dolomite Sorel cement, as substrate, was described (Riera et al. 2018). The choice of this appropriate substrate was fundamental for biofilm development at the surface, which affected the subsequent colonization by multicellular eukaryotes and lastly its efficiency in bioremediation and environment protection. In a different case, polyjet technology allowed the fabrication of bionic corals capable of growing microalgae with high spatial cell densities of up to 10^9 cells/mL (Wangpraseurt et al. 2020). Corals have been evolved as optimized photon augmentation systems, but their efficiency is still limited by the light attenuation. In this particular example, a platform that mimics morphological features of living coral tissue was fabricated (Fig. 6.4a). The printable material was a bioink based on microalgae mixed with a photopolymerizable gelatin-methacrylate hydrogel and cellulose derived nanocrystals. Similarly, the artificial skeleton was also 3D-printed with a PEG diacrylate-based polymer doped with cellulose-derived nanocrystals. The fabricated bionic coral increased the photon residence time since light travels through the algal culture, which increases the light absorption for a given density of microalgae. However, a comparison with commercial photobioreactors was not possible, because the printed bionic coral was only at a centimeter scale. Nonetheless, this 3D bioprinting process allows the encapsulation of different algal species and supports their growth in a customized habitat. This contribution will certainly inspire the scientists for the development of novel systems to improve the efficiency of photon augmentation process by algae.

In another work, combining FDM technology with microalgae cultivation, it was possible to prepare free-floating cultivation instead of the usual attachment to fixed structures. 3D-printed polymeric structures, named bioballs, were fabricated and seeded to determine the effect of their buoyancy on algal growth (Fig. 6.4b) (Praeger and de Nys 2018). Three polymeric materials with different densities were

investigated (ABS, PLA, and PP), which affected their buoyancies and lastly their position in the water. An improvement in the cultivation process of the species with specific needs was noticed. For example, bioballs with negative buoyancy can be used for species that prefer low-light environments, while structures with positive buoyancy can be used for species with high-light requirements (Praeger and de Nys 2018). Overall, 3D printing can offer an innovative solution for the simultaneous cultivation of multiple species based on the printing of structures with different buoyancies.

Despite these attractive features, 3D printing technology has not been widely used to fabricate advanced functional laboratory-scale bioreactor components or develop new cultivation strategies, which is proven by the very few examples founded in the literature (Table 6.2). Much more time investment in the research and development must be done concerning the reusability, scalability, printing times, and the environmental impact of 3D-printed living systems. On the one hand, 3D printing makes possible to design, optimize, and fabricate structures dimensioned to the purpose at a reduced cost like artificial coral reefs, while on the other hand, it is necessary to evaluate the impact of the printing materials, such as monomers and cross-linking agents, in the aquatic environment.

Additionally, the development of new non-toxic ink materials and 3D geometries that favor long-term cell functionality is an important factor for the creation of stable and effective biofilms. The combination with GMOs will certainly boost the field applications, but their use for large volume bioremediation applications is still limited by the uncontrolled risk of exposition to the environment and human health.

6.3.2 3D Technologies for (Bio)monitoring

Biomonitoring is an appealing tool for the assessment of environmental imbalance, in which a wide range of biological methods are used to detect pollutants and to monitor the concentration and pathogenicity of microorganisms in wastes and in the environment.

Biosensors have been used in this field due to their simplicity, low cost, and ability to develop portable devices. Biosensors are devices that convert a biological response into a physical, chemical, or electrical signal, which is measurable. The development of biosensors involves the integration of a sensing element, which can be whole cells, enzymes, or antibodies, with a physic–chemical transducer to detect specific molecules of pollutants. Organic acids, glucosinolates, aromatic hydrocarbons, pesticides, and pathogenic bacteria are some of the representative examples of these pollutants.

3D printing technology has contributed to these advancements in environment (bio)monitoring, providing practical solutions to scientific problems. Several devices can be easily and quickly printed with suitable control in design and geometry, resorting to different printable materials. The most trending applications can be divided into three main groups: operating and supporting components, fluidic

Table 6.2 3D printing technologies used in bioremediation applications

3D printing technology	Printer supplier	Material	Device type	Degree of integration	Application	Ref.
Polyjet	3D discovery	Bioink (hyaluronic acid, κ-carrageenan and fused silica mixed with *Pseudomonas putida*)	Bioreactor	Biofilm	Degradation of phenol	Schaffner et al. (2017)
Polyjet	–	PEG diacrylate-based polymer doped with cellulose-derived nanocrystals	Bionic coral	Coral skeleton	Fabrication of an artificial coral	Wangpraseurt et al. (2020)
		Bioink (gelatin-methacrylate and cellulose-derived nanocrystals mixed with microalgae)		Coral tissue		
FDM	Prusa Mk2S LulzBot Taz 6	PLA ABS, PP	Bioballs	Structure	Investigation of buoyancy in algae cultivation (*Ulva Tepida*)	Praeger and de Nys (2018)
SLA	Pegasus Touch	Photosensitive resin	Thumb-sized motor	Structure	Remediation in situ of underwater by *Bacillus subtilis*	Yu et al. (2017)
-	D-Shape	Dolomite sorel cement	Artificial reefs	Substrate	Monitoring of biofilm colonization	Riera et al. (2018)

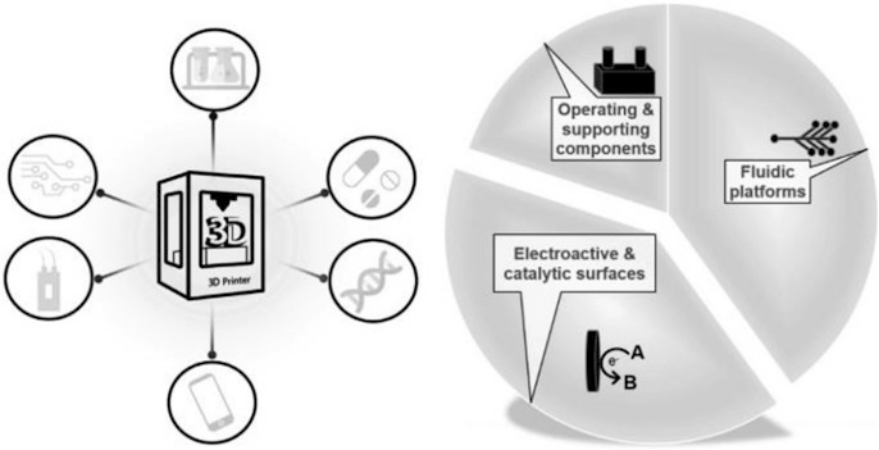

Fig. 6.5 Representation of the most trending applications and roles of 3D printing in (bio)-monitoring. Reproduced from Manzanares Palenzuela and Pumera (2018). Copyright © 2018 with permission from Elsevier

platforms, and electroactive and catalytic surfaces (Fig. 6.5). Smartphone interfaces, support structures (such as housing for devices), labware, microfluidic devices, and even electrodes for electroanalytical applications are the representative examples, which are summarized in Table 6.3.

6.3.2.1 Operating and Supporting Components

The development of real-time and portable instruments is required for the cost-effective monitoring of the environment quality in water, air, and soil systems. The great potential of biosensors associated with the main significant advantages of 3D printing (speed, cost, flexibility, tangible design, and product testing, quality, and consistency) has been drawing the attention of scientists to develop and self-produce operating and supporting components.

An interesting example refers to the development of a smartphone biosensor based on colorimetric detection of zearalenone, a mycotoxin produced by several species of *Fusarium* fungi (Chen et al. 2017). The smartphone is equipped with a custom-designed and 3D-printed accessory for the readout of colorimetric assays, which was made by FDM with PLA as printable material. The proposed smartphone biosensor allowed the monitoring of this specific contaminant in the concentration range of 1.6–25.0 μg/L. A similar supporting component was proposed to be attached to a smartphone to develop a sensing platform for quantitative detection of thiram (a dithiocarbamate pesticide) in tap water by fluorescence response (Chu et al. 2020). The constructed device enabled the determination of this pesticide with a detection limit of 14.0 μg/L. The low cost of the printed accessories and the usefulness as a

Table 6.3 3D printing technologies used in (bio)monitoring applications, including operating and supporting components, fluidic platforms, and electroactive surfaces

3D printing technology	Printer	Material	Device type	Degree of integration	Application	Ref.
Operating and supporting components						
Polyjet	Objet 30, stratasys	PMMA	MFC—microbial fuel cell	The membrane-less single-chamber MFC in an open air	Monitoring glutaraldehyde, nickel (II), and chromium (III)	Agostino et al. (2020)
Polyjet	Objet260 Connex1, stratasys	Photopolymer (VeroBlackPlus)	Disposable wireless sensors	Housing	Monitoring temperature, humidity, and H_2S	Farooqui et al. (2017)
Polyjet	Objet24, stratasys	–	JetLid, tipwaster	Housing and parts	Monitoring temperature, lighting, humidity, presence, motion, and switch states	Courtemanche et al. (2018)
FDM	PowerWasp, WASProject	PLA	nEMoS—nano environmental monitoring system	Housing	Monitoring air temperature, relative humidity, radiant temperature, wind speed and direction, lighting, and CO_2 concentration	Salamone et al. (2015)
FDM	Shining 3D	PLA	Colorimetric reader	Accessory piece to attach in the cell phone	Monitoring zearalenone in environment	Chen et al. (2017)
FDM	Zortrax M200	Z-ABS	Portable device for field deployment of living biosensors	Full device	Fabrication of a portable device for field deployment of living biosensors	Movizzo et al. (2019)
FDM	–	PLA	Humidity sensor	Housing	Monitoring humidity	Yusof et al. (2019)

(continued)

Table 6.3 (continued)

3D printing technology	Printer	Material	Device type	Degree of integration	Application	Ref.
SLS	–	Graphite-reinforced nylon	PiSpec	Housing	Monitoring SO_2 levels	Wilkes et al. (2019)
–	–	–	Smartphone sensing platform	Housing	Monitoring thiram (pesticide)	Chu et al. (2020)
–	–	PMMA	MEMS—microelectromechanical system	Channel in the device	Detection of nanoparticles in the air	Kwon et al. (2019)
–	–	PE	Impinger device	Housing	Collecting bio aerosols	Powers et al. (2018)
Fluidic platforms						
SLA	MiiCraft	BV-001 (acrylic)	Flow system (FIA)	Knotted reactor	Differentiation of silver ions and nanoparticles for their assessment	Su et al. (2016)
SLA	Form1+	PMMA	Flow system (MPFS)	Fluidic platforms (Column reservoir for resin, mixing coil, and detector cell)	Monitoring of lead	Mattio et al. (2017)
SLA	3DS Projet HD 3500	ABS	Chromatographic column	Column housing and packing	–	Fee et al. (2014)
SLA	Miicraft	BV-003 (acrylic)	Lab-on-chip	Full device	Monitoring of urinary protein	Chan et al. (2016)
SLA	Felix 3.0	PLA	Photodiode detector	Slit and housing	Monitoring of zinc and copper complexes	Cecil et al. (2017)
SLM	Realizer SLM50	Titanium	Chromatographic column	Column structure	Separation of protein and peptides	Gupta et al. (2016)

DLP	Miicraft 100	BV-007 (acrylic)	Flow system (MSFIA-LOV)	Lab-on-valve unit	Monitoring of lead and cadmium	Mattio et al. (2018)
Electroactive and catalytic surfaces						
SLM	Concept laser GmbH	Stainless-steel	Electrochemical sensor	Electrode body (electroplated with a metal)	Monitoring of lead and cadmium	Lee et al. (2017)
SLM	Concept laser GmbH	Stainless steel	Electrochemical sensor	Electrode body (electroplated with a metal)	Monitoring of phenolic compounds	Cheng et al. (2017)
SLM	Concept laser GmbH	Stainless steel	Electrochemical sensor	Electrode body (electroplated with a metal)	Monitoring of nitroaromatic compounds	Tan et al. (2017)
SLM	Concept laser GmbH	Stainless steel	Electrochemical sensor	Electrode body (electroplated with a metal)	DNA biosensing	Loo et al. (2017)
SLM	Concept laser GmbH	Stainless steel	Electrochemical sensor	Electrode body (electroplated with a metal)	Monitoring of paracetamol	Liyarita et al. (2018)
SLM FDM	Concept laser GmbH FlashForge (dreamer)	Stainless steel PLA	Water electrolyzer	Electrode body Housing	Production of H_2 (renewable energy)	Ambrosi and Pumera (2018a)
FDM	–	Composite material (carbon nanofibers, graphite flake microparticles, and polystyrene)	Electrochemical sensor	Electrode body	Monitoring of lead	Rymansaib et al. (2016)

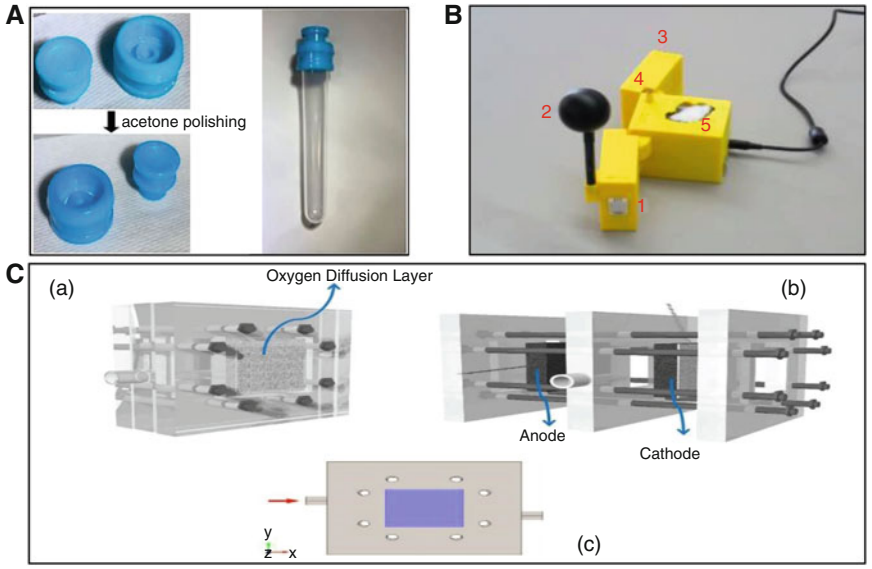

Fig. 6.6 Representative examples of 3D printing applications in the fabrication of support and housing structures for analytical devices. (**a**) Environment-friendly bioassay device. Left: The 3D-printed housing and plunger before and after exposure to an acetone vapor bath for 1 h; Right: The fully assembled bioassay device attached to a 14-mL culture tube. Reproduced from Movizzo et al. (2019). Copyright © 2019 with permission from Elsevier. (**b**) Assembled 3D-printed device to assess the indoor environmental quality, called nEMoS. The numbers correspond to different sensors. Reproduced from Salamone et al. (2015). (**c**) Schematic view of the squared single-chamber MFC biosensor. (a) assembled device, (b) exploded view, (c) y–x plane of intermediate layer. Reproduced from Agostino et al. (2020). Copyright © 2020 with permission from Elsevier

point-of-use device can provide scalable and cost-effective instruments based on 3D printing for real-time and in situ detection.

Genetic engineering allowed us to fabricate living biosensors based on GMOs to detect specific pollutants more efficiently. However, its release into the environment is prohibited, which hinders the application of GMO-based biosensors for in situ monitoring. Nonetheless, 3D printing technology offered a solution for this limitation, because a robust portable housing device was fabricated by FDM, using ABS as printable material (Movizzo et al. 2019) (Fig. 6.6a). The printed structure was designed to contain an engineered *Escherichia coli*, which was further attached at the top of a common culture tube and successfully used for the detection of the chemical signal of *Pseudomonas aeruginosa*, an opportunistic pathogen (Wolozny et al. 2019). The freeze-dried bacteria can be loaded into the printed housing structure, sealed, and be ready for use or transported to the point-of-use. No application in the environmental field was described even though this 3D-printed structure enables the use of a GMO-based biosensor safely. This contribution

combined with the versatility of genetic engineering can revolutionize the biosensing systems, regarding the development of novel and cost-effective monitoring systems.

Another promising approach refers to the fabrication of microbial fuel cells (MFCs), in which the conversion of the chemical energy stored in organic matter into electricity is performed directly, through reactions catalyzed by electroactive microorganisms. In MFC-based biosensors, microbial metabolism is the driving force for the chemical energy conversion into electrical energy. Thus the sensing and signal transduction steps are integrated into a single element, without the need of a transducer and an external power source. Resorting to 3D printing technology, a single-chamber MFC was printed by polyjet and used for freshwater toxicity monitoring (Fig. 6.6c) (Agostino et al. 2020). A linear range was obtained for glutaraldehyde in a concentration level between 5 and 1000 mg/L, while nickel(II) and chromium(III) could be detected at concentrations above 2 mg/L. The implementation of such a device can offer a low-cost and self-contained system for in situ detection of water contaminants.

Although biosensing systems represent an important application for biotechnology, other practical solutions to monitor environmental conditions are also described (Table 6.3). The real-time monitoring of the environment is beneficial, as it can provide early warnings in crises, such as floods, forest fires, and industrial leaks, which can save lives and property. In this context, a disposable and compact 3D-printed sensor with integrated microelectronics was developed and dispersed in the environment for large area-monitoring applications (Farooqui et al. 2017). The developed wireless sensor incorporates multiple fully inkjet-printed sensors, based on a photopolymer material, to monitor humidity, temperature, and H_2S gas levels. Another example refers to the development of a custom-designed spectrometer based on smartphone sensor technology, called PiSpec, for the monitoring of volcano gas (Wilkes et al. 2019). The optical components were mounted into a housing structure printed by SLS using graphite-reinforced nylon as material. This device provides a linear range at concentration levels between 0 and 4000 mg/L of SO_2, with a lower limit of detection of 50 mg/L.

The ability of 3D printing technology to fabricate small structural elements can also be combined with microcontrollers, like simple and small computers. The purpose is to develop novel hardware solutions, such as 3D-printed housings and parts, to monitor temperature, light, humidity, animal presence, motion, and switch states (Courtemanche et al. 2018) or even liquid levels in bottles and carboys (Courtemanche et al. 2018).

The evolution of AM processes has provided new means of open design making possible a faster development of integrated and complete devices. Herein, an "all-in-one" device, called nano Environmental Monitoring System (nEMoS), was developed to assess the indoor environmental quality. The housing structure was fabricated by FDM using PLA as printable material (Fig. 6.6b) (Salamone et al. 2015). The device was equipped with different sensors to monitor air temperature and relative humidity, radiant temperature, wind speed and direction, lighting, and CO_2 concentration. Finally, the collected data is sent to a cloud server for storage, thanks to a Wi-Fi shield mounted on the nEMoS.

A recent trend in 3D printing is the search for novel materials with enhanced properties for the fabrication of functional devices. Accordingly, a novel polymeric composite material was used for the fabrication of a low-cost and light-weight humidity sensor resorting to FDM technology (Kalsoom et al. 2020). The new composite material consisted of ABS loaded with boron-doped diamond and lithium chloride. While the first is a well-known electrically conducting material and works as the electrode material, the second is a classic electrolyte material widely used in humidity sensors. The customized 3D-printed sensor doesn´t need additional electrodes or conducting materials, which represents the main advantage over other humidity sensors. However, this fabricated device was only used for monitoring the humidity of an N_2 supply, but other practical applications can be further investigated.

6.3.2.2 Fluidic Platforms

In a laboratory scale, the combination of software design with 3D printing technology allows the fabrication of fluidic platforms in a more affordable and fast way, which can be used for environmental pollutants monitoring. Any change in the design can be carried out without external workshops that increase the experimental time and the price per unit. A simple example is the design of different channel geometries in microfluidic devices for experimental optimization. The most attractive contributions of 3D printing in fluidic platforms comprise sample collection, pre-treatment, mixers and reactors, separation, and detectors (Cocovi-Solberg et al. 2018). The fabrication of functional elements, such as valves, pumps, and multiplexers, has also been described (Weisgrab et al. 2019). Various review papers have been devoted to exploring the potential of 3D printing on the fabrication of cost-effective platforms in both millifluidic, microfluidic, and lab-on-chip configuration (Cocovi-Solberg et al. 2018; Gross et al. 2017; Salentijn et al. 2017; Weisgrab et al. 2019; Yazdi et al. 2016).

A 3D-printed reactor to sample pre-treatment was fabricated by SLA and inserted in a flow system for quantitative assessment of silver ions and nanoparticles in municipal wastewater samples (Su et al. 2016). A lab-on-valve unit, printed by DLP, was also described for the quantification of lead and cadmium in natural waters, such as spring, city canal, and well waters (Mattio et al. 2018). Other printing techniques (polyjet and SLA) were also investigated during the fabrication of the lab-on-valve unit, but worst results were obtained, namely clogging problems and uneven surfaces in printed structure (Mattio et al. 2018). This particular feature clearly shows the differences in the accuracy and precision of different 3D printing techniques.

A more complete flow system was designed and produced for the spectrophotometric determination of lead in natural waters (Mattio et al. 2017). The sample pre-treatment, mixing coil, and detector units were printed by SLA using a poly (methyl methacrylate) (PMMA) as the printable material (Fig. 6.7a). The flow system exhibited a linear response from 3 to 120 μg/L, with a detection limit and a sampling rate of 2.7 μg/L and 4 samples/h, respectively.

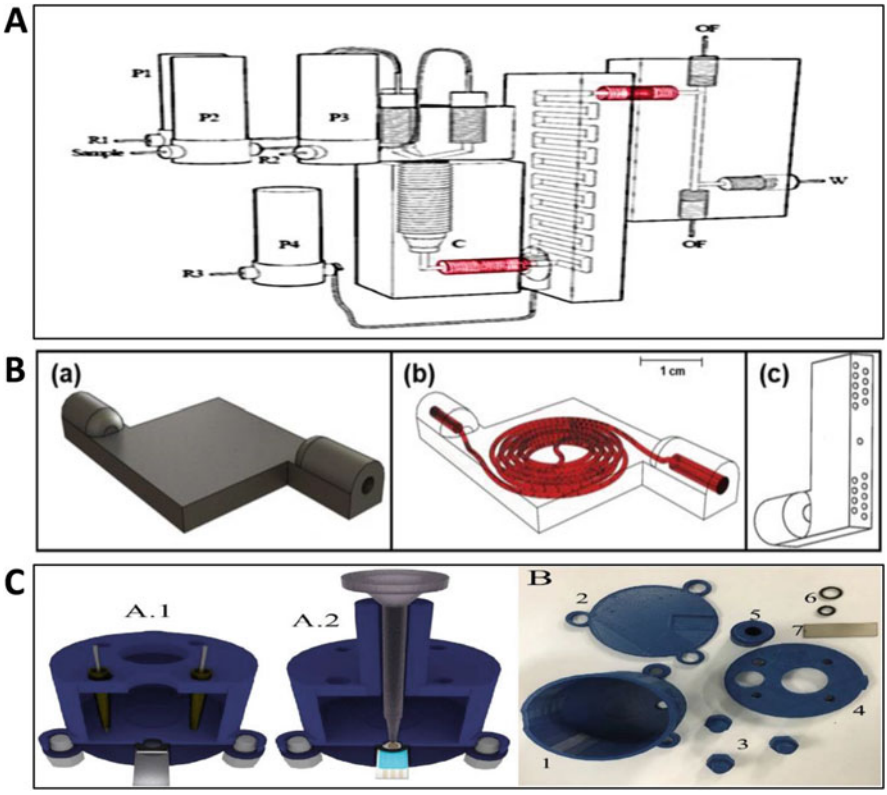

Fig. 6.7 Representative examples of 3D printing applications in the fabrication of fluidic devices. (**a**) Schematic view of the flow system with the three 3D-printed units, which includes pre-treatment, mixing coil, and detector parts. In red, the screws and corresponding screw threads to assemble the units. Reproduced from Mattio et al. (2017). Copyright © 2017 with permission from Elsevier. (**b**) Design of titanium alloy column hardware: (a) image of the 3D-printed column hardware, (b) clear view of the design demonstrating the double-handed spiral channel, (c) transverse section demonstrating the three-dimensional orientation of the channel. Reproduced from Gupta et al. (2016). Copyright © 2016 with permission from Elsevier. (**c**) Schematic view of the 3D-printed cell (transversal cut view) used for batch (1) and flow (2) electrochemical measurements. (B) Components of the 3D-printed cell. Reproduced from Cardoso et al. (2018). Copyright © 2018 with permission from Elsevier

In a different trend, the contribution of 3D printing technology in the field of separation science is opening a new world of possibilities. The creation of highly customized devices, novel packing materials, and structures, with complex geometries, are the most remarkable applications (Umme Kalsoom et al. 2018). As a representative example, a titanium alloy chromatographic column in the form of a double-handed spiral (Fig. 6.7b) was 3D-printed by SLM (Gupta et al. 2016). The channel was then filled with a porous monolithic stationary phase based on methacrylate by thermal polymerization (Gupta et al. 2016) or packed with reversed-phase octadecylsilica particles (Sandron et al. 2014). The high thermal conductivity of

titanium (compared with stainless steel printed columns) and the planar structure of the column enabled the combination with a heater/cooler module for rapid temperature gradients. The monolithic-type column was then used for the separation of proteins and peptides obtained after the digestion of *Escherichia coli*, one of the most common microorganisms used in bioengineering (Gupta et al. 2016). Also, the fabricated columns were compact, portable, and robust, which combined with software design tools, enables the construction of new complex geometries, which were inaccessible so far.

Fee et al. (2014) demonstrated the use of 3D printing to prepare porous media with precisely defined packing morphologies. It is noteworthy that the ability of the printed polymeric substrates to be used directly (without additional functionalization) for separation, resorting to different polymers with different polar and non-polar functionalities (Cocovi-Solberg et al. 2018). This example clearly shows the high impact of 3D printing, extending the possibility to fabricate chromatographic columns in a single step for portable and low-cost chromatographic systems.

An interesting contribution of 3D printing on separation platforms used in the chemical analysis was represented by the fabrication of a single-piece photodiode detector, including integrated slit and housings. This was possible by resorting to the FDM printing technique and by using PLA as printable material (Cecil et al. 2017). The printed detector was used as proof of concept for the analysis of zinc and copper complexes in river water samples after separation by capillary electrophoresis. Another remarkable solution for remote environmental deployments and point-of-care medical use regards the integration of the flow and detection system in the so-called "lab-on-chip" systems. The fabrication of such devices can be largely simplified and boosted by 3D printing technology (Weisgrab et al. 2019; Yazdi et al. 2016). Accordingly, a microfluidic chip, including pump and valves that can be operated manually without any bulky equipment, was 3D-printed by SLA using an acrylic material (Chan et al. 2016). The on-chip colorimetric assay connected to a smartphone was applied for the urinary protein quantification, but the potential for applications in the environmental field is very high.

The use of 3D printing is also an attractive solution for producing suitable flow-cells, particularly in the case of electrochemical detection (Fig. 6.7c) (Snowden et al. 2010) in which the fabrication of the electrodes was also possible by 3D printing using different conductive materials (Cardoso et al. 2018).

6.3.2.3 Electroactive and Catalytic Surfaces

AM techniques can be applied to composite materials containing a catalyst, electroactive, or reactive component, combined with a suitable material for their incorporation into a 3D-printed device, to detect and monitor target species (Ambrosi and Pumera 2016; Kitson et al. 2016; Symes et al. 2012).

Conductive blends based on carbon nanofibers and graphite flake microparticles added to polystyrene polymer were prepared and used for the fabrication of

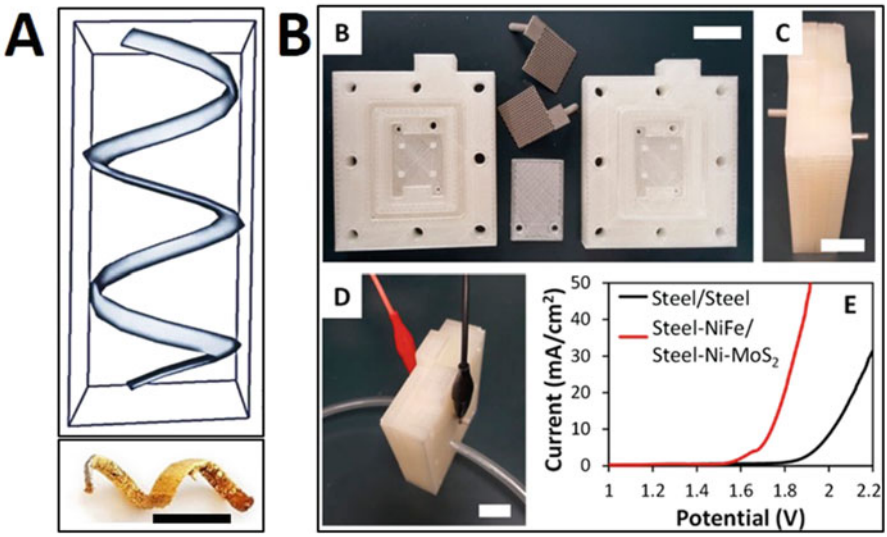

Fig. 6.8 Representative examples of 3D printing applications in the fabrication of catalytic, electroactive, and/or reactive objects for analytical devices. (**a**) 3D-printed helical-shaped electrodes. Top: Schematic view of the electrode design by CAD software. Bottom: Picture of the 3D-printed electrode after Au electroplating. Scale bar: 10 mm. Reproduced from Cheng et al. (2017). Copyright © 2017 with permission from Elsevier. (**b**) Water electrolyzer. (b) Pictures of the printed components and (**c**) the respectively assembled water electrolyzer; (**d**) assembled electrolyzer with liquid tubing and electrical connectors. Scale bar: 20 mm. (**e**) LSV curve of the electrolyzer recorded after filling with KOH 1M and using the bare printed steel electrodes (black) and the steel electrodes modified with NiFe and Ni-MoS2 catalysts (red). Reprinted with permission from Ambrosi and Pumera (2018a). Copyright © 2018 American Chemical Society

electrodes by FDM (Rymansaib et al. 2016). The composite material provided good conductivity and the 3D-printed stick-like structures were used for the electrochemical detection of lead by anodic stripping voltammetry.

Sensing platforms for different electrochemical applications based in stainless steel 3D-printed electrodes were fabricated in helical-shaped structure by SLM (Fig. 6.8a) (Ambrosi et al. 2016; Cheng et al. 2017; Lee et al. 2017; Lölsberg et al. 2017; Tan et al. 2017). The simultaneous determination of lead and cadmium in aqueous solution by anodic stripping voltammetry is a representative example, in which the detection limits of 3.53 and 9.35 μg/L were attained, respectively (Lee et al. 2017). The combination with electrodeposition methods to modify the electrode surface by coating it with a thin gold film or a bismuth film improved the analytical performance. Similar helical-shaped structures were printed and electroplated with gold for the detection of phenolic (Cheng et al. 2017), nitroaromatic compounds (Tan et al. 2017) and biologically active compounds (Liyarita et al. 2018) in aqueous solutions as a proof-of-concept, which represent attractive solutions for their determination in environmental samples. Moreover, the

same electrodes were also used for DNA biosensing by voltammetry using the ability of methylene blue as an electroactive mediator, which was sandwiched into the DNA structure (Loo et al. 2017). The designed biosensing system based on DNA hybridization process showed a linear range from 1 to 1000 nmol/L.

Gauze-shaped stainless steel electrodes were also used as electrochemical devices. They were modified by electrodeposition of the catalyst materials (platinum, nickel, and iridium oxide) after their fabrication by SLM technology (Ambrosi and Pumera 2018b). Besides, combining the metal 3D printer to fabricate the electrodes with a commonly used FDM printer to fabricate polymeric structures based on PLA, the construction of full electrochemical cells in different sizes is an attainable goal. Following this combination trend, a prototype water electrolyzer was recently fabricated using SLM to produce the metallic components (electrodes) and FDM to construct the handling components (cells) of the electrolyzer (Fig. 6.8b) (Ambrosi and Pumera 2018a). The generated molecular hydrogen can thus be used as renewable and green energy.

The electrode substrates used in electrochemical applications are mainly based on gold and carbon materials, obtained by sealing them into polymeric supports. However, the fabrication of disposable electrodes of this type is still expensive, increasing the prices of in situ and point-of-use analysis. Nonetheless, 3D printing technology is making a breakthrough in the fabrication of low-cost electrodes with different materials, making it suitable for electrochemical lab purposes.

6.4 Future Trends

Environmental pollution is increasing at an alarming rate due to rapid industrialization and globalization, causing irreversible damages to the planet. An attempt to minimize such damages regards the use of cost-effective remediation processes, which include physical, chemical, and biological methods. The last group, also known as bioremediation, is more safe, specific, and friendly to the environment, because the organic waste is converted by biological organisms into stable and non-toxic products. The monitoring of target and hazardous pollutants through biosensors is also a valuable strategy included in bioremediation methods.

3D printing technology has been contributed with attractive and effective solutions in the environmental biotechnology field, regarding the improvement of the quality of water, soil, and air. 3D printing is a booming field nowadays that revolutionized the fabrication of three-dimensional objects. The use of different processes, printers, and materials, combined with software designs, allows the fabrication of complex and customized objects, inaccessible so far. From bioremediation strategies with "living ink materials" to the monitoring of toxic pollutants in the environment through printed structures and devices, 3D printing technology represents a paradigm shift in biotechnology. The 3D-printed structures and materials for bioremediation processes are at an early stage, and further work needs to be performed regarding the scalability, efficiency, cost, safety, and stability of such

bioprinted objects. The search for new ink materials capable of integrating living cells with supporting hydrogels can be the solution to produce a printable ink that can be further transformed into customized 3D geometries. Also, the combination with programmable synthetic biological methods of microorganism can create "living materials" with controlled 3D shape, microstructure, and dynamic metabolic response. True "minibiofactories" could be created in a way inaccessible so far. The development of new devices, such as the thumb-sized motors, improved by the use of 3D printing, can contribute to in situ remediation applications. Overall, the development of highly efficient and cost-effective technologies for bioremediation of waste and natural waters can be achieved more affordably.

Concerning the use of 3D printing in the (bio)monitoring applications, the achievements obtained so far are remarkable. Recently, several 3D-printed structures have been fabricated resorting to different printing technologies and materials for the monitoring of hazardous substances in the environment. Operating and supporting components, fluidic platforms, and electroactive surfaces are the most representative examples. Nonetheless, more complex and even full devices can be developed for easy and reliable monitoring. The constant improvement of print resolution and increase variety of printable materials, including functional and composite materials, will certainly draw the attention of scientists and engineers to develop new analytical devices.

3D printing is now an emergent topic that made a paradigm shift in the fabrication of three-dimensional objects, transversal to several areas. In environmental biotechnology, there is a window of opportunity to explore the full potential of this technology in the development of highly cost-effective strategies for remediation and monitoring of natural samples.

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Chapter 7
Biofuel: Marine Biotechnology Securing Alternative Sources of Renewable Energy

Jakir Hossain and Roksana Jahan

Contents

Abstract Due to the excess demand for fuels and the subsequent impact of global warming issues, the establishment of alternative environment-friendly energy is a prime concern to the scientific communities. Thereby, renewable energy in the form of biofuel is gaining research momentum and finding its way into the energy processing for development and consumption. Biofuel is potentially thought as one of the greatest sources of renewable energy in use currently unlike fossil fuels

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such as natural gas, coal, and petroleum. Therefore, this chapter describes an overview of biofuel production from marine algal sources by applying biotechnological approaches meta. Algae can produce a plethora of biofuels including biodiesel, biogas, biomethane, biobutanol, bioethanol, syngas, bio-oil, etc. In that case, marine algae can be a potential and reliable biomass source because ocean has an untapped vast algal resource that could reduce land cost and efficiently synthesize organic carbon through photosynthesis. The chapter elaborates on the potential of marine algae biomass as a renewable feedstock for biofuel production. Moreover, this chapter has compiled various marine sources involved in biofuel production, along with their properties, some important biofuel production procedures, and prospects and challenges of biofuel production from marine sources and commercialization. Hence, this section could provide a baseline summarization in biofuel production from marine algal sources through biotechnological advances.

Keywords Marine bio-resources · Blue biofuels · Renewable bio-energy · Sustainable approach · Marine biotechnology

7.1 Introduction

From the beginning of civilization, the primary energy in which human is depending on is only fossil fuel. The world energy requirement is raising quickly with increasing excess consumption of fossil due to increasing population and commercial industries. But regrettably, the surges of fossil fuels and oil reserves are being exhausted rapidly. Besides, these fossil fuels contribute to negative effects in the environment like emission of harmful gases, climatic changes, rising sea levels, loss of biodiversity, etc. Due to the excess demand for fuels and the subsequent impact of global warming issues, the establishment of alternative environment-friendly energy is a prime concern to the scientific communities. Thereby, renewable energy in the form of biofuel is gaining research momentum and finding its way into the energy processing for development and consumption. Biofuel is a cost-effective and environment friendly alternative to fossil fuels especially high pricing petroleum. It is being projected that renewable energy especially biofuels would become prominent in the energy mix led by the invention of sophisticated technologies. Researchers are doing experiments continuously to produce biofuel from renewable biomass sources as an alternative effective approach to reduce the use of non-renewable fuels.

Biomasses are living or dead organisms containing carbon that is utilized for biofuel production. Biodiesel, biogas, bio-alcohol, bio-oil, syngas, etc. are some concerning biofuels for energy supply. Biofuels are produced from agricultural crops like maize, soy, rapeseed, palm, or microalgae, macroalgae, seaweeds, etc. In the recent past, a review covered by Pogson et al. (2013) focused on the long-term cost and environmental impact of biofuel production using terrestrial biomass and determined that although terrestrial energy crops are less costly and economical, the

problem is an alarming risk to food security. Because the same resource (arable land) will face a tight competition between energy crops and food crops production. Conversely, the biomass which has highest production per unit area is more economical and considerable as a huge amount of biomass is needed for biofuel production commercially. In that case, marine algae can be a potential and reliable biomass source because ocean has an untapped vast algal resource that could reduce land cost and efficiently synthesize organic carbon through photosynthesis.

The bioenergy produced from marine renewable sources is being a sustainable alternative energy which received warm appreciation in many fields such as public, industries, and government policies. Moreover, biofuel produced from marine sources offers various advantages of providing good content of energy production, consumes high carbon dioxide, and provides a cheap fuel source. However, the production process and chemical transformation are being an expensive process and therefore commercial supply of biofuel on large scale is not yet successful. Hence an economic and efficient production process is essential to commercialize marine biomass-based biofuels. Many critics are paying curiosity about the prospect and the rise of a variety of biofuels because of the economic and environmental benefits. Therefore, this chapter describes an overview of biofuel production from marine algae sources by applying biotechnological approaches. This chapter has compiled various marine sources involved in biofuel production, along with their properties, some important biofuel production procedures, and prospects and challenges of biofuel production from marine sources and commercialization.

7.2 Biofuels and Its Types

If any of the molecules produced during carbon fixation provides energy in a mechanical setting, it is called as fuel. Biofuels are a renewable energy source, made from organic matter or wastes, that can play a valuable role in reducing carbon dioxide emissions. Biofuel is any kind of fuel that is produced from biomasses such as plants, algal materials, animal wastes, organic matter, or any other organism and can play a starring role in lessening carbon dioxide discharges (Demirbas 2009).

Biofuel produced from biomasses of different sources can be solid, liquid, and gaseous biofuel. On the basis of nature and chemical structure of biomass, biofuels are categorized as first-generation biofuels, second-generation biofuels, third-generation biofuels and somewhat fourth-generation biofuels. Different generations of biofuel with their characteristics are shown in Table 7.1. The first-generation biofuels are being produced from oil crops, food materials, and animal fats by applying conventional technology (Nigam and Singh 2011). This can be corn, sugarcane, wheat, sugar beet, sorghum, etc. As conventional technologies are use to produce this type of biofuels, they are also known as “conventional biofuels.” Some industrial concerns like cost and inadequacy and competition with food crops led to the second-generation biofuels. Feedstocks used for second-generation are not a food crop and they are no longer used for consumption. This involves agricultural

Table 7.1 Different generations of biofuel (modified from Vaishnavi et al. 2020)

	First-generation biofuel	Second-generation biofuel	Third-generation biofuel	Fourth-generation biofuel
Biomass sources	Produced from sugar, starch, vegetable oil, or animal fats. Basic feedstocks are wheat, corn, rapeseeds and grains	Produced from a variety of non-food crops, such as lignocellulosic materials from agricultural, forestry, and industry	Produced from yeast, fungi, and algal biomass	Produced from photobiological and solar biofuels
Examples	Bioethanol, biodiesel, starch-derived biogas, vegetable oils, biomethanol, and boating fuels	FT (Fischer Tropsch) diesel from biomass and bioethanol	Hydrogen and methane gas, bioethanol, butanol, and acetone	Electrofuels, photobiological solar fuels
Technology used	Enzymation	It utilized liquid technology to produce biofuel from solid biomass	Biochemical, thermochemical, and chemical	Not highly developed yet. Basically, synthetic biology tools
Advantages	Reduced global warming emissions and fossil energy consumption	Improved land-use efficiency and environmental performances Availability of widespread and cheap raw material Allow coproduction biofuels, chemical compounds	Eco-friendly and cost-effective	Eco-friendly and resources available
Disadvantages or limitations	Compete with food and feed industries for the use of biomass and agricultural land	Biomass residues used are still at the pre-commercial stage	Usage of large volumes of water	Efficient technology needed for better usage of materials used for fuel energy production

residues, woody crops that are a little more difficult to extract and require advanced conversion technologies for their process. Therefore, second-generation biofuels are called "advanced biofuels." Lignocellulosic processing is a well-recognized second-generation technology. Increased fuel consumption upsurges the challenge of sustainable supply of feedstock and so the scientists look for an alternative resolution concerning these problems.

In the third-generation biofuel, the marine resources, seaweeds, and cyanobacteria are promising sources because they can produce higher yield with

lower resources and lower production cost. Among these algae is the most capable non-food source of biofuel and can highly grow even in saltwater, adverse condition, and also in seawater (Amish et al. 2010). Depending on the technique and the part of the cell used, the algal feedstock can be transformed into different kinds of fuels. The increasing concern about the use of algae to produce biofuel is due to the accumulation of a very high level of lipid that can be then easily transesterified into biodiesel.

7.2.1 Qualities of Sustainable Biofuels

The sustainability of producing biofuels relies on the net energy gain that is fixed in the biofuels and depends on the production parameters, such as the type of land where the biomass is made, the volume of energy-intensive inputs. Sustainability also depends on the energy input for harvesting, transporting, and running the production facilities (Haye and Hardtke 2009). In addition, competition for cultivation land between biomass crops versus food production is also an important issue. The parameters like raw materials, complicating life-cycle assessments, local conditions, and preventing any valid global statement are responsible for biofuel sustainability (Davis et al. 2009; Farrell et al. 2006). If the energy balance of the biofuel is substantially positive only then the large-scale biofuel production can be considered sustainable (Haye and Hardtke 2009). The biofuel production would be certainly sustainable when it is eco-friendly (less emission of greenhouse gas, insulation of huge quantity carbon, not polluting the soil, air, water, and biodiversity, etc.), be acceptable in society and economically feasible. Lora et al. (2011) listed the basic criteria and sustainability indices for a sustainable biofuel (Table 7.2).

Table 7.2 Criteria and sustainability indicators for sustainable biofuels (Lora et al. 2011)

Criteria	Sustainability indicators
1. To be carbon neutral, considering the necessity of fossil fuel substitution and global warming mitigation 2. Not to affect the quality, quantity, and rational use of available natural resources as water and soil 3. Not to have undesirable social consequences as starvation because of high food prices 4. To contribute to the social-economic development and equity 5. Not to affect biodiversity	1. Economic indicators (cost of production) 2. Output/input relation (net energy analysis) 3. Substituted fossil fuel per hectare 4. Avoided GHG emissions (CO_2 savings) 5. Evaluation of Environmental impacts using impact categories indicators 6. Carbon emissions due to land-use changes 7. Renewability indicators (exergy or emergy accounting)

7.2.2 *Benefits of Third-Generation Biofuel over First- and Second-Generation Biofuels*

Due to some concerning issues such as reported displacement of food crops, effects on the environment and climate change, the sustainability of many first-generation biofuels has been progressively questioned over (Singh et al. 2011). Since the first-generation biofuels' sources are mainly food, oil crops, and animal fats, the increasing energy need makes the competition in food and fuel crops production for utilizing arable lands, high water, fertilizer requirements, etc. (Nigam and Singh 2011). Due to these negative criticisms about the sustainability of many first-generation biofuels, the potentiality of the so-called second-generation biofuels that have raised attention is manufactured from lignocellulosic feedstocks, agriculture residues, municipal wastes, and grasses because it emits little greenhouse gases and does not contradict with food supply needs. Still, second-generation biofuel production experiences some limitations to accomplish commercial deployment, though considerable progress continues to overcome the technical and economic challenges (Sims et al. 2010).

Conversely, algal biomass culture for biofuels production has gained much attention because algae can produce higher energy yield and need less space for growth than any conventional feedstocks. Moreover, the marine algal biomass cultivation is not limited to fertile or arable land. So that algae as a feedstock for third-generation biofuel would not compete with food for animals and it could grow within a short time at minimal inputs with a variety of nutrient and carbon sources. So, GreenFuel Technologies Corporation considered algae as the fastest growing plant in the world (Girardet and Mendonça 2009). The benefits of using marine algae as alternative sources for biofuel include: obtained from renewable resources, sustainable, cheap, reduce our reliance on foreign energy, reduce greenhouse gas emission. Moreover, this is the fourth largest energy resource available in the world (Saxena et al. 2009).

7.3 Marine Sources for Biofuel Production

Marine resources like macroalgae, microalgae, seaweeds, fungi are extensively diverse to use as renewable sources for biofuel production. But, until now maximum works are highlighted on one species of brown algae (*Laminaria japonica*), subsequently some species in *Sargassum*. Because this species is conventionally used and hence widely cultured and researched in many countries (Mazarrasa et al. 2014). In 2014, *Laminaria japonica* is produced roughly half of the total seaweed production in China, which is presently the biggest seaweed cultivating country in the world. In contrast, in the case of red and green algae, the maximum researched species are *Gracilaria* sp. (red algae) and *Ulva* sp. (green algae) which are also high production

Table 7.3 Marine resources used in biofuel production (Baskar et al. 2018)

Macroalgae	Microalgae
Acrosiphonia orientalis	*Dunaliella tertiolecta*
Ulva fasciata	*Isochrysis galbana*
Ulva lactuca	*Botryococcus braunii*
Enteromorpha compressa	*Chlamydomonas reinhardtii*
Caulerpa peltata	*Chaetoceros calcitrans*
Valoniopsis pachynema	*Euglena* sp.
Bryopsis pennata	*Spirogyra* sp.
Caulerpa racemosa	*Phormidium* sp.
Padina tetrastromatica	*Cyanobacteria*
Dictyota adnata	*Tetraselmis suecica*
Lobophora variegata	*Scenedesmus obliquus*
Sargassum wightii	*Nannochloropsis oculate*
Centroceras clavulatum	*Phaeodactylum tricornutum*

species annually in Asian countries, like Japan, Indonesia, and Philippines. The list of algae commonly used in biofuel production is summarized in Table 7.3.

7.4 Algae Harvesting Technology

Biomass harvesting tends to be the most energy-requiring process because of the algal concentration, smaller size, and surface charge. Filtration, flocculation, settling, and centrifugation are the most common approaches to harvest algae biomass. Based on the size and density of algae, target product, and the production procedure, the harvesting methods are selected to have the ultimate product.

Filtration is used as one of the best commonly methods, but it is only applicable comparatively for larger microalgal species (>70 μm) and is considered unsuitable to smaller strains (<30 μm). Mohn (1980) confirmed that the filtration process can accomplish 245 times more concentration factor than the original concentration of *Coelastrum proboscideum* to get a sludge that contains solids around 27%. To harvest the algal strain of small-sized cells membrane, it is suggested to apply microfiltration and ultrafiltration/centrifugation methods (Petrusevski et al. 1995).

On the contrary, flocculation and settling are thought to be low-costing methods which need short time energy only for mixture of the cells with a coagulant. Flocculants decrease the negative charge of the algal surface and avoid them from sticking to the suspension (Molina et al. 1999). Algae responses vary expressively with some flocculants. The effectiveness and dosage of a particular flocculant differed immensely from one species to another. Some algae get accumulated and settled down when pH increases that is regulated by CO_2 aeration or lime addition (Demirbas 2011a). Brennan and Owende (2010) found multivalent metal salts such as $FeCl_3$, $Al_2(SO_4)_3$, and $Fe_2(SO_4)_3$ as suitable flocculants.

Lastly, only the centrifugation method is found reasonable for high-value products (Molina Grima et al. 1999). Because this is a highly energy consuming technique, though the nonstop centrifugation method has been discovered that is more profitable if the systems are assembled on a larger volume (Briggs 2004). Ultrasound induced aggregation with increased sedimentation can also be considered to collect microalgal biomass (Brennan and Owende 2010) and this approach is effectively applied in a study conducted by Bosma et al. (2003) which found 92% segregation proficiency and a heavy concentration factor with 20 times more than the original concentration.

7.5 Algal Oil Extraction for Biofuels Production

The sustainability of algae-based biofuel is established by the process of algal oil extraction as it is an expensive approach. All algae cell contains a sturdy cell wall that causes oil extraction more convoluted. The algae need to get dried before the oil extraction process (Heger 2009). Widjaja et al. (2009) uncovered that during the lipid extraction process from algal biomass, the drying temperature could influence the lipid composition and its content. Freeze drier process can hold the original lipid composition while drying at higher temperature reduced the TAG content. Still drying at 60 °C can cause a slight decrease in the lipid composition. Ultrasonication process does not have any considerable impact during the extraction of lipid but appropriate pulverization facilitates extracting the lipid content from the algal cells. Using proper harvesting technology, the algae is segregated from the growth medium, and oil is obtained by any of the mechanical or chemical methods.

The chemical method includes: (1) hexane solvent method, (2) Soxhlet method, and (3) supercritical fluid extraction. The use of chemical solvents may result in safety issues, health problems, and environmental pollution. The supercritical extraction is both costly and energy-intensive as it requires a high-pressure device. For convenient and effective oil extraction, a combination of mechanical pressing and chemical solvents can be used by many manufacturers. The other method is enzymatic extraction in which enzymes are utilized to damage the cell walls and this improves the extraction process. But, in this case, the cost is highly exclusive in contrast to chemical extraction. The solvent extraction method is not only a rapid but also an effective method that is used on dried biomass directly (Mata et al. 2010). In this method, oil is extracted from microalgae by cleaning or washing repeatedly using organic solvents. Several solvents like ethanol, hexane, or mixture of hexane–ethanol, cyclohexane, benzene, etc. are applied and these are successful to extract fatty acids up to 98% (Becker 2004). Supercritical fluid extraction method uses those substances that have both liquid and gaseous properties (i.e. CO_2) while exposing to rising temperatures and pressures. This quality helps to be used as an extracting solvent and there are no residues left behind after the system is taken back to normal atmospheric pressure and standard room temperature (Mercer and Armenta 2011).

The mechanical method includes: (1) expression press and (2) ultrasonic assisted extraction. In both the methods dry algae can only be used which is energy exhaustive. Cravotto et al. (2008) used UAE (ultrasound-assisted extraction) and MAE (microwave-assisted extraction) methods to isolate lipids from plant sources. They also used a cultivated marine microalga containing much DHA (docosahexaenoic acid) and revealed that either single or mixed methods can significantly upgrade the extraction of the bioactive substances with greater proficiency and lower reaction periods with moderate costs and minimum toxicity. Widjaja et al. (2009) showed that algae grown in nitrogen lacking culture media result in higher lipid content and gradually change the lipid composition from FFA-rich lipid (free fatty acid) to mostly TAG contained lipid. Consequently, cooperating between increasing lipid accumulation and time of harvesting is essential to attain better lipid percentage and higher productivity. Moreover, the osmotic shock treatment can be used to crack the cells in solution to release cellular components and oil. This requires low-energy input but it gives the lowest efficiency. In a project by the US Department of Energy's Aquatic Species Program (ASP), solvent extraction costs are found three times higher for algal oil than for soybean oil. It is possibly due to the higher moisture percentage of the paste in the experiment (Sheehan et al. 1998). Pressing and filtration (mechanical dewatering) can be more inexpensive than heating but the real key is having a few steps and simple scalable extraction (Molina Grima et al. 1999).

7.6 Biofuels Production

The algae are transformed into many kinds of renewable biofuels like biodiesel, biogas, bioethanol, biomethane, biohydrogen, bio-oil, and syngas. Algae can be converted into different biofuels depending on technique and part of the cell used in the process. There are many steps followed for producing liquid biofuels from algae like microalgae, macroalgae, seaweeds, fungi. The lipid obtained from algal biomass is converted into biodiesel and following the extraction of lipid, the carbohydrate contents of algae are fermented into bioethanol and butanol fuel and so on.

7.6.1 Biodiesels Production

Biodiesel is a combination of fatty acid methyl/ethyl esters produced from the transesterification of algae oil, vegetable oil, or any animal fats. These feedstocks contain 90–98% triglycerides (TAGs) and a small amount of monoglycerides and diglycerides, FFAs (free fatty acids, 1–5%), and little amounts of other by-products like phosphatides, phospholipids, carotenes, tocopherols, and sulfur compounds and some water (Bozbas 2008). Biodiesel is thought to be one of the most demanding alternatives of fossil fuels because of its similarity in physical and chemical

properties with commercial petroleum diesels. Besides, biodiesel performs like commercial fossil diesel by emitting lower emissions. Moreover, biodiesel has some environmental advantages, for instance, highly biodegradable, lower emissions of toxic and carcinogenic gases (Sheehan et al. 1998). Biodiesel is used up more effectively and reduces the emissions of carbon monoxide, unburned hydrocarbons, and particulate things such as smut, sludge, and so on (Kumar et al. 2015).

The most common feedstocks used for biodiesel production are lipids of vegetable seeds, organic wastes, and marine biomass of algae and other organic matter. Yet, the most reported potential feedstock to produce lipid is marine microalgae. Some prominent marine microalgae and its chemical composition are summarized in Table 7.4. As macroalgae do not contain triglycerides, they are not widely used for biodiesel production. To date, biodiesel from macroalgae is sparingly reported and yields very low compared to microalgae (Huihui et al. 2015). Moreover, marine algal feedstocks do not compete with others animals' foodstuffs and resources. The marine algal feedstocks are available in larger quantities and are considered sustainable by increasing commercial cultivation without any negative impact on the environment.

7.6.1.1 Methods of Biodiesel Production

There are many kinds of oils from different sources that are used to produce biodiesel through transesterification or esterification process. Algal biodiesel production includes harvesting biomass, drying, oil extraction, purification, and further transesterification of oil. Balat (2011) has illustrated the processes of biodiesel production and Lin et al. (2011) have discussed the pros and cons of all these processes of biodiesel production. Both studies found transesterification as the most auspicious solution to the high viscosity problem and biodiesel produced from the transesterification with methanol is almost similar to the conventional diesel in its main characteristics and compatibility. Presently, transesterification is the most recognized method to produce biodiesel from biobased oils because of its better conversion efficacy and low costing.

Transesterification

Transesterification (alkali catalysis) is a common process for most of the biodiesel production systems for lipid conversion. The alkali process is more efficient and lower corrosive than the acid process. That is why it a favorable catalysis process to be used in maximum commercial biodiesel production. Generally, KOH, NaOH, or CH_3ONa are very much popular catalysts that are used with any alcohols (methanol or ethanol) and any oils. The transesterification performs well when the free fatty acids and moisture of the lipid are less than 0.1% and the amount of phosphorus is less than 10 ppm. Nevertheless, base catalysts are very much sensitive to free fatty acids and moisture content. But oil feedstock contains high FFAs that cause soap

Table 7.4 Chemical composition of microalgal biofuel sources (% of dry matter) (modified from Zabed et al. 2019)

Microalgae species	Lipid (%)	Protein (%)	Carbohydrate (%)
Euglena gracilis	4–20	39–61	14–18
Chlorella protothecoides	55	10–52	10–15
Chlamydomonas reinhardtii	21	48	17
Chlorella vulgaris	14–22	51–58	12–17
Dunaliella salina	6	57	32
Dunaliella bioculata	8	49	4
Scenedesmus dimorphus	16–40	8–18	21–52
Scenedesmus obliquus	35–55	50–56	10–17
Spirogyra sp.	11–21	6–20	33–64
Anabaena cylindrical	4–7	43–56	25–30
Spirulina maxima	6–7	60–71	13–16
Spirulina platensis	4-9	46–63	8-14
Synechococcus sp.	11	63	15
Chaetoceros calcitrans	39	58	10
Chaetoceros muelleri	33	44–65	11–19
Porphyridium cruentum	9–14	28–39	40–57

formation. The soap formation has adverse effects on the production process and decreases the production of biodiesel. Moreover, NaOH and KOH also cause water and soap formation that reduce the reaction rate. Therefore, sodium methylate or sodium methoxide is pretty good to use as a catalyst than NaOH or KOH, but it is more costly in fact. Sodium methoxide is used as 30–50% solution with methanol for safe uses and added around 0.3–0.5% of the oil biomass. Anyway, according to Barnwal and Sharma (2005), the concentration of the catalyst differs from 0.5% to 1% of oil content (w/w). Reaction temperature is another critical variable in the transesterification process. The recommended optimum reaction temperature is 60 °C though it can vary depending on the catalyst types and different conversion rates. In general, the temperature should be within 25–120 °C (Barnwal and Sharma 2005; Marchetti et al. 2007).

Esterification

Alkali catalysis performs better if the FFAs content in the feedstock is less than 1% of the content of the oil. Before transesterification, chemical neutralization is done to remove FFAs with a base compound, for example, NaOH or physical deacidification is done with a vacuum. Anyway, it is not recommended because some oil is lost during this pretreatment. Fats and lipids containing high FFAs are used to produce biodiesel by acid esterification process. In that case, the formation of soap is not a challenge as no alkali metals are in the reaction medium. Besides FFAs, triglycerides are also transesterified by acid catalysts, but it must take a couple of days to complete

that is why it is not considered suitable for industrial esterification. Yet, the entire process can take around one hour at 60 °C. So, the process of esterification of FFAs to alcohol is comparatively quick. An issue to remember is that the produced water should be removed continuously from the reaction medium by phase separation for better reaction rates.

In acid esterification, more acid around 5–25% and higher alcohol:FFAs ratio (20:1 to 40:1) are necessary. Like the alkali esterification, extra alcohol enhances the triglyceride conversion but regaining of glycerol is far critical. According to Marchetti et al. (2007), alcohol and raw material ratio should be optimum. When the conversion of the FAs (fatty acids) to alcoholic esters has been completed, then the water, alcohol, and acid mixture is removed by settling or centrifugation. After that, clean alcohols and basic catalysts are incorporated to the remaining transesterification reaction process. Therefore, esterification should be run after transesterification to get better results and full oil conversion.

Enzymatic Conversion

Enzymes act as catalyst to produce biodiesel from oils. Currently, lipase has gained attention as one of the most used catalysts for enzymatic catalysis of oils into biodiesel. Lipases are a common group of enzymes that are generally used to catalyze the reactions, for instance, hydrolysis, acidolysis, and alcoholysis. Besides these, lipases also catalyze the transesterification and esterification reactions (Marchetti et al. 2007). The reactions are run at 35–45 °C for 4–40 h. Still, there is no single standardized enzyme that can be used with different feedstocks for biodiesel production. Still now, there is a far difference between the cost of existing techniques and the industrial application, although reuse of the immobilized enzymes reduces the cost relatively. Unfortunately, considering the economic perspective this process is not cost-effective for biodiesel production from microalgae.

Non-Catalytic Conversion

Non-catalytic conversion can be considered to some extent like to enhance the reaction of lipids with alcohol or to improve the miscibility of the oil–alcohol step and to reduce the drawbacks of the mentioned methods. Some commonly used non-catalytic methods are supercritical conversion, microwave-assisted conversion, or ultrasound-assisted conversion (Bharathiraja et al. 2014).

Supercritical Alcohol Conversion It is a relatively advanced and more appropriate method (Warabi et al. 2004), but it is not sure that this process is more efficient and faster than transesterification and esterification to convert oil into biodiesel (Marchetti et al. 2007). This is a very simple process and can be completed within a very short time (2–4 min). Since there is no need for catalyst, biodiesel purification is more simple, easy, and eco-friendly (Demirbas 2005). Reaction time and

temperature, catalyst loading, stirring rate, and alcohol/oil molar ratio are considered to identify the optimum conditions for the conversion process (Meher et al. 2006).

Microwave-Assisted Conversion Microwave-assisted conversion works under microwaves and the reaction is completed within a short time by a huge reduction of by-product quantity (Hernando et al. 2007). Furthermore, this process produces a high quantity and quality of the products quickly which helps to reduce the production cost significantly (Nüchter et al. 2000).

Ultrasound-Assisted Conversion It is a good method that secures maximum mixing and increases liquid–liquid mass transfer (Ji et al. 2006). This process also enhances the surface area for interacting between alcohol and oil (Stavarache et al. 2006). Ultrasound supplies the activation energy needed for initiating the reaction that increases the mass and heat transfer of the solution and causes up the reaction rate and better production (Adewuyi 2001).

7.6.1.2 Biodiesel Separation and Purification

After finishing the process, biodiesel is found in a mixture of extra methanol, glycerin, and catalyst. Self-phase separation happens due to the specific gravity of the compounds in the mixture like the rules of thumb. Gravity separation is helpful to separate the biodiesel from the by-products (glycerin and methanol). Yet, emulsion formation is induced if the feedstock is not purified that causes the separation far tough. So, to face this difficulty, saturated salt (sodium chloride) or centrifugation is used to segregate the emulsion that intensifies the phase separation process. Further, the concentration of methanol in the reaction is reduced for good phase separation. Distillation, glycerin, and methanol used in the process could be purified generally. When phase separation is completed, the remaining methanol in the process is eliminated through evaporation. In the end, the remaining of the microalgal biomass after biodiesel production process can be used further to get other biofuels like biobutanol, bioethanol, or bio-oil (liquids) (Gouveia and Oliveira 2009; Miranda et al. 2012) or gaseous biofuels like biomethane, syngas, and biohydrogen (Ferreira et al. 2013). If that could be done, then the overall cost would be reduced significantly.

7.6.1.3 Some Issues Considered During Biodiesel Production

In order to increase the yield, new techniques like ultrasound irradiation assisted transesterification were used to form emulsion of oil and alcohol and the cavitation formed during this process accelerates the rate of the reaction. It was also observed that biodiesel from wet biomass is ten times lower from dry biomass and it implies the negative effect of water on transesterification (Huihui et al. 2015). Thus, the dehydration process is necessary to achieve high yield. As these steps increase the total production cost, direct transesterification or in situ extraction is carried out in

which the oil-bearing material directly contacts with alcohol instead of reacting with extracted oil and thus eliminates the two-stage process of biodiesel production.

Microalgae can accumulate a significant number of triglycerides amounting to 20–50% of its cellular weight (Chen et al. 2015), though it depends on the type of strain and cultivation condition. The microalgae selected for biofuel production is firstly grown under optimal growth condition. Then they are put on a restricted diet nutrient growth media resulting in increased oil production. TAGs synthesis in these species can be improved by some conditions subjected to the growing microalgae, for instance, causing stress to the microalgae with temperature, pH, salinity, nutrient starvation, and age of cultured algae. Increased TAG content enhances the production and effectiveness of biodiesel.

In order to enhance the economics of biodiesel production using microalgae, genetic modification and molecular level engineering receive keen focus to increase its photosynthetic efficiency, biomass growth rate, oil content, and reduces photoinhibition. To attain a consistent annual yield of oil, photobioreactors should be used that provides a controlled environment to increase the microalgal biomass required for making biodiesel.

7.6.2 Bioethanol Production

Bioethanol is one of the highest utilized alcoholic biofuels and is a vital promising biofuel worldwide (Chia et al. 2018). Ethanol is thought as a booster of octane for gasoline. 40% ethanol mixing with gasoline can cause 3.0–4.4% less gasoline consumption, induce the efficacy of internal combusting of engines, and also reduce the emissions of CO_2 around 19 to 35 metric tons annually.

Bioethanol from macroalgal feedstock is also a liquid algal transportation fuel. As macroalgae are rich in carbohydrates and contain only little lignin, they are considered suitable for the fermentation process for producing bioethanol (Hamelinck et al. 2005). Some prominent marine algae sources for bioethanol production are reported that include brown algae: *Laminaria hyperborean* (Adams et al. 2009), *Alaria crassifolia Kjellman* (Yanagisawa et al. 2011), *Laminaria japonica* (Kim et al. 2011), *Sargassum* spp. (Lee et al. 2011); Red algae: *Gracilaria verrucose* (Kumar et al. 2013), *Kappaphycus alvarezii* (Khambhaty et al. 2012); Green algae: *Ulva* spp. (van der Wal et al. 2013), *Chaetomorpha linum* (Schultz-Jensen et al. 2013). As these organisms are grown in an aquatic environment, the buoyancy helps its upright growth without lignin crosslinking and so they contain hardly the same type lignin crosslinking compounds in their cellulose. Due to the lower amount of lignin, it contains enough sugars (not less than 50%) that can be applied for the fermentation of bioethanol.

7.6.2.1 Marine Algae-Based Bioethanol Production Process

The process of bioethanol production is similar to the technological process of common ethanol production. The bioethanol production from marine biomass includes a series of sequential steps that are algae drying, crushing, pulverization, liquefaction, saccharification, ethanol fermentation, and refinement. Among them, some major steps are discussed below.

Liquefaction

After the algal ingredients being extracted, liquefaction is needed for enzyme treatment or microbial fermentation. The methods of this liquefaction are extracting sugars from dried powder, using enzymes on live algae to break the polysaccharides in cells or cell walls, and then liquefying live algae in intense heat and pressure.

The extraction of sugars from dried powder reduces the energy balance because it requires a large amount of energy for drying and pulverization. The enzyme treatment method involves the liquefaction of algal structural polysaccharides through the treatment of cellulase or the digestive enzymes of algivorous mollusks. Liquefaction is achieved by treating the dried, powdered form of kelp or other brown algae using enzymes. The enzymes break the fibrin in the cell wall or alginic acid and reduce the molecular weight of the mucopolysaccharides (alginic acid) between cells to decrease their viscosity. The red algae such as *Gelidium amansii* is treated with sodium chloride for removing the lignin. After that, b-galactosidase and xylanase are used for liquefaction and saccharification. To reduce the molecular weight of proteins and other polymer compounds, the intense temperatures and pressure are used in liquefaction. However, this method faces difficulties to treat a high amount of algae because of its capacity issues with the internal pressure vessels (Huang et al. 2011).

Saccharification

Saccharification processes are acid hydrolysis, degradation under high temperature and pressure, and enzymolysis. Acid hydrolysis method can be done by treating with 3% H_2SO_4 for 60 min at 120 °C. Polysaccharides are broken into monosugars by acid hydrolysis, but the retention of monosugar can also be decreased by extreme degradation. Besides, too much breakdown reduces monosugars' recovery rate ultimately lowering the recovery rate of ethanol. Sulfuric acid should be removed after hydrolysis. Thereby, alkali neutralization can be used to remove sulfuric acid. The type of enzyme used in enzyme-based saccharification process depends on the components and bonding of the algal sugar (Demirbas 2009).

Ethanol Fermentation

Fermentation is the conversion of monosugars into ethanol where at first, complex components must be reduced to low molecular weight. The sugars (glucose and mannitol in brown algae, and glucose, galactose, and xylose in green and red algae) is the main component in ethanol fermentation. Microorganisms like yeasts and bacteria have a major role during the fermentation of ethanol. The common enzymes found available in these microorganisms are not so reliable in fermentation. Recently, a growing concern in genetically improved yeasts with a wider range of substrate specificity has gained a new hope for the future. Anyway, some yeasts which are used to produce ethanol from glucose can be *Saccharomyces cerevisiae*, *Pachysolen tannophilus*, and *Pichia angophorae* and bacteria like *Zymomonas mobilis*. Ethanol is synthesized from galactose too. The high amount of galactose can be found in red algae. The sugar present in maximum brown algae can entirely be converted into ethanol and this is a remarkable advancement in ethanol production from marine algal biomass because the brown algae are one of the most abundant resources (Takeda et al. 2011; Wang et al. 2011; Lee and Lee 2012).

7.6.2.2 Other Issues Related to Bioethanol Production

In the production of bioethanol from macroalgae, pretreatment has a vital role in saccharification and fermentation processes. This is because as the carbohydrates in the macroalgae are not freely available and so mechanical or acid pretreatment could enhance the reaction surface and helps locked sugars in the structural polysaccharides to be more available for hydrolytic enzymes. The acid hydrolysis pretreatment is reported to be highly cost-effective but suffers from a drawback of glucose decomposition that occurs during hydrolysis (Horn et al. 2000). Thus, saccharification is enhanced by a combination of acid and enzymatic pretreatment but it is highly necessary to use suitable enzymes to obtain high efficiency in hydrolysis and enzyme recovery (Demirbas 2009). Therefore, the high efficient hydrolysis process and efficient fermentation are the two major issues in using macroalgae as the feedstock for bioethanol production. The red alga, *Gelidium amansii* consists of cellulose, glucan, and galactan and can be an efficient feedstock for bioethanol production (Horn et al. 2000). The other brown algal species such as *Alarie*, *Saccorhiza,* and *Laminaria* consists of laminarian and mannitol as main energy-storing materials and so they are widely used in bioethanol production using mannitol and laminarian as substrates (Horn et al. 2000).

Microalgae also synthesize large amounts of carbohydrates in different combinations in each species that can be fermented to produce bioethanol. Besides lipids, carbohydrates are the main components that store energy. The microalgal biomass also needs to be pretreated for the efficient extraction of fermentable sugars. For this, easily handling energy method and cost-effective hydrolysis method can be used. Enzymes or diluted or concentrated acids are commonly used for hydrolysis of algal biomass. Acid concentration, temperature, and algal loading are the important

parameters to be considered for the efficient release of fermentable sugars from the biomass (Harun and Danquah 2011). High levels of polysaccharides accumulate in the complex cell wall in green algae such as *Spirogyra sp.* and *Chlorococum sp.* and this starch accumulation can be used in the production of bioethanol (Harun and Danquah 2011). It is reported that the *Chlorococum sp.* could produce 60% more bioethanol concentrations for the sample. Therefore, these suggest that same biomass source (microalgae) could be used to produce both lipid-based biofuel and ethanol biofuel at the same time increasing economic benefits (Harun and Danquah 2011).

In order to enhance the production of bioethanol, several attempts have been reported in the development of genetically modified microalgae by introducing ethanol-producing genes (Ross et al. 2008). Some private company describes the ability of microalgae in bioethanol production photosynthetically and introduced photosynthetic bioethanol production protocol. However, the technology is under development and investigated for the commercial application of microalgae-based bioethanol production. Another advantage of using microalgae is they are good producers of hydrogen; therefore, biohydrogen can be produced as a pollution-free renewable green fuel.

7.6.3 Biobutanol

Butanol is being consumed as a transportation fuel for around 100 years. So biobutanol can be a potential biofuel and even it can replace ethanol as a gasoline additive due to its low vapor pressure and higher energy density (Potts et al. 2012). The production of butanol from algal biomass could also be more energy-efficient than ethanol (Huesemann et al. 2010). It is nonpolar and long hydrocarbon like gasoline that is why it is suitable for use in gasoline vehicles without any modification. Additionally, the vaporization heat of butanol is little more than that of gasoline (Hönig et al. 2014). So, the gasoline blended with butanol does not cause cold start problems and could be utilized as 100% biobutanol fuel in place of gasoline (Pospíšil et al. 2014). In general, biobutanol has high miscibility, low volatility, high energy contents from 33.07 MJ Kg^{-1} (Klass 1998) to 36.1 MJ Kg^{-1} (Laza and Bereczky 2011), and density of 810 Kg m^{-3} (Pfromm et al. 2010).

Unlike biodiesel, the main feedstock used biobutanol production is carbohydrates. Carbohydrates can be monosaccharides, disaccharides, polysaccharides, or oligosaccharides on the basis of the length and composition of feedstock (Gloria et al. 2013). Algae contain more carbohydrates than lipids and carbohydrate content in algae differs substantially among the algal species. Some algae like chlorophytes have carbohydrates in the cell wall that are composed of mostly cellulose and soluble polysaccharides (Domozych et al. 2012). Starch or glycogen is found in most green algae and cyanobacteria (Singh and Olsen 2011; Chen et al. 2013). Some prominent species of green microalgae like *Chlorella* sp., *Dunaliella* sp., *Chlamydomonas* sp.,

and *Scenedesmus* sp. are being considered potential to produce biobutanol industrially (Singh and Olsen 2011).

7.6.3.1 Biobutanol Production

Biobutanol is being produced massively by the fermentative process. In general, biobutanol can be produced by ABE process that is acetone, butanol, and ethanol production process. The ABE fermentation process is accomplished in three main steps: (1) pretreatment of algae biomass, (2) fermentation, and (3) recovery.

Algae Pretreatment for Biobutanol Production

Algae pretreatment is a crucial phase because the process breaks polymer crystalline structure (like cellulose and starch) into simple sugars that are fermentable. So, this can result in faster hydrolysis and higher production (Mosier et al. 2005). A study showed that using intact algae without pretreatment generates lower biobutanol because of lower conversion rates (Wang et al. 2016). Therefore, a proper pretreatment method can increase subsequent hydrolysis and fermentation (Sun and Cheng 2002). In general, the microalgal starch can be transformed into biofuel directly by applying dark and anaerobic fermentation, though the biofuel production would be much lower (Ueno et al. 1998). Thus, algal feedstock needs treating with a pretreatment method to enhance the production. Thus, the selection of a suitable method for algae pretreatment needs to consider the cost of the production. The pretreatment process can be done using different three methods such as hydrolysis/saccharification, nourishment, or sterilization (Hemming 2011). Yet, saccharification is the most popular and effective process that is used for the conversion of carbohydrate polymers into simple fermentable monomers.

Hydrolysis/Saccharification Saccharification is the most important pretreatment phase in the fermentation of microalgal biomass especially lignocellulosic or cellulosic compounds. The process is done for the saccharification of raw feedstocks by enzyme digestion, alkaline, thermolysis, and acid hydrolysis. These processes can be divided into three main sections such as enzymatic saccharification, physical saccharification, and chemical saccharification.

These pretreatment saccharification methods have a certain economic cost that depends on several parameters including (1) alkaline or acid reagent, (2) electricity cost, (3) time of thermal pretreatment and working temperature, (4) surfactant loading during enzymatic hydrolysis, (5) type of hydrolytic enzymes used, and (6) type of feedstock used (Hernández et al. 2015). Regarding pretreatment costs, different methods may be sorted, from high to low costs as (1) enzymatic pretreatments (using amylases and cellulases), (2) chemical pretreatment (alkaline and acid), and (3) physical pretreatment (microwaving, sonication, high-pressure homogenization, and heat) (Talebnia et al. 2010; Tao et al. 2011).

Enzymatic saccharification is done by utilizing the hydrolytic enzymes like cellulases, amylases, and glucoamylases. Microalgae cell walls contain heavily cellulose and very few hemicelluloses but no lignin at all. So, lignin-degrading enzymes are not needed for this enzymatic saccharification process. Enzymatic saccharification shows some advantages like low cost of tools, higher glucose production without toxic by-products or sugar degradation products (Cara et al. 2007). Moreover, it requires low energy because of its low temperature and high selectivity of components present in microalgae (Mubarak et al. 2015). A comparative study showed that fermentation of enzymatic pretreated algae with xylanase and cellulase produced 9.74 g L ABE but, fermentation of acid-/alkali-pretreated algae produced 2.74 g L 623 -1 ABE only (Ellis et al. 2012). But, the enzymatic digestion process is a costly one that reduces the wide-ranging application of ABE production (Kumar and Murthy 2013).

Chemical saccharification is characterized by its short reaction time, though it needs higher temperature, pressure, and acid like H_2SO_4, HCl, and HNO_3 or base like NaOH, KOH, and Na_2CO_3. Moreover, it makes some inhibitor like furfural and 5-hydroxymethylfurfural that can downregulate the fermentative reaction (Mussatto et al. 2010). To stop producing these inhibitors and to increase saccharification efficacy, the appropriate reaction parameters like temperature, residence time, and moisture content are followed (Okuda et al. 2008). Optimizing these parameters, fermentable sugars production will be amplified (Castro 2014). Castro (2014) found 166.1 g kg^{-1} sugars from dry biomass of butanol-producing bacteria *Clostridium saccharoperbutylacetonicum* using acid hydrolysis. In case of treatment cost, the acid/alkali saccharification can be low costing yet than that of enzymatic saccharification (Choi et al. 2010). Nevertheless, a combined approach of acid hydrolysis and enzymatic digestion could obtain higher production (Park et al. 2012; Castro 2014).

Physical saccharification means the application of physical force to increase the hydrolysis and fermentation of carbohydrates (Talebnia et al. 2010; Tao et al. 2011). Still now, this pretreatment process is not much analyzed for microalgae biomass with the exception of macroalgae or seaweeds or lignocellulosic biomasses (Laghari et al. 2014). Nonetheless, most effective physical pretreatment like microwave and sonication was used in some cases. Microwave application is more popular for biomass transformation than conduction or convection heating. Because, it is a more direct, fast, and stable method that can directly interact with the heated substrates with an electromagnetic field to produce heat (Macquarrie et al. 2012). Moreover, ultrasonication can increase the rate of hydrolysis for simple fermentable sugar (Zhao et al. 2013).

ABE Fermentation

The ABE (acetone, butanol, and ethanol) fermentation is done by utilizing some microorganisms like bacterium (*Clostridium acetobutylicum*) that can produce saccharolytic butyric acid. Difference from yeast, clostridia can produce alcohol

from many carbohydrates like hexoses, pentose, or same type carbon sources (Yoshida et al. 2012). Furthermore, some disaccharides like sucrose, mannose, and polysaccharides such as starch can also be fermented by clostridia (Campos et al. 2002). *Granulobacter saccharobutyricum*, *Amylobacter butylicus*, *Bacillus orthobutylicus,* and some other microorganisms can be used in biobutanol fermentation (Dürre 2007). Anyway, most of the *Clostridium* sp. like *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* can be effective for the fermentation of biobutanols (Gao 2016). ABE fermentation is butyric acid fermentation that is done by anaerobic metabolism of bacteria. ABE fermentation has two steps, i.e. acidogenesis and solventogenesis.

Acidogenesis works at the time of exponential period of bacterial growth. In this process, carbohydrates are converted into granulose and accumulated inside the cells whose structures contain α-1,4-linked polyglucan sugar (Shaheen et al. 2000). In this phase, organic acids like acetate and butyrate are produced from re-assembled monosaccharides. These organic acids diminish the pH value in the medium that excites the solventogenesis phase (Li et al. 2011a). In the end, solventogenesis was found to be started only at pH less than 5.1 (Millat et al. 2013).

On the contrary, solventogenesis works at the end of exponential period to the early stationary period of bacterial growth in the cytoplasm. Then acid production inside the cytoplasm becomes slow and the excreted acetate and butyrate are converted into the acetone and butanol. Finally, as by-products acetone, butanol, and ethanol were found at 3:6:1 ratio (Qureshi et al. 2006). Anyway, about 1–2% butanol can inhibit bacterial growth by disrupting the cell membrane (Jin et al. 2011). Then, the cells synthesize endospores for survival. Because, the endospores can survive in different stress conditions, for instance, UV light, heat, drought, or frost, etc. After getting proper conditions, the spores grow again (Wang et al. 2014). In that way, ABE fermentation can be regulated.

Butanol production is different from ethanol production in the case of substrate fermentation. Therefore, biobutanol production from microalgae could be more effective compared to the production of methanol or ethanol.

7.6.4 Marine Biogas

Biogases are important trendy renewable biofuels produced from various sources of organic biomass. Biohydrogen, biomethane, bioethane are the most promising gaseous biofuel candidates. Biogas contains mainly CH_4 and CO_2, along with other compounds like H_2S, NH_3, water vapor, and certain trace elements. The effective composition of biogas depends on the nature of feedstock used in the production process and the reaction conditions used to digest the feedstock. The production of biogas by anaerobic digestion of algae is a new attention because of high polysaccharides (agar, alginate, carrageenan, laminaran, and mannitol), no lignin, and low cellulose content in the algae feedstock used. Macroalgae especially seaweeds are the best source of feedstock for biogas production. Some studies

showed the application of some algae species to produce biogas that are *Scenedesmus*, *Spirulina*, *Euglena*, and *Ulva* (Ras et al. 2011; Zhong et al. 2012; Saqib et al. 2013). Besides these, a few more sources can be red algae, *G. vermiculophylla* (Tedesco et al. 2014); brown algae, *Macrocystis pyrifera* (Gurung et al. 2012), *S. latissimi* (Vivekanand et al. 2012), *Durvillaea antarctica* (Gurung et al. 2012). Moreover, microalgae can also be considered to produce biogas along with other carbonaceous feedstocks.

7.6.4.1 Anaerobic Digestion and Production Process

In general, anaerobic digestion (AD) means the fermentation of compound organic matter in the absence of oxygen, which causes decomposition of organic matter to produce CH_4, CO_2, H_2, and some volatile fatty acids (VFAs). The organic components in the macroalgae like carbohydrate, protein can easily be converted into biogas by anaerobic digestion. Anaerobic digestion is a multi-step process, and the four major steps are hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

Hydrolysis treatment is the first step of anaerobic digestion in biogas production. In this rate-limiting step, the complex organic matter is dissoluted or disintegrated or broken down into simple monomers and this increases their bioavailability to the fermentative bacteria. Various pretreatment processes like milling, maceration, thermal pretreatment are reported for efficient breakdown of cell wall and biogas production (Bird et al. 1990). At first, using the enzymes released from some definite anaerobes the insoluble organic compounds and heavyweight molecular substances like lipids, proteins, and carbohydrates are hydrolyzed into soluble compounds. The prevalent bacteria in this step are facultative anaerobes of the genera *Clostridium*, *Bacteroides*, *Butyrivibrio*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, and members of the *Enterobacteriaceae* family (Amani et al. 2010; Christy et al. 2014). An alarming issue is that more saline, sulfur, and halogens present in the production system prevent the production and growth of anaerobic microorganisms and also induce to grow fouling agents. Therefore, both water and weak acid pretreatment are necessary to eliminate a significant amount of mineral contents. This results in a high energy yield. This also helps in using biomass directly without drying and so any kind of microalgal biomass can be used as feedstock in the anaerobic digester system. A pretreatment can significantly increase the hydrolysis proficiency and enhance the methane synthesis capacity of the used feedstock. The pretreatment step is applied on the basis of the applied feedstock, energy needs, and the viability for use in large-scale production (Carrere et al. 2016). The common pretreatment methods like acid–base hydrolysis or mechanical pretreatments include using autoclave, homogenizers, microwaves, sonication, and also enzymatic methods. The important parameter to consider in biogas production is the ratio of C/N. The algae biomasses have a low ratio of C/N and it may inhibit the methane yield because it is undesirable for anaerobic digestion. To overcome this problem, the co-digestion of algae was used successfully to achieve a high C/N ratio and enhances

the methane production by decreasing the levels of ammonia under its inhibitory levels (Mussgnug et al. 2010).

The second phase is acidogenesis that is the principal step. In this step, monomers are converted into higher organic acids, alcohols, aldehydes, and some gaseous products. The soluble compounds are converted with the help of enzymes released by the acidogenic bacteria. Obligate and facultative (fermentative) bacteria work on the monosaccharides found in sugars and convert them to organic acids (lactate, propionate, butyrate, propionate, and acetate) and alcohols (ethanol or methanol) by adding with CO_2 and H_2. Fatty acids and amino acids found in lipids and proteins can be used as carbon sources for anaerobic bacteria. Some important bacterial species working on this step can be *Bacillus*, *Clostridium*, *Micrococcus*, *Pseudomonas*, *Lactobacillus*, *Salmonella*, *Corynebacterium*, *Eubacterium*, *Escherichia coli,* etc. (Christy et al. 2014). There are some organic acids formed in this phase, but acetate and butyrate are selected to generate methane gas.

The next one is acetogenesis, where acetogenic bacteria convert higher organic acids to acetate and hydrogen by the process of acetogenesis. Acetogenic bacteria are obligate anaerobes that grow slowly at pH 6 optimally (Christy et al. 2014). Some important acetogenic bacteria found in this step are *Syntrophomonas wolfeii*, *Syntrophobacter wolinii*, *S. fumaroxidans*, *Pelotomaculum* sp., *Smithella* sp., and *Clostridium aceticum* (Amani et al. 2010). Hydrogen evolved in this phase is caused by the accumulation of electron sinks as higher acids and alcohols. Acetogenic bacteria catalyze to convert these electron sinks to acetate, CO_2, and H_2 (Christy et al. 2014). The evolved hydrogen is toxic for the acetogenic bacteria that is why a low partial pressure of hydrogen is recommended. A syntrophic relationship is seen between hydrogen-evolving acetogenic bacteria and hydrogen-consuming methanogenic one, and this relationship regulates the proficiency of biogas production (Weiland 2010). A higher concentration of hydrogen helps in methane formation, where lower concentrations of hydrogen help in the formation of acetate from CO_2 and H_2 through homoacetogenic bacteria. Some recognized homoacetogenic bacteria are *Acetobacterium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus*, and *Sporomusa* (Saady 2013). Anyway, homoacetogens could develop methanogens in an anaerobic method at low temperatures and adverse environments (Ye et al. 2014).

Methanogenesis is the final step which is mainly methane-producing phase. Methanogens metabolize acetic acids and hydrogen into methane and carbon dioxide (Cantrell et al. 2008; Brennan and Owende 2010; Romagnoli et al. 2010). Archaea regulate the phase because of their special metabolism capacity of using acetate, CO_2/H_2, formate, or other methylated carbons that can be a source of energy and carbon for methane production (Enzmann et al. 2018). Methanogenic organisms found in anaerobic digestion can be hydrogenotrophic methanogens or acetoclastic methanogens. Acetoclastic methanogens cause acetate decarboxylation and generate methane and CO_2. Few species like *Methanosarcina barkeri*, *Methanococcus mazei*, *Methanothrix soehngenii* are capable of acetoclastic methanogenesis (Weiland 2010). Methanogens determine the efficacy of the anaerobic digestion process. So, it is necessary to regulate the production parameters in anaerobic digestion that can

favor methanogens. Finally, biogas (a mixture of CO_2 and CH_4) and the residual digestate are found as the resulting products. Then, the digestate can be solid and liquid fractions. The solid digestate can be applied as a biofertilizer because it is easy to handle and contains higher bioavailable nitrogen for plants. On the contrary, the liquid portion contains leftover organic acids and other macronutrients like NH_3 and phosphorus. This process is also associated with several challenges in the biogas production such as, inappropriate C:N ratio and high level of ammonia in pretreatment process and the presence of alkaline metals in macroalgae. These may inhibit the anaerobic process. Therefore, to get an increased yield of methane, an appropriate C:N ratio should be maintained and a lower ratio may result in the accumulation of ammonia in the bio-reactor that reduces the yield of biogas finally.

Overall, some issues can be considered regarding biogas production from marine sources. There are some factors like the requirement of suitable space, infrastructure, and heat required for the digesters may control the biogas yield (Collet et al. 2011; Jones and Mayfield 2012). Some proteins of algae cells can enhance ammonium synthesis that causes low carbon–nitrogen ratio. The C-N ratio can affect the production by inhibiting the growth of anaerobic microorganisms. Anaerobic microorganisms are also downregulated by the sodium ions. So, salt-tolerant microorganisms can be a better option for the anaerobic digestion of algae biomass (Brennan and Owende 2010; Jones and Mayfield 2012).

7.6.5 Biomethane Production from Marine Microalgae

Biomethane is considered as one of the most encouraging renewable fuels which has a great possibility to cause a transition of existing fossil fuel-dependent energy toward a sustainable energy for the future. Methane combustion emits a lower CO_2 compared to other traditional hydrocarbon fuels. But the ratio of molar weight (16.0 g/mole) to combustion heat (891 kJ/mole) reveals that methane produces more heat/unit weight than any other hydrocarbons (Shuba and Kifle 2018). Biomethane can be generated from various sources of biomass such as food wastes, agricultural residues, animal manure, forestry residues, energy crops, microalgae, organic-rich wastewaters, organic fraction of municipal solid waste, and industrial organic waste by the anaerobic digestion (Cucchiella and D'Adamo 2016; Jankowska et al. 2017). But, among them, microalgae are considered to be more suitable feedstock as they grow faster (5–10 times), have higher biomass production, and also are suitable to cultivate in the nonarable lands and nutrient-rich wastewaters. Moreover, microalgae are very potential to consume CO_2 so that accumulation of CO_2 in the atmosphere is reduced (Stephens et al. 2013; Ward et al. 2014). Microalgae contains enough biodegradable compounds, for instance, carbohydrates (4–57%), lipids (2–40%), and proteins (8–71%) of total solids (Prajapati et al. 2013) that can generate more biomethane around a theoretical yield of 0.42, 1.01, and 0.5 L STP CH_4/g, respectively (Guiot and Frigon 2012).

The anaerobic digestion is a widely accepted method for producing methane (CH4) from algal biomass (Ho et al. 2018). The anaerobic digestion to produce methane from microalgae can be two types: liquid AD (L-AD) and solid-state AD (SS-AD). The basic principles of these techniques are similar but can vary with the physical conditions of the system, especially moisture content of biomass (Li et al. 2011b). The methane production is almost the same in these two methods. But volumetric productivity can be higher in SS-AD than that of A-AD (Brown et al. 2012). The basic protocol to produce biomethane by anaerobic digestion of microalgae includes several steps such as cultivation, harvesting, pretreatment, and then anaerobic digestion of the microalgae. Besides, the biomethane production varies significantly on the basis of selecting suitable algal strain because microalgae show wide variation in their biomass composition.

7.6.6 Biohydrogen Production

Algal biohydrogen is a common commodity nowadays that is used as gaseous fuels or electricity generation. Biohydrogen production has various processes such as biophotolysis and photofermentation (Shaishav et al. 2013). Biohydrogen can be produced from various marine algal sources. Park et al. (2011) positively recommended *Gelidium amansii* (red alga) as the potential biomass source for biohydrogen production using anaerobic fermentation and the study produced 53.5 mL hydrogen per 1 g of dry algae with a production rate of 0.518 L H_2/g VSS/day. Moreover, Shi et al. (2011) found 71.4 mL hydrogen from per 1 g dry algae (*Laminaria japonica*) by anaerobic sequencing batch reactor for 6 days of hydraulic retention time maintaining mesophilic condition (35±1 °C), pH 7.5. So, to maximize the biohydrogen production pretreatment method should be optimized importantly (Park et al. 2011; Shi et al. 2011). Saleem et al. (2012) decreased the lag period in hydrogen production from microalgae (*Chlamydomonas reinhardtii*) using an optical fiber for an internal light source and they found maximum hydrogen production rate using exogenic glucose.

Some of microalgae such as BGA (blue green algae) contain glycogen in their cell instead of starch. In this case, oxidation of ferredoxin is caused by the hydrogenase enzyme to produce hydrogen in the anaerobic situation. Nevertheless, this enzyme helps in the detachment of electrons too. Hence, some scientists have emphasized to identify the enzyme activities having interactions with ferredoxin and the other metabolic activities for microalgal photobiohydrogen synthesis (Yacoby et al. 2011; Rajkumar et al. 2014).

7.6.7 Bio-Oil and Syngas Production

Bio-oil is produced in the liquid phase of anaerobic digestion of algal biomass at high temperature. The proximate composition of bio-oil can be varied on the basis of different feedstocks and processing parameters used (Iliopoulou et al. 2007; Li et al. 2008). Some parameters like biomass composition, pyrolysis temperature, water, ash content, and vapor residence time can regulate the productivity of bio-oil (Fahmi et al. 2008). Anyway, crude bio-oil is not used as fuel because it contains water, oxygen content, unsaturated and phenolic moieties too. So, some treatments are needed to enhance its combustion quality (Bae et al. 2011). Bio-oils are treated to generate power with the help of an external combustion process using steam Rankine cycles and Stirling engines. Yet, power generation can also be done by internal combustion by applying diesel and gas-turbine engines (Chiaramonti et al. 2007). Existing evidence shows that few studies have been conducted on algae pyrolysis compared to lignocellulosic biomass. Some pyrolysis processes were applied to reduce their inherent disadvantages of carrier gas flow and excessive energy inputs, but high yields of bio-oil were found in fluidized-bed fast pyrolysis (Oyedun et al. 2012). Demirbas (2011b) examined the suitability of marine microalgae to produce bio-oil and got better quality than the wood one. Porphy and Farid (2012) conducted a study to produce bio-oil from the pyrolysis of extracted lipids from microalgae (*Nannochloropsis* sp.) by applying 300 °C. This contains 50% acetone (wt), 30% methyl ethyl ketone (wt), and 19% aromatics (wt), i.e. pyrazine and pyrrole. Choia et al. (2014) also conducted a study on pyrolysis of *Saccharina japonica* (brown algae) at 450 °C and found almost 47% bio-oil production.

Syngas is a mixture of some gases such as CO, CO_2, CH_4, H_2, and N_2 that can be generated by normal gasification. Gasification transforms biomass into the combustible gas mixture at high temperatures (800–1000 °C) with the help of partial oxidation. This combustible gas mixture is known as syngas or producer gas. The syngas has a calorific value of 4–6 MJ m^{-3} (McKendry 2002). It mainly contains a mixture of H_2 (30–40%), CO (20–30%), CH_4 (10–15%), ethylene (1%), nitrogen, carbon dioxide, and water vapor (Saidur et al. 2011). This gas is consumed to emit heat or convert into electricity and in gas-turbine systems (McKendry 2002). The syngas could be also used to generate methanol and hydrogen fuel for transport and other applications (Saidur et al. 2011), although the production cost from methane and marine biomass is theoretically estimated at 1.5–4 times more compared to the production cost of fossil fuel gas. In the gasification process, pyrolysis is done initially in a reaction producing char, then gasified with a gasifying agent such as O_2 or H_2O to produce syngas. Here, biomass reacts with oxygen and steam water to produce syngas. Different marine algal feedstocks like *Ulva lactuca* (Nikolaison et al. 2012), *S. latissimi* (Cherad et al. 2013), *S. japonica* (Kwon et al. 2012) can be used for syngas production through the gasification process.

7.7 New Opportunities for Biofuels and Advantages of Producing Biofuel from Marine Algae

There is a growing concern for increasing energy requirements on a global scale. To satisfy these excess energy needs, marine biotechnology was dedicated to providing an important contribution in very different ways. Moreover, the global production of oil was saturated and people are searching for an alternative option to fossil fuel. Therefore, biofuel is considered as a leading energy source for the future. The biofuel production from marine algae indicates a huge potentiality to be an alternative energy source replacing fossil fuel. Growing evidence suggest that marine microalgae and macroalgae, especially seaweeds are suitable sources for biofuel production. Microalgae can contain enough hydrophobic compounds that are converted into biodiesel. So, biodiesel production from microalgal tri-acylglycerides is much more focused on the biofuel industry. Some of the other advantages include:

1. The marine algal biomass are a huge and superior feedstock that act as an alternative to terrestrial biomass for bioenergy production (Chen et al. 2015).
2. These algae uptake enormous greenhouse gas and release extra oxygen while growing (Bharathiraja et al. 2015).
3. They are non-edible sources and so there exists no competition.
4. The algal species are a highly biodegradable resource with rapid bioremediation and are non-toxic (Chen et al. 2015).
5. They show rapid growth and result in high growth yield and increased productivity (Abbasi and Abbasi 2010).
6. They have high energy conversion efficiency by photosynthesis (Huber et al. 2006).
7. Prevent eutrophication and pollution in the aquatic ecosystem (Huber et al. 2006).
8. They can easily be adaptable to a wide range of climatic conditions (Bharathiraja et al. 2015).
9. These biofuels act as sustainable and environment-friendly fuel and are highly effective to meet the present energy demand (Ziolkowska and Simon 2014).
10. The biofuel from algae has great reactivity and decreases hazardous emission (Abbasi and Abbasi 2010).
11. Diversification of fuel supply.

7.8 Challenges and Disadvantages of Using Algae and Algal Biofuel

There are many arguments for and against the use of algae and algal biofuel. Marine algae cultivation for bioenergy production is a great challenge indeed. Even though this technique has the potential to deliver clean energy, before commercial use

science should find solutions to address some of the serious drawbacks associated with this technique. The main challenges or/and disadvantages of marine algal biofuel are mentioned below:

1. Understanding of microalgal biodiversity is needed to decipher at the molecular level and on a global scale.
2. Achievement of a net energy gain along the whole production chain necessary to convert microalgal biomass into biofuels.
3. Achievement of full sustainability of the whole production chain in terms of regional and global impact.
4. Increased initial production cost for growing, harvesting, collection, transportation, storage, and pretreatment (Bharathiraja et al. 2015).

Lack of monitoring and algal growth control for fuel production (Sharma et al. 2013).

1. Use of extra water for algae processing.
2. Low ash fusion temperature (Ross et al. 2008).
3. Limited practical experience in biofuel production (Ziolkowska and Simon 2014).

7.9 Conclusions

Globally, there is a much potential for biofuel market. Biofuel from marine algae has the potential to replace fossil-based petroleum, seems technically feasible and conversion of extracted lipid to biodiesel is relatively easy. The commercial-scale production of algal biofuels requires careful consideration of several issues that can be broadly categorized as: selection of high oil and biomass yielding algal species, cultivation and harvesting technology, water sources, and nutrient and growth inputs. The promising and clear potential of algal biofuels for contributing to environmental, social, and economic sustainability needs to be transformed into a sustainable reality. As yet, there is no commercial production of such biofuels due to the high production costs and technical issues concerning post-cultivation processing. Therefore, for strengthening the global economy, mitigating climate change, increasing the feasibility, and reducing the production cost, the sector still requires technological development. Finally, it is expected that the prospects for liquid biofuel production from autotrophic marine microalgae will much improve in the near future, especially using genetically modified microalgae.

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Chapter 8
Modified or Functionalized Natural Bioadsorbents: New Perspectives as Regards the Elimination of Environmental Pollutants

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Contents

Abstract One of the greatest concerns affecting the environment today is the presence of pollutants in large bodies of water. This is mainly caused by industrial effluents that are discharged without any treatment to nearby water sources and that produce a wide variety of potentially dangerous pollutants. In this area, adsorption plays a very important role owing to their low-cost operation and high performance. The efficiency of adsorption is indicated by the quality of the adsorbent material. For this reason, in recent years, the research community has focused its attention on the study of biomasses as adsorbent materials, because most of them are wastes that have no utility. They have been produced from agricultural and livestock waste without any previous treatment to the modification and functionalization of biomasses or extracts obtained from them. In this chapter, different types of modified and functionalized materials that can be used as bioadsorbents in the treatment of contaminated water are presented, as well as the different types of treatments that the materials can receive to improve their adsorbent characteristics, depending on the substance to be adsorbed. Furthermore, the importance and approach with which the functionalized materials are studied are highlighted, since these represent the new scope of the bioadsorbent materials used in environmental remediation.

Keywords New materials · Modified bioadsorbents · Functionalized bioadsorbents · Bioadsorption · Environmental remediation

8.1 Introduction

The existence of environmentally toxic and dangerous pollutants is currently one of the most serious problems faced by humanity (Kyzas and Kostoglou 2014). A clear example of this is the water resources damaged by substances produced from various anthropogenic activities, such as accelerated industrialization and unplanned

urbanization (Varghese et al. 2018). Much of the wastewater produced by domestic and industrial services is discharged into the environment without receiving adequate treatments. This is common in underdeveloped countries and, in some cases, developed countries (Liang et al. 2018). These effluents may contain a high level of pollutants that are potentially dangerous for aquatic organisms, human health, and the environment, in general, as they destabilize the balance of ecosystems.

Effluents may be contaminated by several different types of pollutants, such as dyes (Robledo-Padilla et al. 2020), heavy metals (Tejada-Tovar and Villabona-Ortiz 2015), phenols (Zbair et al. 2018a), pesticides (Badawy et al. 2018), and pharmaceutical products (Kar et al. 2018). However, most control and sanitation entities do not have either the resources or the technologies required to deal with these pollutants, since many treatment processes are expensive, have high energy requirements, and may even generate more toxic by-products that are difficult to dispose (Barakat 2011). This is a challenge for organizations with limited financial resources; therefore, numerous approaches have recently been studied to develop cheaper and more effective substance treatment and removal technologies.

Various technologies that enable the separation and removal of harmful organic and inorganic compounds from wastewater have been developed in recent years (Cui et al. 2016; Quesada et al. 2019), one of the best of which is adsorption owing to its high efficiency, low cost, technological simplicity, quick response, and respect for the environment (Wang et al. 2018; Asuquo et al. 2018). Some of the various factors that influence the application of adsorption are the nature of the adsorbent, concentration of hydrogen ions (pH) in solution, its temperature, its resistance to abrasive materials, and its adsorbent regenerative capacity (Quesada et al. 2019). During this process, the adsorbent affinity for contaminating species (adsorbate) facilitates their attraction to the adsorbent and is linked to it through different physical and/or chemical mechanisms (Cuizano and Navarro 2008). Currently, the most important characteristic being sought in adsorbents is, therefore, high porosity, which results from a greater surface area and enables faster adsorbate elimination, because the degree of adsorption is proportional to the surface area available for adsorption (Tien 1994; Dąbrowski 2001). Selecting the appropriate adsorbent is, therefore, essential before conducting any study, and a wide variety of materials with which to achieve effective adsorption have consequently been studied, such as activated carbon, resins, zeolites, agricultural and industrial residues, biological materials, and others (Crini et al. 2019).

The development, modification, and functionalization of natural adsorbent materials is another interesting aspect owing to the availability of biomasses, their renewable and generally safe nature, and the fact that these materials have the ability to bond with others, thus increasing the efficiency of adsorbate removal. Biomasses have, therefore, become the focus of attention of researchers worldwide (Alvarez et al. 2012). These researchers have shown that the structural components of biomasses have the ability to bound and adsorb several different natural compounds (Lavado Meza and Oré Jiménez 2016), and various natural adsorbents have been used in several research works (Ahmaruzzaman 2008; Rafatullah Mohd et al. 2010; Yang et al. 2018).

This chapter presents different types of natural and improved bioadsorbent materials that could be used as alternatives for the removal of polluting substances.

8.2 Naturally Occurring Adsorbent Materials

An ideal material for the adsorption of contaminants must meet various requirements, such as low cost and high adsorption and regeneration capacities, and the approaches must always be cost-effective (Mahfoudhi and Boufi 2017). The functional groups that natural bioadsorbents have in their structure play a significant role during the natural adsorption of pollutants of a different nature. It is also important to take into account other mechanisms involved in the process, such as electrostatic interactions, hydrogen bonds, and ion exchange. Several examples of natural biomasses used as bioadsorbent materials are presented in Fig. 8.1. Recent studies on bioadsorbent materials of natural origin without prior chemical or physical treatment are presented in this section.

Multiple natural materials resulting from agro-industrial activities and which are already available in the environment are used as potential adsorption materials owing to their low cost and mechanical resistance and the presence of compounds in their structure, such as functional groups capable of binding with different adsorbates.

Marine biomass provides important resources for the treatment of environmental pollutants, which is performed through bioadsorption (Tondwal and Singh 2018). Chitosan is an effective adsorbent material owing to its high amino acid content and hydroxyl functional groups, and its adsorption potential is significant for the retention of various water contaminants (Hajji et al. 2015). It has also been used to remove heavy metals (Mende et al. 2016), tints (Subramani and Thinakaran 2017), and phenols (Francis et al. 2020). Shrimp shell has been investigated for the bioadsorption of various metallic ions and is capable of removing high percentages of Fe (63.4%) and Cr (62.2%) (Rech et al. 2019), in addition to the metallic ions and

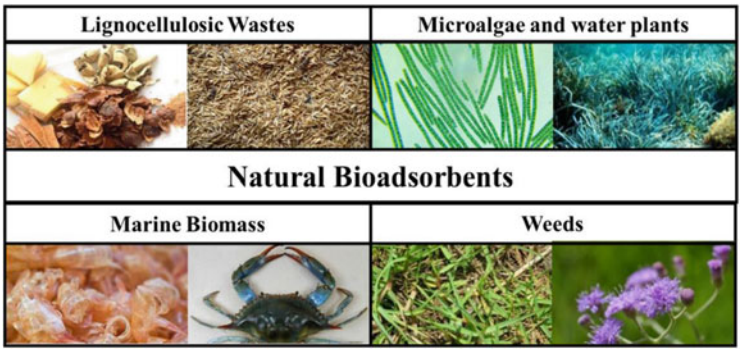

Fig. 8.1 Some natural biomasses used as bioadsorbent materials

acids present in acid mine drainage (Núñez-Gómez et al. 2019). Furthermore, a structural analysis of the *Callinectes sapidus* (blue crab) biomass has revealed that it has several useful metal oxides with which to adsorb pollutants, especially in the case of Cd, Ni, and Pb during the flow control of aqueous stream (Foroutan et al. 2019). Marine aquatic plant fibers, such as *Posidonia oceanica*, also have favorable characteristics for the removal of heavy metals from an aqueous medium (Boulaiche et al. 2019), in addition to the removal of dangerous dyes, such as crystal violet and methylene blue (Sulyman and Gierak 2020).

Microalgae biomass has recently been used to remove various contaminants. Indigo blue dye has been reported to exhibit high adsorption capacities in the *Spirulina platensis* biomass, which maintains an average adsorption of 91% from 25 mg L^{-1} to 100 mg L^{-1} dye concentrations (Robledo-Padilla et al. 2020). This microalga has also been used as a bioadsorbent for other dyes, such as reagent red RR-120 (Ayachi et al. 2019), FD&C red no. 40, tartrazine (Ben Torkia et al. 2018), and acid blue 9 (Ben Torkia et al. 2020); it is also superior as a bioadsorbent for the retention of metal ions, such as Cr (III) (Rezaei 2016), Cu (II) (Gunasundari and Senthil 2017), and Zn (II) (Zinicovscaia et al. 2018). The use of *Artrospira* (Spirulina) has been tested for the multielement adsorption of Cr (III), Mn (II), and Mg (II); it has also been applied as an animal food bioactive additive, which enables it to act as a valuable metal ion carrier (Michalak et al. 2020).

Lignocellulosic residues have been widely used in wastewater treatment processes, thanks to their high availability, and their adsorption capacity owing to their lignocellulosic structure (amines and carboxylic acids) is chemically related to various contaminating species (Tzvetkov et al. 2016). These favorable characteristics of biomasses make them attractive as adsorbents for resources. Some of the lignocellulosic biomasses that have been widely evaluated as natural adsorbents are rice husk (Chowdhury et al. 2016; Kumar et al. 2017) and sugarcane bagasse (Asif and Chen 2017; Kaur et al. 2020), as they exhibit high mechanical resistance and chemical stability and contain abundant cellulose and hemicellulose lignin (Nagaraj et al. 2017; Milani et al. 2018), which are favorable for adsorption. Experiments have also been conducted on tea waste for the removal of Cr (VI) (Cherdchoo et al. 2019) and methyl blue in aqueous media (Liu et al. 2018). Nutshells were studied for the removal of Cr (VI) and were evaluated in fixed-bed and continuous-flow columns for small- and large-scale industries (Banerjee et al. 2018). Coconut and banana peel, ground coffee, and eucalyptus bark have shown to be capable of removing Pb (II) and Ni (II) and are applicable to metal ions in wastewater treatment (da Silva Correia et al. 2018). Ground coffee has also been evaluated for the adsorption of Cr (VI) (Cherdchoo et al. 2019), heavy metals from mining waste (Ayala and Fernández 2019), and Cd in solution (Kim and Kim 2020). Moreover, banana peel has been widely used in the treatment of waters contaminated with Cd (II) (Chen et al. 2018b) and in the decontamination of Cu and Pb (Vilardi et al. 2018). Lignocellulosic biomass residues, such as chestnut peel, araucaria bark, cactus, and palm, have been used as natural adsorbents of crystal violet dye (Pang et al. 2019). Other lignocellulosic residues can be used as efficient adsorbent materials for the removal of industrial dyes from water, orange albedo can be used for the elimination of cationic dyes (Silva et al. 2019a), and corn silk can be used for the elimination of

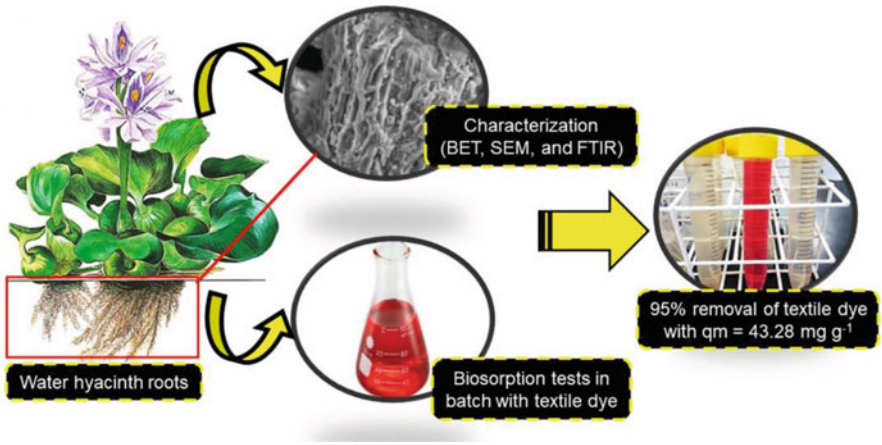

Fig. 8.2 Adsorption of BF-4B red reactive textile dye using the roots of *Eichornia crassipes* with 95% removal and a maximum adsorption capacity of 43.38 mg g^{-1}. Reproduced from Rigueto et al. (2020). Copyright © 2020 with permission from Elsevier

Blue Reactive 19 and Red Reactive 218 (Değermenci et al. 2019). Eggshell has recently been studied for use in the degradation of dyes, such as Direct Blue 78 at a concentration range of 25–300 mg L^{-1} (Murcia-Salvador et al. 2020).

Weeds such as *Cyanthillium cinereum* (L.), H. Rob and *Paspalum maritimum* contribute to the reduction of water contamination caused by toxic substance retention (Silva et al. 2019b); moreover, they have achieved high percentages of elimination, with maximum adsorption capacities of 76.34 mg g^{-1} and 56.18 mg g^{-1}, respectively. The raw fibers of piassava (*Attalea funifera*) are used to remove color from effluents containing methylene blue and dyes with similar characteristics (Marques et al. 2019). The roots of the aquatic plant *Eichornia crassipes* can be used as an alternative for dyes such as the red reagent BF-4B in aqueous media treatment (Fig. 8.2) (Rigueto et al. 2020); the dry biomass of this aquatic plant can also retain heavy metals (de Freitas et al. 2019).

8.3 Modified Bioadsorbent Materials

The use of bioadsorbents has many challenges with regard to the attempt of improving each of their applications (Johari et al. 2016). As mentioned in this chapter, studies on bioadsorbents started with materials that had not been modified (of natural origin). The characteristics of these materials have subsequently been improved, depending on the requirements, and this led to the emergence of modified and functionalized materials.

In recent years, many natural bioadsorbents have been modified, with the aim of converting them into materials that are more appropriate for a certain substance and thus improving the adsorption process (Akbas and Yusan 2020). Modified

bioadsorbents have easily exposed active sites, 3D structures, and mechanical properties that make them highly desirable and ideal for an efficient compound removal (Anastopoulos et al. 2019). Some of the studies on modified bioadsorbent materials, along with their respective treatments and applicability, are described in the following sections.

8.3.1 *Bioadsorbent Materials Modified by Chemical Activation*

Chemical activation treatment involves the modification of the surface characteristics of a material through the elimination or entrapment of functional groups, thus exposing more active sites on which the adsorbent material binding with the substances can be retained (Karaoglu et al. 2011).

Chemical methods include treatments with various organic and inorganic compounds, including acid and base treatments. Chemical modification through alkaline and acid hydrolysis treatments increases the adsorption capacity and performance of multiple lignocellulosic materials. Acid treatments increase surface acidic functional groups, which improves chelation capacity with metallic species, thus providing carboxylic and phenolic functional groups in the fiber structure. Meanwhile, basic treatments increase the adsorption capacity of organic compounds (Gautam et al. 2014), as they provide basic active sites (predominantly carboxylate ions). For example, chemical activation using alkaline sodium hydroxide solution has been applied to pine bark, to increase its adsorption affinity for metal ions (Arim et al. 2018), and to aquatic plants, such as *Eichhornia crassipes*, to produce an improved adsorbent (cellulose xanthate) (Neris et al. 2019; Sayago et al. 2020). Also, the chemical activation of cashew nut shells with sulfuric acid has enhanced the adsorption capacity of this adsorbent, in the adsorption of heavy metals (Yahya et al. 2020). The coffee solid waste has also been modified with citric acid, resulting in an increase in its adsorption capacity (Botello-Gonzalez et al. 2019). The same effect has been experienced by sea grasses, such as *Posidonia oceanica*, modified with acetic acid (Elmorsi et al. 2019). Other biomasses such as banana peel, eucalyptus bark, maize cob and maize leaf have been treated with alkaline and acidic chemical activation, increasing the adsorption of Pb (II) and Ni (II) ions (Santos et al. 2019).

Rice husk has been the basis of several investigations on modified materials owing to its high availability in the form of a production waste and especially its high lignin content (Soltani et al. 2015). The adsorption capacity of rice husks has been increased to remove nickel and reactive yellow dye (Azadi et al. 2018; Rachna et al. 2019) and to enable adsorption of imidazolinone herbicides (Kaur et al. 2020) through the use of thermal–chemical treatments with alkaline solutions. This material has also been modified using citric acid to adsorb Cd (II) (Wang et al. 2019b). In the study by Bhatti et al. (2020), rice husk modification has been evaluated through

chemical treatments with different substances, such as nitric acid, hydrochloric acid, sodium hydroxide, and sulfuric acid. The treatment of rice husk with hydrochloric acid has been shown to significantly increase its adsorption capacity compared to that of native biomass. The combination of various technologies to increase the adsorption performance of rice husk has also been investigated. For example, the synthesis of a double-network hydrogel based on a rice husk lignin, chitosan, and polyacrylamide solution using the free-radical polymerization process obtained exceptional yields for the treatment of heavy metals in individual and ternary systems (Ma et al. 2019). Chitosan and rice-shelled lignin have good adsorption properties, as they provide reactive functional groups in alkaline solution (Nair et al. 2014; Ma et al. 2019). Furthermore, polyacrylamide provides an entrapment matrix with which to constitute a stable structured hydrogel adsorbent (Ma et al. 2017).

Marine wastes that are rich in chitin and proteins are potential bioadsorbent materials (Wysokowski et al. 2014), and studies on treatments that improve their properties, such as specific surface area, have consequently been conducted (Simsir et al. 2017). Shrimp shells have been treated through deproteinization and deacetylation, followed by hydrothermal carbonization (hydrochar) and acid washing, and their methyl orange adsorption performance has been improved through electrostatic interactions (He et al. 2020). The raw biomass of marine wastes (thorns and shells) can also be treated with acid and alkaline activation, and the resulting chemical nitrogen residues (amine and acetamide) are considered as the parameters responsible for substance binding (Duran et al. 2017; Maachou et al. 2019; Teshale et al. 2020).

8.3.2 Activated Carbon

One of the modified materials most frequently used for the removal of industrial pollutants is activated carbon (Zbair et al. 2018b). Its adsorption capacity depends on the precursor material, along with the activation and carbonization methods employed during its preparation (Habila et al. 2015). Activated carbons are porous solid materials with high thermal stability, hydrophobic property, and high chemical resistance (Marsh and Reinoso 2006). In general, they are mainly microporous; however, in addition to micropores, they contain mesopores and macropores, which are important to facilitate the access of adsorbate molecules to the interior of a carbon particle (Gautam et al. 2014). Traditional activated carbons are commonly produced from truly expensive and non-renewable raw materials, such as coal, lignite, or anthracite (Tan et al. 2017). However, many scientific studies have suggested the use of biomass residues, especially lignocellulosic residues (Suhas et al. 2016; Campos et al. 2018; Villar da Gama et al. 2018), to produce porous activated carbons (Gil et al. 2014; Ruiz et al. 2017; Rojas et al. 2019; Alcaraz et al. 2019; Marsagishvili et al. 2020; Trakoolsa and Yoochatchaval 2020). With regard to the activation procedures, the chemical method is preferred over physical activation, as it requires lower temperatures, and highly developed microporosities are obtained

in a single step (Yahya et al. 2015), indicating higher yields (Budinova et al. 2006). One of the many agents used is zinc chloride, which improves the development of pores in the carbon's structure (Ruiz et al. 2017).

The activation of non-pyrolyzed lignocellulosic residue using sodium carbonate at 900 °C has led to greater porosity and yields to eliminate siloxane and volatile organic compounds compared with commercial samples (Santos-Clotas et al. 2019). The chemical activation of dry carbonized rice husk and corncob samples has been performed at 350 °C using sodium hydroxide and hydrochloric acid, with a maximum fluoride removal efficiency of 91% and 89%, respectively (Gebrewold et al. 2019). Campos et al. (2020) performed the chemical activation of the corncob residues using phosphoric acid. Li and Xiao (2019) have also shown that the modification of rice husk charcoal can be performed using potassium carbonate, which produce favorable results. Zinc chloride-activated copyrolyzed rice straw charcoal has been used to remove metals (Fan et al. 2019), and similarly, potassium hydroxide as a carbon activation element has been evaluated for the adsorption of drugs, such as paracetamol (Spessato et al. 2019).

8.3.3 Biochar

Thermochemical conversion is employed for the efficient generation of high added-value by-products obtained from biomasses, such as biochar, bio-oil, and gas (Zhang et al. 2015; Wang et al. 2017b). Biochar is a carbon-rich by-product that is synthesized by using pyrolysis and the thermal decomposition of organic compounds in the absence of oxygen at a relatively low temperature (Brown 2011). Biochar is considered to be an economical and ecological adsorbent and has been used to adsorb various water pollutants, such as pesticides, heavy metals, and dyes, representing a competitive material against raw agro-industrial wastes (Liu et al. 2015; Rajapaksha et al. 2016). The modification of the biochar surface through chemical and physical treatments further improves its adsorption capacity and increases its active surface area by incorporating highly active functional groups for the adsorbates, thus allowing its wide application (Liu et al. 2015; Tan et al. 2017; Wang et al. 2017a). The properties of a product depend on the material used and the temperature of pyrolysis, indicating that biochars produced at high temperatures have a greater adsorption surface area, porosity, and mineral content and a smaller number of functional groups (Li et al. 2017). It has been shown that biochar porosity increases from approximately 0.056 to 0.099 $cm^3\ g^{-1}$ at temperatures between 500 and 900 °C, with an increase in adsorption surface area (Chen et al. 2014). The surface pore size and surface area also depend on the pyrolysis temperature and ranges from nano, micro, to macropores (Ahmad et al. 2014).

The use of numerous biomass waste materials in biochar production has recently been reported. Studies have been conducted to develop innovative biochars with high porosity and paracetamol (PRC) adsorption capacity from pure glucose derivatives (spherical biochar) and grapefruit skin residues (non-spherical biochar), which are obtained through pyrolysis at 900 °C and 700 °C, respectively (Tran et al. 2020).

The application of these treatments additionally improves yields and adsorption capacity. For example, the active surface sites of biochar prepared through rice husk acid modification were increased by up to ten times (Sarkar et al. 2019), whereas biochar production conducted through biomass steam activation increased the adsorption surface area (Sewu et al. 2019).

The high yields of compound removal provided by biochars have, in general, led to their wide use with contaminants such as heavy metals, dyes, and drugs, among others. Heavy metals have been treated by developing biochar from *Torreya grandis* walnut shell (Huang et al. 2019), rice husk and magnesium oxide (Xiang et al. 2018), *Typha orientalis* modified using shrimp wastes (Yin et al. 2019), fungus substrate (Wu et al. 2019), rice straw and sewage sludge (Gao et al. 2019), and *Camellia sinensis* residues (Guo et al. 2020). Meanwhile, Macroalgae biochar (Chen et al. 2018a), fungus substrate and *Saccharina japonica* (Sewu et al. 2017), crab shell (Dai et al. 2018), and sewage sludge (Fan et al. 2017) have been used to treat dyes. Other examples are nitrate removal with biochar derived from macadamia nutshell (Bakly et al. 2019), phosphate removal with rice husk modified using lanthanum hydroxide biochar (Xu et al. 2019), carbon dioxide adsorption with cashew biochar (Garg and Das 2019), natural organic matter adsorbed with pineapple biomass biochar (Yazdani et al. 2019), and sulfonamide removal with biochar and hydrochar based on ground coffee (Zhang et al. 2020b).

8.3.4 Immobilized Bioadsorbent Materials

Material immobilization in a matrix can reduce some adsorption deficiencies, such as low density, small particle size, and low rigidity and mechanical resistance exhibited by native biomass. Immobilization is a technique that provides bioadsorbents with the support required to endow a material with greater mechanical resistance, porosity, rigidity, and, in addition, stability for several cycles under different conditions (Nadeem et al. 2016). It is especially useful for those adsorbents based on microorganisms (Velkova et al. 2018).

Several studies have been conducted to evaluate immobilized biomass performance by concentrating on characteristics, such as adsorption capacity, stability, and recycling, during which the efficiency of immobilized materials increases compared with that of native biomass (Liu et al. 2016; Busquets et al. 2016). Bioadsorbent immobilization can be performed using various entrapment materials, such as alginates (Qi et al. 2019; Zhang et al. 2020a; Verma et al. 2020), polyacrylamide (Yue et al. 2019), polysulfone (Rangabhashiyam and Vijayaraghavan 2019), polyvinyl formal (Ramteke and Gogate 2016), cellulose (Ciftci and Cevrimli 2016; Zhou et al. 2019), polyvinyl alcohol (Kew et al. 2016), and carboxymethyl cellulose sodium salt (Skrzypczak et al. 2019).

Several immobilized materials have been used for the treatment of contaminants, one of which is corncob biomass immobilized in calcium alginate pearls, which is capable of removing 64.52% of heavy metals (Manzoor et al. 2019). Biomass wastes

obtained from the production of polyglutamic acid trapped in sodium alginate have also been used with the same aim (Zhang et al. 2020a). In addition, microbial biomass immobilization technologies have been tested, such as the use of sawdust to immobilize fungal biomass (Przystaś et al. 2018) and *Escherichia coli* biomass in polysulfone (Kim et al. 2016). Immobilization protects the biomass for adsorption and also improves enzyme production for the degradation of substances.

8.4 Functionalized Bioadsorbent Materials

As stated in the previous section, modified materials are those on which treatments are performed, such as basic acid chemical activation, carbonization, and calcination, among others. These are basically treatments that modify the structure and surface of the adsorbent to expand the surface areas and pore volumes specific to each material (Mistar et al. 2020), thus providing the new material with more active sites. In these cases, the treatment does not include the addition of activating substances on the adsorbent surface.

Functionalized materials also have specific treatments according to the type of molecules that it is desired to retain in the adsorbent material. The objective of these treatments is to obtain super materials with a large number of functional groups on the surface of the material that strengthens its selectivity and adsorption capacities (Huacai et al. 2016; Pereira et al. 2020). This is achieved by mixing different materials or compounds derived from other adsorbents to take advantage of the characteristics of each one in a single material. It should be noted that, with the evolution of bioadsorbent materials, the latest studies focus on the different types of material functionalization; moreover, improved conventional adsorbents have even been reported, which are obtained by adding compounds of natural origin that provide a new material with repowered characteristics (Wang et al. 2019a).

This section refers to various studies in which materials proven as bioadsorbents have been functionalized. Owing to the great variety of existing materials, the following classification is presented, arranged as presented in Fig. 8.3:

- Natural: Biomaterials or the functionalization of some of their main polymers.
- Activated carbon: The functionalization of biomaterials that have undergone carbonation and activation processes.
- Biofunctionalized metal–organic framework (MOFs): Functionalization in which MOFs are involved with compounds of natural origin or bioadsorbents.

The first two categories are defined as follows:

- Nanomaterials: Functionalization in which the final product has nanometric sizes.
- Magnetized: Functionalization in which the aggregates endow the final products with magnetic characteristics.

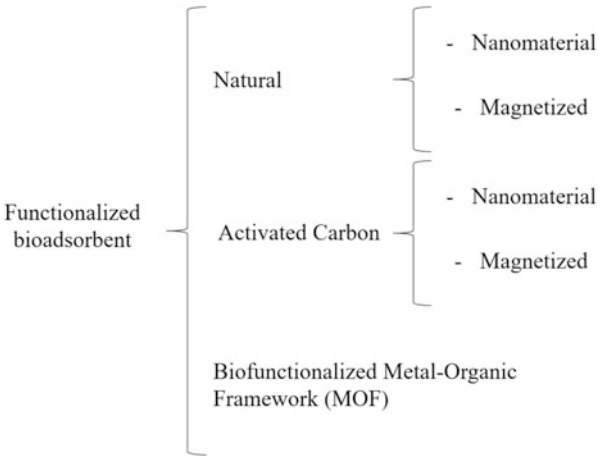

Fig. 8.3 Classification of functionalized bioadsorbents

8.4.1 Natural

To improve the characteristics of bioadsorbent materials and reduce the operating costs, different raw materials have been used, and substituent groups have been added for their functionalization. The lotus stick (Liu et al. 2019) and the pineapple crown leaf (Gogoi et al. 2018) were functionalized using acetic acid and eggshell with mineral sericite (Choi 2019), so as to increase the number of OH groups on the adsorbent surface. This enabled the materials to increase their capacity to adsorb heavy metals, such as lead and chromium. Hydrogen peroxide has also been used to increase the amount of OH groups, as in the case of coconut husk, which was used in mercury adsorption (Johari et al. 2016). Methylene blue dye was removed through the functionalization of sesame straw with citric and tartaric acids (Feng et al. 2017). It is also common to observe materials that have been functionalized with aminopropyltriethoxysilane, such as corncob to adsorb reagent red 141 (Carijo et al. 2019) or cocoa shell to adsorb CO_2 (Bargougui et al. 2018), during which cobalt immobilization was also performed to expand the sites of adsorbate adhesion. Cocoa shell is a very versatile material that has been studied with different types of functionalization, including modification with diazonium salts (Fioresi et al. 2017) and cobalt nanoparticles (Bargougui et al. 2018). Values of up to 1668 mg g^{-1} have been achieved for gold adsorption during which, as reported by Lin et al. (2018), corn bract functionalized using 2-aminothiazole and chloroacetyl chloride was used. As in the previous case, Godiya et al. (2020) inserted amino groups onto egg albumin, but with the use of polyethylene amine, which made it possible to adsorb sodium diclofenac.

In general, biomasses, which mainly consist of agro-industrial waste used as adsorbents, as is the case of pineapple crown leaves, have rough surfaces. However, leaf roughness that initially appears to be smooth in its natural state (Fig. 8.4a)

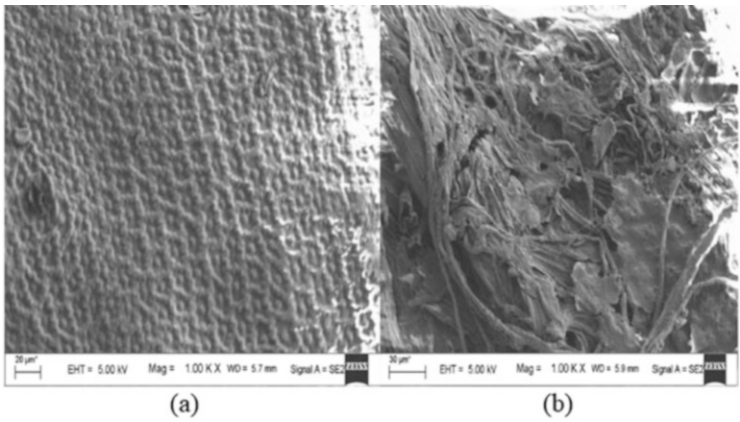

Fig. 8.4 SEM of crown leaf: raw (**a**) and modified (**b**). Reproduced from Gogoi et al. (2018). Copyright © 2018 with permission from Elsevier

increases when functionalized, as the grafting of OH groups leads to the emergence of large pores on the surface, which facilitate the adsorption process (Fig. 8.4b).

Adsorption capacity is mainly determined by the type of substituent groups present on the surface of the material, and these are supplied by the biopolymers of which they are composed (Guleria et al. 2020). Lignocellulosic materials are mainly composed of two large polymers, cellulose and hemicellulose, with a strong lignin coverage (Guo and Chen 2005; Guleria et al. 2020). Because lignin prevents cellulose hydroxyl groups from interacting with adsorbates, some authors have chosen to extract and functionalize cellulose. Cellulose sources can be very varied, such as aged cotton (Guo and Chen 2005), *Hibiscus esculentus* fibers (Guleria et al. 2020), chemomechanical pulp (Wang et al. 2019a), and filter paper (Li et al. 2020), among others. Cellulose is the most abundant biopolymer in nature and is composed solely of β-cellulose, indicating that its structure is endowed with a large number of hydroxyl groups (Salama et al. 2018). When extracting cellulose, techniques such as graft copolymerization with acrylic acid, acrylated vinyl, and n-isopropylacrylamide have been applied in the adsorption of heavy metals (Guleria et al. 2020; Li et al. 2020). The addition of grafts loads the surfaces of materials with amide and carboxylic groups (Fig. 8.5). Another approach in which to repower the amount of carboxylic groups in cellulose is the addition of sodium chloroacetate, which, as reported by Wang et al. (2019a), has managed to remove large amounts of lead. Cellulose has also been used in its oxidized and silica gel form (Salama et al. 2018), thereby improving the capacity to adsorb dyes through the silanol and silicon oxide groups.

Other polymers that have been used as adsorbents owing to their abundance include chitosan and chitin. Chitin is the second most common biopolymer in nature, following cellulose. It is composed of N-acetylglucosamine and is therefore a structure that is loaded with acetyl and amine groups (Lima et al. 2019). The partial deacetylation of chitin results in the acquisition of chitosan, and it is for this reason

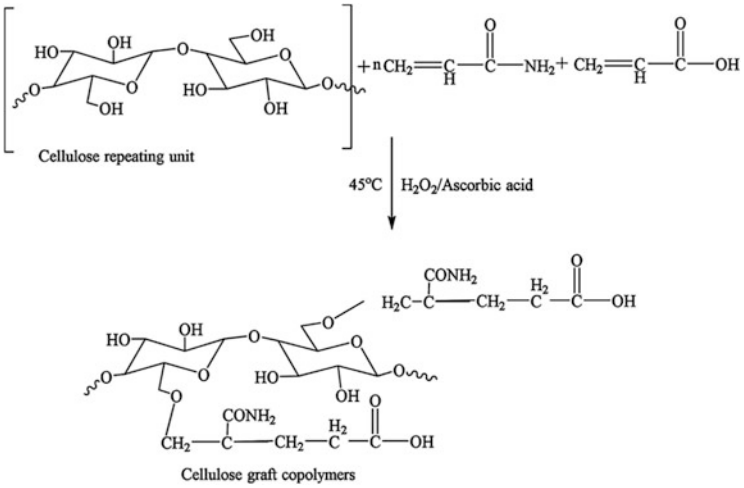

Fig. 8.5 Reaction steps for cellulose graft copolymer synthesis. Reproduced from Guleria et al. (2020). Copyright © 2020 with permission from Elsevier

that this biopolymer is made up of two sections: the acetylated part (N-acetylglucosamine) and the deacetylated part (D-glucosamine) (Salama and Hesemann 2018; Lima et al. 2019). The quality of chitosan is provided by the percentage of its deacetylation, which increases its quality. Both chitin and chitosan have been repowered by adding 3-aminopropyltriethoxysilane to adsorb sunset yellow dyes (Lima et al. 2019) and reactive violet 5 (Pereira et al. 2020). Chitosan is very versatile and has been functionalized in several ways, for example, with 2-hydroxy-1-naphthaldehyde and $CuCl_2$, using the crosslinking reaction to adsorb the pesticide pentachlorophenol (Shankar et al. 2020); with grafts of silk fibroin, polyvinyl alcohol, and malic acid to adsorb lead (Ajitha et al. 2017); and with N-guanidine and silica to adsorb methylene blue (Salama and Hesemann 2018). In the third case, one of the highest adsorption values was achieved with chitosan (539 mg g^{-1}). Chitin has also been functionalized by using grafts copolymerized with acrylonitrile and dimethylformamide, which enabled it to adsorb arsenic by means of linkages with amino and cyanide groups (Hanh et al. 2015).

As mentioned above, the subdivision of nanomaterials has been added to natural materials, which refers to those functionalization products resulting in nanometric sizes (1 nm = 10^{-9} m), of which the materials most frequently studied are cellulose and chitosan. Nanomaterials are used in adsorption owing to the need of surface area improvement of the existing absorbents (macroadsorbents). The main characteristic of nanomaterials is their large surface areas, which is caused by their large number of active sites and functional groups in very small spaces, thus increasing the number of adsorbate–adsorbent-type interactions (Song et al. 2017).

In this respect, α-cellulose nanofibers have been functionalized with Fe_3O_4 and immobilized with cobalt nanoparticles, with an average size of 15 nm (Kadam et al. 2019), which provide them with great adsorbent and magnetic properties.

Cross-linked keratin sponge and lyophilized cellulose nanocrystals have also been extracted to achieve high adsorption capacities of reactive black 5 dye (1201 mg g^{-1}) and direct red 80 dye (1070 mg g^{-1}) (Song et al. 2017). Furthermore, chitosan has been functionalized, and the nanospheres and nanofibers of this material have been obtained by mixing with other polymers, such as poly(methacrylic acid), to adsorb cadmium (Huang et al. 2013); polyamide to adsorb reactive black 5 and ponceau 4R (Dotto et al. 2017); and grafting poly(acrylic acid), glutaraldehyde, and Pb 2 as a common ion to adsorb lead (Lin et al. 2018). Chemical coprecipitation and crosslinking with Fe_3O_4 nanoparticles have also been employed to remove humic acid (Zulfikar et al. 2016). The average size of the produced chitosan nanoparticles is 50 nm, with an adsorption capacity of up to 734.3 mg g^{-1} for heavy metals (Huacai et al. 2016). Ravi and Sundararaman (2020) synthesized a nanomaterial from an eggshell to adsorb chromium. This nanoadsorbent had a size of 24 nm and was obtained via ultrasonic coprecipitation with iron oxide.

Another division of natural materials is that of magnetized materials. These are materials that, as a result of functionalization, exhibit magnetic properties and are generally subjected to treatments that principally involve metallic compounds based on iron (Fan et al. 2011; Edathil et al. 2018). Chitosan is one of the most versatile materials to magnetize since, although it is a fairly large polymer, it can easily bound to metal salts (Jin et al. 2017). Magnetized materials are generally intended for the adsorption of heavy metals, i.e., thiourea-chitosan magnetized with Fe_3O_4 and silver-printed ions to remove silver from aquatic sources (Fan et al. 2011), or chitosan–tripolyphosphate magnetized with Fe_3O_4 and reinforced with SiO_2 to remove copper from aqueous solutions (Jin et al. 2017). Other mixtures of chitosan that can remove metals have also been prepared, such as a mixture of chitosan magnetized pearls with cysteine/glutaraldehyde schiff to adsorb copper and chromium (Abou El-Reash 2016). In this particular case, a conglomerate of nanoparticles is formed. However, the surface area of the material and amount of available active sites on it are not affected. Furthermore, in the study conducted by Dos Santos et al. (2019), chitosan magnetized with cobalt ferrite was synthesized, which allowed the adsorption of indigotine blue dye. Materials of an agricultural origin have also been used, although in few studies, which include coconut mesocarp and sawdust magnetized with ferric nitrate and cobalt nitrate to remove lead and cadmium (Cunha et al. 2018), as well as coffee residues magnetized with ferrite nanoparticles to remove lead (Edathil et al. 2018). Magnetized bioadsorbents tend to be more attractive to adsorbates that have some metal in their structure.

8.4.2 Activated Carbons

Studies related to the functionalization of activated carbons are limited as it is necessary to interact with some characteristic groups of raw products (without carbonization) to functionalize a material (Jia-liang et al. 2016). However, despite

the existing limitations, some authors have chosen to functionalize this material by including inorganic salts or organic compound grafts linked by means of a metal. An example of this is activated carbon synthesis with chitosan since, according to Danalıoğlu et al. (2017), it is necessary to include, by means of simple coprecipitation, magnetite interactions that allow binding with chitosan, through the addition of magnetite. The resulting material exhibited magnetic characteristics owing to the presence of iron on its surface. This functionalization allowed the adsorption of large amounts of dangerous drugs. Another example of this is the preparation of carbonized rice husk functionalized with cyclodextrins and magnetite, which, unlike the previous case, was performed by means of thermal coprecipitation. The resulting material was used to adsorb methylene blue and exhibited adsorption capacities of up to 438.6 mg g^{-1} (Jia-liang et al. 2016).

Agro-industrial residues, such as sawdust and cocoa husk, have been evaluated as carbonized adsorbents and functionalized with polymers bound by using metal ions. Nisar et al. (2020) used polyethylene nanocomposites linked to iron, nickel, and cobalt salts to functionalize sawdust carbon. This treatment made it possible to form a complex between polyethylene (PE) nanoparticles, carbon (C)., and metals (PE–C–Fe, PE–C–Ni, PE–C–Co), which translated into much larger pore sizes with magnetic characteristics owing to the presence of metals on its surface. Carbon obtained from carbonized cocoa shell was grafted with aminopropyltriethoxysilane, forming a complex bound by cobalt particles. This functionalization made it possible to obtain a surface with an abundant amount of amine, amides, silicon, and cobalt groups, which allowed CO_2 to be adsorbed. Furthermore, activated carbons obtained from chicken feathers and eggshell (Rahmani-Sani et al. 2020) and from residues of *Nigella Sativa L.* (Abdel-Ghani et al. 2019) were magnetized with ferrous chloride, ferric chloride, and ferrous sulfate, and in both cases, it was possible to obtain a surface with larger pores and a large number of functional groups based on iron which, therefore, exhibited magnetic properties. In the study by Rahmani-Sani et al. (2020), the charcoal obtained after functionalizing chicken feathers and eggshells was evaluated with regard to its adsorption capacity for heavy metals, such as lead, cadmium, copper, zinc, and nickel, whereas the study by Abdel-Ghani et al. (2019) evaluated the adsorption capacity of *Nigella Sativa L.* residues with regard to the coomassie bright blue dye. Please note that adsorbent magnetic properties also play a very important role in the recovery of materials after adsorption in highly dispersed systems.

8.4.3 Biofunctionalized Metal–Organic Framework (MOF)

Biofunctionalized metal–organic frameworks (MOFs) are currently considered to be among the most innovative materials, since they have opened up a new interdisciplinary field owing to their numerous uses and great variety of ligands that allow them to form countless complexes (Zhao et al. 2020). They are crystalline hybrid solids capable of forming structures in one, two, and three dimensions and can be

synthesized according to specific characteristics. One of their main characteristics is their large surface area, which is superior to those of most porous materials (Zhu et al. 2018; Zhao et al. 2020). Despite the fact that these materials are synthetic, they have been considered within functionalized bioadsorbents, since when combined with biopolymers, they behave like bioadsorbents.

The most popular MOFs are those that are zeolite-based, which are denominated as zeolitic imidazolate frameworks (ZIFs). ZIFs have extremely rough structures on which there are transition metals, such as Zn, Co, Fe, and Cu, which form a tetrahedral structure with the imidazolate bonds (Zhu et al. 2018). Their characteristics are similar to those of zeolites, but with much larger surface areas, thus making them attractive for adsorption processes. ZIFs, like MOFs, are denominated according to the order in which they have been synthesized (hence, their name), followed by a hyphen and the identification number. ZIF-8 was synthesized by Omar Yagui (Park et al. 2006), and its metallic part is composed of Zn (II). It exhibits great thermal stability and an approximate specific permanent surface of 1810 $m^2\ g^{-1}$, which makes it an ideal material to be functionalized, principally through the use of cellulose and chitosan. In studies conducted by Zhu et al. (2018), ZIF-8 was functionalized with cellulose nanofibers to adsorb heavy metals, as also occurred in the study by Shanshan et al. (2018). However, to allow adsorption of gases, such as CO_2, silver ions were added. ZIF-8 has also been grafted with chitosan nanoparticles by Wang et al. (2019c) (Fig. 8.6) and chitosan pearls (Zhao et al. 2020) to allow it to adsorb dyes and drugs. Among all the aforementioned cases, adsorption capacities were quite high (up to 600 mg g^{-1}). ZIF-67 was first synthesized by Omar Yagui and his team (Banerjee et al. 2008). Its metallic part is made up of cobalt and is isomorphic with ZIF-8, indicating that its surface area and thermal stability are similar. ZIF-67 has been grafted with cellulose nanofibers to adsorb heavy metals (Zhu et al. 2018), with cellulose and cobalt nitrate to adsorb methyl orange (Song et al. 2020), and with chitosan nanoparticles to adsorb tetracycline (Wang et al. 2019c). The biopolymers grafted onto ZIFs provide them with characteristics that they do not naturally exhibit, such as the ability to form hydrogen bonds, thanks to the OH groups conferred, and principally by using cellulose and chitosan. However, in some cases, when metals are added, they occur on grafted polymer active sites.

Another functionalized MOFs is MOF-5, which was one of the first to be synthesized. Its metallic part is composed of zinc, and its organic part is benzodicarboxylate ($Zn^3\ (BDC)^2$), to which Yang et al. (2017) added cellulose filled with calcium carbonate and used it for the adsorption of gas mixtures (N_2, CH_4, H_2, and CO_2). Rehman et al. (2019) used amine- and piperazine-functionalized γCD-MOF to adsorb CO_2, and Vahidi et al. (2017) used the same functionalization, but on UiO-66, which is zirconium-based. The functionalization of these MOFs was performed principally to adsorb gases, since they quickly interact with cellulose and amine functional groups. The grafting of compounds of a natural or organic origin endows the functionalized MOFs with OH, NH_2, and NH groups, which are characteristic of bioadsorbents. Since they have such high specific surfaces, MOFs are ideal structures for functionalization, since they can be impregnated with a large

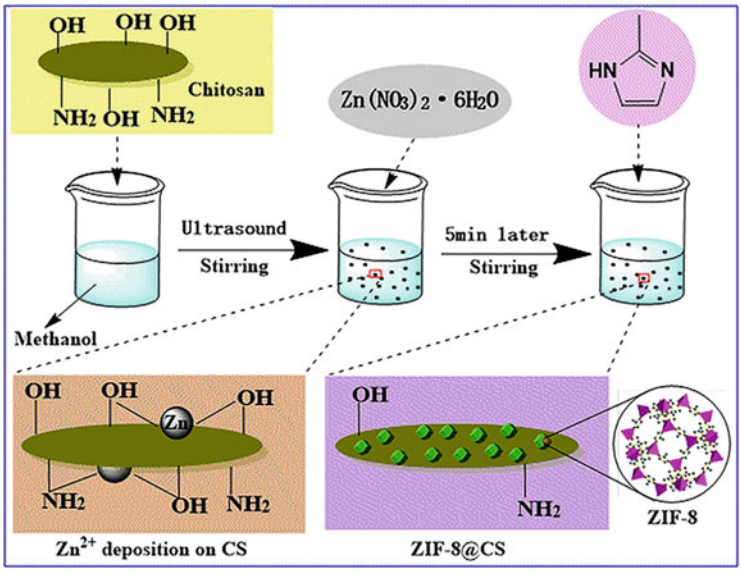

Fig. 8.6 Synthesis route for hybrid ZIF-8@Chitosan. Reproduced from (Wang et al. 2019c). Copyright © 2019 with permission from Elsevier

amount of functionalizing substances, and their large surfaces are covered by grafted functional groups. The functionalization of bioadsorbents with MOF leads to a field of research in obtaining new materials. Since the adsorption capacity of both materials can be used to obtain a new adsorbent. This allows to increase the selectivity and affinity of the new biomaterials obtained, being able to be used in the adsorption of different types of adsorbates in complex matrices. This improves the efficiency and profitability of the adsorption process and represents a possible decrease in operational costs. Although functionalised materials with MOFs are a topic that has been gaining interest nowadays, there are still challenges that need to be further investigated, such as increasing the efficiency of functionalisation processes and synthesising new MOFs.

8.5 Conclusions and Future Prospects

Wastewater treatment is one of the biggest problems today owing to uncontrolled and exponential pollution caused by urbanization and industrial development. This has raised concerns, and municipal treatment systems have been challenged to mitigate this situation and thereby comply with the regulations established by environmental control entities. The conventional technologies used for wastewater treatment to date have high operating costs and have not met the requirements established in standards owing to the low efficiency of the treatments and the fact

that other pollutants can be generated and released into the environment, which can be even more dangerous than the treaties themselves.

This chapter presents an overview of the different types of conventional and unconventional adsorbents used for this purpose. Adsorption is a promising technology that has so far been shown to have the capacity to treat effluents containing different polluting species, with high removal efficiency. Adsorption through the application of natural adsorbents, such as agro-industrial and aquaculture biomasses, is one of the potential alternatives with regard to the removal of pollutants from aqueous solutions and wastewater. These methods may, however, be limited in terms of their adsorption capacity and the fact that they are not being selective in the case of certain pollutants, as they have heterogeneous surfaces, and in many cases owing to the lack of functional groups that provide adequate adsorption mechanisms. In this context, the modification of natural adsorbents via physical and chemical processes has been shown to improve treatment capacities, taking advantage of the flexibility of the materials to be subjected to structural modifications and, therefore, obtain controlled surfaces. This occurs with coals and biochar, which have conditions that enhance the adsorption capacity of various pollutants, such as metals, dyes, drugs, pesticides, etc. The materials presented as ecological adsorbents are, therefore, advantageous for sustainable development management and principles owing to the possibility of being reused for several cycles.

However, at waste level, and especially in industry, a complex situation is taking place owing to the overgeneration of effluents with conventional and emerging pollutant loads, which are far from the maximum indicators normally reported and allowed. Because of this, the opportunity to develop functionalized and synthesized materials has been initiated, such as activated carbons, development of nanoparticles, magnetized materials, and complex structures, such as MOFs, which may even be specific to molecules that are not retained with previous treatments properly performed using adsorption.

In general terms, it is evident that industrial and urban evolutions are the main sources of effluents with high loads of conventional pollutants, and new elements are incorporated every day as a result of the development and innovation of different branches of industry. Adsorbent materials must, therefore, evolve, as well as new trends consisting of synthesizing elements that will, together, have high surface rates of adsorption, with combined removal actions for a broad spectrum of pollutants and also of a selective nature. The development of technologies that can be used to identify the main functional groups of new contaminants in order to define the structure of the adsorbents associated with them is, therefore, necessary. Furthermore, and owing to the principles of sustainable chemistry, these new materials are being developed to be fused with natural biomasses, which contributes to cost reduction, thus improving the design engineering for these treatments and optimizing resources.

These new materials will undoubtedly be at the center of various future commercial activities. However, their development requires further research directed toward mechanisms and the testing of these materials on real industrial effluents.

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Chapter 9
Electrochemical Biosensing of Algal Toxins

Luis Angel Zambrano-Intriago, Célia G. Amorim, Joan Manuel Rodríguez-Díaz, Maria Conceição B. S. M. Montenegro, and Alberto N. Araújo

Contents

Abstract Due to harmful algal blooms, the production of cyanotoxins have become a public and global concern worldwide. This problem has occurred more frequently, owing to global warming, and especially as a result of eutrophication from wastewater, fertilizers, earth movement, fossil fuels, and improper solid waste

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management. Several cyanobacteria produce dangerous and persistent toxins that can contaminate water bodies, restricting this resource for consumption or recreation. These toxins are capable of producing fatal diseases at the neurological, cardiological, muscular, hepatic, respiratory, and intestinal levels, which can present their harmful effects in a short time. For this reason, several regulations and detection methods have been developed, for control and analysis. However, control requires expensive equipment, such as liquid chromatography coupled to mass spectrometer, and highly qualified staff. In this context, owing to the impact of these toxins because of their quick action, a great interest in electrochemical detection methods has arisen, especially the development of biosensors, because they are inexpensive, simple to use, and fast to perform. In this chapter, the recent progress in algal toxins detection using electrochemical biosensors has been approached, so the overall system is covered by components, such as electrochemical transduction, supporting materials and electrodes, recognition receptors, building, and detection mechanisms.

Keywords Electrochemical sensors · Eutrophication · Algal toxins · Immunosensors · Aptasensors

9.1 Introduction

Harmful algae blooms (HABs) had been in past centuries sparse events mainly noticed and studied by biologists. True ecological public concern only started in the recent decades. In 2014, the inhabitants of Toledo (Ohio) woke up with alarming news concerning the water supply. They were told to avoid any contact with it: drink, cook, took showers, wash hands, and also avoid boiling water usage. More than 300 reports accounted on health incidents reflecting more frequent toxic blooms across the globe, 4 years later (Hance 2020). The outbreaks were of concern for populations, management teams of water supply networks, and for local authorities' regarding tourism in some cases. An illustrative example is Sete-Cidades, a beautiful freshwater lake located at the volcanic crater of S. Miguel island (Azores' archipelago), divided into two interconnected sub-units, Lagoa Azul and Lagoa Verde (S. Miguel Island—Azores) (Santos et al. 2005). In the mid-twentieth century, some forested areas of their steep watersheds were transformed into grasslands and pastures. Such disturbance along with the regional rainy climate increased soil erosion and leakages leading up to nutrients accumulation in the lake through years; the eutrophication process firstly increased the phytoplankton population and lastly replaced by cyanobacteria blooms and at dawn of this century. The events jeopardized all lake activities and its sightseeing interest and had mensurable deleterious effects over the local biota. The increase in extent and persistence in many locations of HABs is now directly ascribed to eutrophication (Watson et al. 2015; Griffith and Gobler 2020). The potential relationship between HABs and the

accelerated eutrophication induced by anthropogenic activities as well as the range of toxic compounds segregated into the water bodies became edge topics of public interest (Anderson et al. 2002).

In this chapter, the anthropogenic causes leading to eutrophication and HABs are briefly reviewed as well as the main groups of cyanotoxins produced by cyanobacteria. As the effects of acute poisoning of cyanotoxins are well-known; it has been the established guidelines concerning their monitoring in drinking water. So, in this chapter, it will be described and discussed as the main proposals regarding in situ immunosensors and aptasensors as analytical tools for environmental monitoring.

9.2 Anthropogenic Contribution to Eutrophication

Any increase in C, N, P, and Si nutrients or their relative proportions induces the eutrophication process and shift to nuisance bloom-forming algae. It directly translates into changes at the base of the trophic chain with a higher abundance of fast-growing species, particularly phytoplankton. The opacity caused on the surface turns water bodies anesthetic and induces photosynthesis depletion in bottom water layers, bringing ecosystem exhaustion at benthic zone, and killing the marine life. The global warming climate increases the stratification of the water column turning those phenomena more evident (Cloern 2001; Carpenter et al. 1998; Smith and Schindler 2009).

The rock weathering by acid rainfall in pluvial seasons is the main natural source of dissolved silicon in watercourses, in the form of silicic acid. Dissolved silicon is an important nutrient for diatoms, radiolaria, and sponges blooming during spring, and it confers rigidity to their cellular structure. In autumn, new blooms occur, because the silicon concentration increases again in river estuaries, partly from recycling biogenic silica from previous blooming. Besides flushing by intense flow, decreases in silicon concentration is not usual, because physical processes, such as adsorption, on suspended particles are residual.

The seasonality is also present in the dynamics of phosphorus, an element often associated with the red tides and intense spontaneous eutrophication in freshwaters. This nutrient is involved in a complex set of inputs, cycling, and removal only partially understood. Phosphorus accesses the hydrosphere through watercourses both in inorganic and organic forms with origin in diffuse sources, including anthropogenic ones, such as sewage and agriculture discharges. Both, the sediments at the bottom of the water bed and suspended particulate matter provide some degree of inorganic P buffering by promoting rapid adsorption, followed by internalization through slow diffusion into the particle where it becomes inaccessible (main reservoir). In saline-turbid waters, P is re-dissolved by competitive adsorption of other ions. In freshwaters with low or medium salinity, dissolution of P depends on the recycling of sediments, biological uptake, and groundwater inputs. In turn, the N cycle is furthermore complex due to the diversity of possible redox states, −3 to +5,

and chemical forms: dissolved inorganic, organic, gaseous, and particulate. As it is linked to other cycles, like C and P, any human influence turns out to have a global impact. Changes in the N:P ratio affect the phytoplankton growth and community structure. In particular, the increase of the N:P ratio fuels eutrophication in many N-limited coastal systems (Glibert et al. 2008). The relative importance and magnitude of the processes involved in the N cycle are only partially unveiled. The global impact of anammox has only recently been postulated, and technologies based on isotopic techniques and molecular biology are in use to follow sources, reservoirs, interconversion, and various forms of recombination. Most of the information is obtained through nitrate measurements, but it seems that the organic forms could also be very important.

Human activities and migration to the periphery of hydrographic space together with the direct intervention on catchments have significantly imbalanced the natural silicon, nitrogen, and phosphorus cycles at a global scale (Seitzinger et al. 2005). While detrimental impacts from focal and diffuse sources of N and P are traditionally imputed to fuel combustion and agricultural activities, new forms of contamination by bioactive synthetic chemicals are nowadays challenging the procedures implemented to manage discharges and wastes, reaching to direct interaction with the aquatic ecosystem (Gogate and Pandit 2004; Aksu 2005) The N:P ratio has steadily increased in Europe during the last 20 years, especially in the North, Mediterranean, and Atlantic seas, mainly due to agriculture practices (Blaas and Kroeze 2016). Despite the efforts to lower both nutrients, policies to reduce phosphorus have been more successful than those tackling nitrogen, and the management of point sources seems more effective than those controlling pollution from diffuse sources. Direct human intervention in the bed of aquifers mainly constructing dams and deploying irrigation systems, coupled to high N and P values, favors extemporaneous toxic blooms of cyanobacteria in summer, red tides, as opposed to chlorophyte blooms due to low Si:N and N:P ratios. Such harmful algal blooms generally reduce the biodiversity of the coastal ecosystems, turning them prone to further additional stress, such as climate change (Cloern 2001). The result of biodiversity loss and climate change has been recognized as one of the earth-system processes for which safe boundaries have been overstepped (Grizzetti et al. 2012). Eutrophication lowers the resilience of freshwater and coastal ecosystems to future anthropogenic stress and climate change and compromises the provision of essential goods and services, such as drinking water, fish supply, and recreational activities (Carpenter and Brock 2006; Hobbs et al. 2006). Hence, management and strict control of water bodies are now mandatory for further sustainable societal growth in the relationship with the environment.

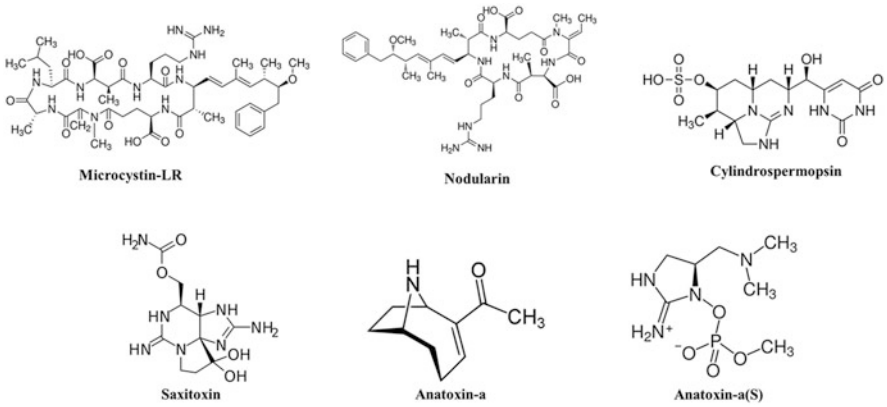

Fig. 9.1 Chemical structures of cyanotoxins

9.3 Algal Toxins and the Importance of Analytical Control

Cyanotoxins (Fig. 9.1) segregated by blue-green cyanobacteria are certainly between the most common harmful compounds associated with HABs events (Carmichael 1992). Environmental regulatory agencies, like EPA (Environmental Protection Agency), recognize five cyanotoxin groups in the list of contaminating candidates: three groups with hepatotoxic effects, microcystins, nodularins, and cylindrospermopsins and two showing neurotoxic activity, namely the anatoxins and the saxitoxins (EPA 2019).

Microcystins and nodularins are commonly detected in either fresh or brackish water. Microcystins are potent and specific heptapeptides that inhibit protein phosphatases 1 (PP1) and 2A (PP2A) by covalent binding (Yoshizawa et al. 1990). The intoxication symptoms vary according to the exposure time and doses. They range from diarrhea, vomiting, goose bumps, weakness, and liver damage up to massive liver hemorrhage likely leading to death (de Figueiredo et al. 2004; Massey et al. 2018). Median lethal doses (LD_{50}) of 43, 235, and 110 μg kg^{-1} were determined in mice bioassays for its 3 variants of microcystin LR, RR, and YR, respectively (Gupta et al. 2003). About 80 microcystins are known these days because besides *Microcystis* sp., multiple cyanobacteria, such as *Anabaena*, *Anabaenopsis*, *Aphanizomenon, Nostoc*, and *Planktothrix* sp., are capable to produce them (Chorus 2001). On the other hand, nodularins have been only associated with *Nodularia spumigena* blooms (Eriksson et al. 1988). Nodularins are pentapeptides of similar structure to microcystins, so they are chemically and toxicologically very similar but do not bind covalently to PP1 (LD_{50} 30–70 μg kg^{-1} in mice) (Pearson et al. 2010; Chen et al. 2013). Besides, other toxicological effects were reported to microcystin and nodularin exposure, such as intracellular glutathione impairment, reactive oxygen species production, lipid peroxidation, mitochondrial inhibition, cell apoptosis, changes in transcription factor, and protein kinase activity, affecting cell

proliferation pathways (Campos and Vasconcelos 2010; Bouaïcha and Maatouk 2004; Jayaraj et al. 2006).

Cylindrospermopsins are two chemical-related hepatotoxic alkaloids, deoxycylindrospermopsin and 7-epicylindrospermopsin, the latter being cytotoxic potentially carcinogenic (LD_{50} 200 μg kg^{-1} in mice) (Bazin et al. 2010; Guzmán-Guillén et al. 2015; Sivonen 2009). They are produced by *Cylindrospermopsis*, *Anabaena*, *Raphidiopsis*, *Lyngbya*, *Umezakia*, *Aphanizomenon,* and *Umezakia* sp. cyanobacteria (de la Cruz et al. 2013). Cylindrospermopsin is capable of interfering in different metabolic pathways, namely inhibition of protein, glutathione, and cytochrome P450 synthesis (Froscio et al. 2003; Runnegar et al. 1995). They affect mainly the liver and also the kidneys, thymus, spleen, heart, and gut lining. Poisoning is reflected in postmortem signs which include dark-colored liver, necrosis, and pale kidneys, spleen, and thymus (Falconer et al. 1999; Chernoff et al. 2011).

Neurotoxins, such as the anatoxin-a, anatoxin-a(s), and saxitoxin, are commonly found in HABs occurring in freshwater bodies. More commonly detected worldwide, the anatoxin-a is produced by multiple cyanobacteria, such as *Anabaena, Microcystis*, *Cylindrospermum*, *Aphanizomenon*, *Arthrospira*, *Raphidiopsis*, *Planktothrix*, *Phormidium*, *Nostoc* and *Oscillatoria* sp. (Osswald et al. 2007). Anatoxin-a (LD_{50} 200 μg kg^{-1} in mice) acts as a potent cholinergic poison, since it binds irreversibly to postsynaptic nicotinic receptors in the central nervous system and at neuromuscular junctions (Thomas et al. 1993; Humbert 2009). The sodium/potassium ion channels become permanently open, causing depolarizing blockage and muscle contraction (van der Merwe 2015; Humbert 2009). The neurotoxin is rapidly absorbed in the intestinal tract, and effects appear within minutes to hours. Symptoms may include loss of muscle coordination, muscle tremors and fasciculations, convulsions, and respiratory distress, the latter being the cause of loss of control over the respiratory muscles (Malik et al. 2020; Osswald et al. 2007). In turn, anatoxin-a(s) is a natural analogue of organophosphate insecticides produced by the *Anabaena flos-aquae* (Patočka et al. 2011). Despite the name, anatoxin-a(s) is not related to anatoxin-a, because it is a unique N-hydroxyguanidine methyl phosphate ester, with a molecular weight of 252 Da (Humbert 2009). The "(s)" in the name refers to salivation, a sign of poisoning observed in rodents after exposure to this toxin (Carmichael and Gorham 1978). This neurotoxin acts as an irreversible acetylcholinesterase inhibitor thus increasing the concentration of acetylcholine in the synapse cleft (Puschner and Roegner 2012). Anatoxin-a(s) (LD_{50} 50 μg kg^{-1} in mice) poisoning causes excessive salivation, urination, diarrhea, and lacrimation and in severe cases can cause chromodacryorrhea, tremors, incoordination, fasciculation, paralysis, cyanosis, bradycardia, and respiratory arrest (Solter and Beasley 2013; Mahmood and Carmichael 1986).

Saxitoxins in conjunction with its analogues are known as paralytic shellfish poisons (PSPs). Saxitoxins are produced in freshwater by cyanobacteria, such as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Planktothrix,* and *Scytonema*. Also, they are produced by dinoflagellates in marine environments by some species of *Alexandrium* (Wiese et al. 2010; Cusick and Sayler 2013). Saxitoxin (LD_{50} 263 μg kg^{-1} in mice with oral route) acts across the blood–brain barrier and binds to site 1 of the sodium channels at the neuromuscular junctions, causing a

blockage of nerve transmission (Borison and McCarthy 1977; Strichartz et al. 1986; Kao and Nishiyama 1965; Wiberg and Stephenson 1960). At relatively low exposures, the poisoning presents moderate paresthesia, with a tingling sensation around the mouth and extremities. However, at higher levels of exposure, it results in numbness of the mouth, throat, and extremities. The most severe symptoms are loss of motor control, twitching convulsion, muscle paralysis, and respiratory failure (Hurley et al. 2014; García et al. 2004). A recent review compiled 115 incidents in humans related to exposure to cyanotoxins in recreational activities between the years 1800 and 2010 around the world (Wood 2016). It accounts for multiple cases of poisoning and chronic disease mainly in children and young adults (Weirich and Miller 2014).

As inferred from the previous paragraphs, the major problem with cyanotoxins is their dangerousness at very low doses, with observed concentrations in freshwater and marine bodies ranging from ng L^{-1} to μg L^{-1} (Gibble et al. 2016; Messineo et al. 2009). As the effects of acute poisoning are known, the WHO has established guidelines concerning the monitoring of these compounds in drinking water, starting with microcystin-LR given its widespread and toxicity. The WHO correlated by the daily water intake (2 L) and the average weight of a person (60 kg) is to impose the maximum allowed concentration of microcystin-LR in drinking water of 1 μg L^{-1} (WHO 2011).

More recently validated official methods for the detection of other microcystins, nodularins, cylindrospermopsins, and anatoxin-a in drinking and environmental water have been proposed (EPA 2015, 2019). Even before EPA publications, different analytical methods ranging from biological to instrumental sophisticated setups were reported by several laboratories and appraised as sensitive, cheap, and easy to reproduce (Sanseverino et al. 2017). Widely preferred are those based in the high-performance liquid chromatography (HPLC), due to capability to isolate water contaminants, and therefore achieving more effective identification and detection. To this, HPLC equipment is commonly coupled to detectors, such as ultraviolet/visible (UV/VIS), fluorescence, and mass spectrometer (MS). Ultraviolet detectors provide the cheapest approach in the analysis of cyanotoxins, such as microcystin (Shamsollahi et al. 2014), nodularin (Kankaanpää et al. 2002), cylindrospermopsin (Welker et al. 2002), and anatoxin-a (Gugger et al. 2005), respectively. Nevertheless, the reliability of results is compromised by the poor ability to distinguish between toxin analogs and other interferences since most organic compounds absorb in the 200 and 300 nm wavelength region. As such, the official methods resort to tandem mass spectrometry for the detection of microcystins and nodularin (Method 544, LC/MS/MS), cylindrospermopsin, and anatoxin-a in drinking water (Method 545, LC/ESI-MS/MS) (Shoemaker et al. 2015; EPA 2015). In this context, the determination of cyanotoxins can be performed down to 0.02 μg L^{-1} which fulfills even the forensic analysis criteria (Msagati et al. 2006).

Cyanotoxins can be also monitored through biochemical methods (Sanseverino et al. 2017). Among the biochemical methods that include enzymatic, immunoassay, and receptor assays are sensitive and suitable for large-scale detection. However, biochemical detection is a complementary, as it is normally used to confirm the presence of cyanotoxins in water bodies. Among the biochemical techniques, the

two most widely used are an enzymatic assay kit (the protein phosphatase inhibitor assay (PPIA)) and an enzyme-linked immunosorbent assay (ELISA). The PPIA is simple and sensitive but can only be applied in the detection of microcystins and nodularin, because only these are inhibitors of serine–threonine protein phosphatase enzymes (Rapala et al. 2002). Within PPIA there are two alternative detection principles: by using radioisotope techniques and radiolabeled proteins, though not suitable for routine analysis (Lambert et al. 1994) and by colorimetric assay using chromophore substrates, such as p-nitrophenyl phosphate (Heresztyn and Nicholson 2001). The technique allows quantification down to the detection limit of 0.01 μg L^{-1} for microcystin-LR equivalent (Hudnell 2008) but is unable to distinguish between toxins and interfering compounds in the blooming samples (Almeida et al. 2006). In turn, ELISA kits can be based on specific monoclonal antibodies (single-element determination) or polyclonal antibodies against microcystin-LR, nodularin, cylindrospermopsin, or saxitoxin (Triantis et al. 2010). The method can be merely qualitative, simple positive or negative tests, or quantitative (Sakamoto et al. 2018) if toxins' concentrations are above 0.1 μg L^{-1} (Sanseverino et al. 2017).

All these analytical methods require expensive equipment, collection of representative samples, transportation to laboratory, and compliance with routine analysis and control objectives. In contrast, sensor technology has been receiving increased attention in detecting toxic contaminants from HABs (Weller 2013). Compared to commonly used methods, they provide low detection limits and high selectivity, beyond fast detection, real-time results, low cost, and portability (Kimmel et al. 2012; Windmiller and Wang 2013).

9.4 Electrochemical Biosensors for Algal Toxins

According to the IUPAC (The International Union of Pure and Applied Chemistry), chemical sensors (Fig. 9.2) are devices with the ability to transform "...chemical information, ranging from the concentration of a specific sample component to total

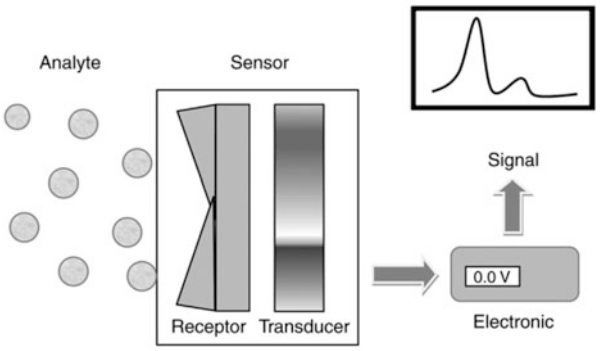

Fig. 9.2 General diagram of a chemical sensor. Reproduced from Simões and Xavier (2017). Copyright © 2017 with permission from Elsevier

composition analysis, into an analytically useful signal" (Hulanicki et al. 1991). Within the subclass of electrochemical sensors (Fig. 9.3a), such devices are made up of a receptor element juxtaposed on the surface of an electrode functioning as transducer, thereby capable of sending an electronic signal for processing and final reading. The most important part of the structure of an electrochemical sensor is the receptor or recognition element (Fig. 9.3b), because it imparts the selectivity needed for the detection of an analyte or group of analytes while suppressing or decreasing interferences of other substances (Banica 2012; Eggins 2002). For biosensor development, different biological materials, such as organisms, nucleic acids, cells, tissues, enzymes, and antibodies, have been immobilized as receptor elements (Fig. 9.3c) (Lu et al. 2019; Bagheri et al. 2019; Reynoso et al. 2019; González-Martínez et al. 2005; Evtugyn 2014; Gründler 2017). They are usually designed to be applied in food and pharmaceutical industry, medical diagnosis, environmental control, etc. (Mishra et al. 2018; Rubab et al. 2018; Stephen Inbaraj and Chen 2016; Sanvicens et al. 2011; Upadhyay and Verma 2015; Metkar and Girigoswami 2019).

One of the major advantages of electrochemical biosensing relies on the versatility of transduction mode regarding the interaction between the analyte and receptor. It can be potentiostatic, if a potential is applied to the sensor surface with the bioelement contacting the sample, resulting in a current change related with the recognition event; galvanostatic, where a current is applied and potential variation is measured; potentiometric, in which the change in potential is measured in a current condition close to zero; or finally as impedance, in which an alternate potential is applied and a capacitive resistance measured (Brett and Brett 1993; Wang 2006). Nevertheless, the most common modes used in algal toxin biosensors in water are potentiostatic (voltammetric and amperometric) and impedimetric (Table 9.1).

Worth of mention is cyclic voltammetry (CV), where the current is plotted as a function of potential scan, being usually the first experiment carried out in a study of chemical reactivity of a compound, biological material, or an electrode surface (Kissinger and Heineman 1983). Specifically, it is capable of providing information on the redox processes and the kinetics of heterogeneous electron transfer reactions, monitoring of reaction intermediates, evaluation of the stability of reaction products, or adsorption processes (Wang 2004). On the other hand, other voltammetric detection methods, such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV), have been used mainly to define the optimal measurement potentials. Furthermore, they have advantages, such as effectiveness, selectivity, lower consumption of species, faster scanning speed, removal of interfering currents, and low detection limits (Wang 2006; Mirceski et al. 2007; Scholz 2013). For this reason, several researchers have applied these methods for the confirmation of the working potentials and the development of the analytical method of quantification of algal toxins. Otherwise, amperometry (AMP) explores the current signal of an analyte obtained at a fixed potential as a function of time. Normally, this potential is defined after a voltammetric study of the whole chemical behavior of the system (Kissinger and Heineman 2018; Bagotsky 2005).

Electrochemical impedance spectroscopy (EIS) is a powerful technique capable of sampling high-frequency electron transfer and low-frequency mass transfer.

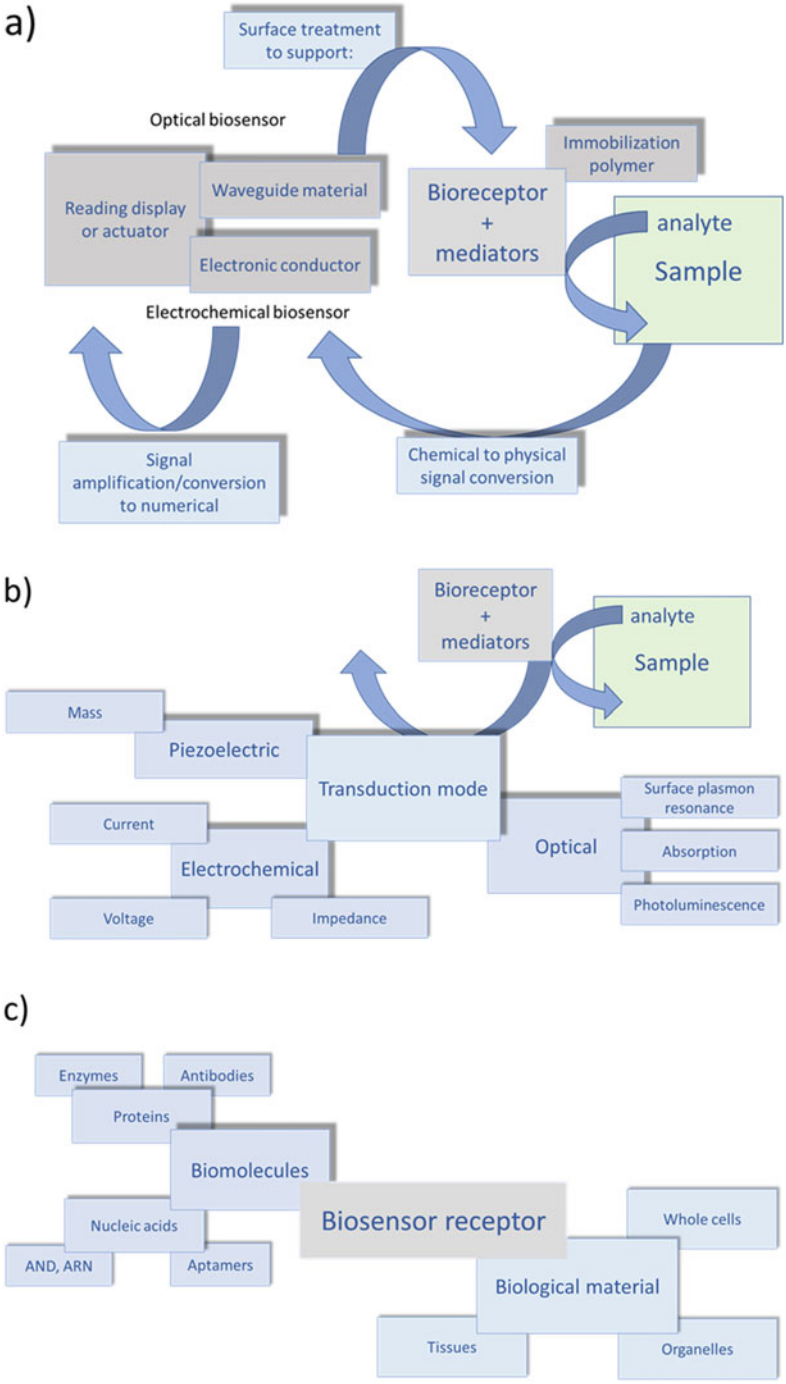

Fig. 9.3 The various aspects of sensor development. (**a**) biosensor types, main components, and working mechanisms; (**b**) selection of transduction mode, i.e., conversion of chemical recognition

Within the context of biosensing technologies, it provides label-free monitoring of immune binding event between antigens (Ag) and antibodies (Ab) on the surface of a biosensor, usually causing changes in impedance at the interface sensor/sample (Ronkainen et al. 2010; Kissinger and Heineman 2018).

Potentiometry (POT) is the least used electrochemical technique for the detection of algal toxins (Bratakou et al. 2017). Potentiometric biosensors measure the change in electrical potential at the working electrode under zero current when the analyte is coupled to the bioreceptor immobilized on the transducer (Ronkainen et al. 2010).

A variety of biosensors for algal toxins have been developed (Table 9.1) with a predominance of those aiming the detection of microcystin toxins, since it is the only algal toxin regulated within the control regulations for drinking water by the WHO, and more frequently present in freshwaters (WHO 2011). In general, the deployment of biosensors is critical when choosing the transducing and detector materials, because most chemical or biochemical reactions take place on the surface of one working electrode. It must be ensured that the material used generates a correct connection between the base electrode and the main substrate with the measuring instrument. Therefore, the working electrode must be made of highly conductive, long-term stable materials (Kimmel et al. 2012). Among the materials commonly used for the development of biosensors for algal toxins are metals, such as gold, platinum, carbon-based materials, and metallic nanoparticles. Moreover, the most widely used biomolecules to date are antibodies, aptamers, and even mixtures of antibodies with enzymes. Some reported biosensors are summarized in Table 9.1.

Most reported biosensors use antibodies as biorecognition elements and conventionally are classified as "immunosensors." In general terms, all materials used for the immobilization of the antibodies must meet certain conditions: highly conductive and be solid so that they do not allow the loss of their biological binding activity. Antibodies must be correctly oriented where the Y-shape should be exposed to the sample and not towards the surface, and the density of the antibodies on the surface cannot be too high in order to minimize steric hindrance (Ronkainen et al. 2010). The electrodes conventionally used for the development of algal toxin immunosensors are made of metals, such as gold, platinum, copper, and carbon forms, such as glassy carbon, graphene, and special materials, such as indium tin oxide (ITO) (Table 9.1).

Within the design of the sensor electrodes, a subclass of biosensors called label-free immunosensors is evidenced (Table 9.1). These allow direct monitoring of immunoreactions by measuring the physical and chemical changes accomplishing the formation of the direct antibody–antigen complex, not requiring the use of labels to facilitate measurements. Instead, they utilize intrinsic physical properties of the analytes, such as molecular weight, size, charge, electrical impedance, dielectric permittivity, or refractive index, to detect their presence in a sample (Ugo and Moretto 2017). Their inherent advantages enable easy detection, higher signal-to-

Fig. 9.3 (continued) event in electrical energy; (**c**) different classes of biological components used selective recognition of the analysis

Table 9.1 Electroanalytical biosensors reported in the literature for algal toxins sensing

Algal toxins	Electrochemical techniques	Biosensor configuration	Sample	Working range (μg. L^{-1})	Limit of detection (μg. L^{-1})	Electroactive probes/ mediator	Reference
Label-free immunosensors							
MC-LR	DPV	mAb/AuNPs/GE	Crude algae and water	0.05–15.00	0.02	Hydroquinone	Tong et al. (2011)
MC-LR	Potentiometric	mAb/thiourea SAM/Ag NPs/GE	Water	10^{-5}–1.00	7.0×10^{-6}	–	Loyprasert et al. (2008)
MC-LR	DPV	Anti-MC-LR/au/MC-LR/ PEG/CNFs/GCE	Polluted water	0.0025–5.00	0.0017	AuNPs	Zhang et al. (2016)
MC-LR	DPV	pAb/IL/AuNPs/polyDPB/ G-AuNPs/GCE	Tap, lake, river, and groundwater	1×10^{-7}–8×10^{-8}	3.7×10^{-8}	–	Ruiyi et al. (2013)
MC-LR	Amperometry	mAb/CNTs-PSS/AFP	Lake water	10.00–40.00	0.6	–	Wang et al. (2009)
MC-LR	Electrical resistance	MC-LR/SWCNTs/SiO_2/ IDE	Tap, river, and lake water	0.001–1.00	0.0006	–	Tan et al. (2015)
MC-LR	EIS	mAb/MWCNTs/cu	Drinking water	0.05–20.00	0.04	–	Han et al. (2013)
MC-LR	DPV	Ab/CNx-MWCNTs/Au/ GCE	Polluted water	0.01–2.00	0.004	o-PD and H_2O_2	Zhang et al. (2011)
MC-LR	DPV	Anti-MC-LR/Ag-nanocubes/ PAMAM/GA/CHIT/GCE	Lake water	0.05–25,000	0.017	$Fe(CN)_6^{4-/3-}$	Fu et al. (2016)
MC-LR	EIS	MC-LR/3DGF	Tap water	0.05–20.00	0.05	$Fe(CN)_6^{4-/3-}$	Zhang et al. (2017)

MC-LR	EIS	MC-LR/GF	Environmental water	0.005–10.00	0.0023	$Fe(CN)_6^{4-/3-}$	Zhang et al. (2018b)
MC-LR	DPV	Ab/AuNPs/MB-Si-CHIT/GCE	Lake water	0.5–25,000	0.1	–	Fu et al. (2013)
MC-LR	EIS	pAb/AuNPs/L-cysteine/GE	Drinking water	0.05–300	0.0182	$Fe(CN)_6^{4-/3-}$	Sun et al. (2010)
MC-LR	EIS	Ab/BSA/ILL/MWCNTs/GCE	Distilled, tap, and bottle water	0.005–1	0.0017	$Fe(CN)_6^{4-/3-}$	Sun et al. (2013)
MC-LR nodularin	Chronoamperometry	Anti-MC-LR IgG/ultraBind membrane/CSPE	Water	0–10.00	0.5	H_2O_2	Lotierzo et al. (2012)
Saxitoxin	Potentiometric	Ab/lipid layer/glass fiber filter/GNS/Cu	Lake water and shellfish	1.00–1000	1.00	–	Bratakou et al. (2017)
Label-based immunosensors							
MC-LR	DPV	HRP-CNP-Ab bioconjugates/MC-LR/GS-CHIT/GCE	Reservoir, tap, and river water	0.05–15	0.016	H_2O_2	Zhao et al. (2013)
MC-LR	EIS	HRP-mAb/MC-LR-BSA/AuNPs/GCE	Fortified water	0.01–100	0.004	$Fe(CN)_6^{4-/3-}$	Hou et al. (2016a)
MC-LR	CV	Multi-HRP-(Fe_3O_4@PDA-Au)-Ab_2/MC-LR antigen/CNT@Co silicate/ITO	Tap and lake water	0.005–50	0.004	H_2O_2	Gan et al. (2016a)
MC-LR	CV	Multi-HRP-(MCSs/Thi@AuNPs)-Ab_2/AuNPs/GH@PDA/CSPE	Lake water	0.01–10	0.0097	H_2O_2/hydroquinone	Gan et al. (2016b)

(continued)

Table 9.1 (continued)

Algal toxins	Electrochemical techniques	Biosensor configuration	Sample	Working range (μg. L^{-1})	Limit of detection (μg. L^{-1})	Electroactive probes/ mediator	Reference
MC-LR	Chronoamperometry	PtRu-Ab_2/Ab_1/GS/GCE	Polluted water	0.01–28	0.00963	H_2O_2	Wei et al. (2011)
Saxitoxin brevetoxin	Amperometry	Glucose oxidase-labeled antobody/antigen-BSA/ Immobilon membrane/platinum surface	Buffer	15	–	Glucose	Carter et al. (1993)
Aptasensors							
MC-LR	EIS	ssDNA-aptamer/GE	Lake, river, and tap water	0.05–10	0.018	$Fe(CN)_6^{4-/3-}$	Lin et al. (2013)
MC-LR	SWV	5′-disulfide terminated aptamers/GE	Buffer	0.01–10	0.0075–0.012	$[Ru(NH_3)_6]^{-3}$	Ng et al. (2012)
MC-LR	SWV	Ferrocene labeling of the 3-′-terminus of the MC-LR aptamer and disulfide labeling of the 5′-terminus /GE	Fish and tap water	0.0001–1	0.0019	$Fe(CN)_6^{4-/3-}$	Eissa et al. (2014)
MC-LR	EIS and DPV	Calf thymus dsDNA/GE	Tap water	0.004–0.512	0.0014	$Fe(CN)_6^{4-/3-}$	Zhang et al. (2018a)
MC-LR	CV	MC-LR 5′-thiol ssDNA aptamer/AgNPs/SDD–Co (II)/GCE	Tap, distilled, and wastewater	0.1–1.1	0.04	–	Bilibana et al. (2016)
Saxitoxin	DPV	3′-amino-modified STX aptamer/MWCNTs/GE	Mussel	0.9–30	0.38	Methylene blue	Hou et al. (2016b)
Cylindrospermopsin	EIS	Amino-substituted aptamer/ thionine-graphene/GCE	Lake water	0.39–78	0.117	$Fe(CN)_6^{4-/3-}$	Zhao et al. (2015)

Cylindrospermopsin	EIS	Disulfide-modified aptamer/GE	Tap water	0.1–80	0.1	$Fe(CN)_6^{4-/3-}$	Elshafey et al. (2014)
Anatoxin-a	EIS	ATX-aptamer/GE	Drinking water	1–100	0.5	$Fe(CN)_6^{4-/3-}$	Elshafey et al. (2015)
Brevetoxin-2	EIS	Aptamer/GE	Shellfish	0.01–2000	0.16	$Fe(CN)_6^{4-/3-}$	Eissa et al. (2015)

Abbreviations: *MC-LR* microcystine, *mAb* monoclonal antibody, *pAb* polyclonal antibody, *AuNPs* gold nanoparticles, *GE* gold electrode, *CSPE* screen-printed carbon electrode, *AgNps* silver nanoparticles, *SAM* self-assembled monolayers, *DPV* differential pulse voltammetry, *PEG* polyethylene glycol, *CNFs* carbon nanofibers, *polyDPB* conducting polymer, *G-AuNPs* graphene–gold nanocomposite, *IL* 1-isobutyl-3-methylimidazolium bis(tri-fluoromethane-sulfonyl)imide ionic liquid, *CNTs* carbon nanotubes, *PS* poly(sodium 4-styrenesulfonate), *AFP* analytical filtration paper, *IDE* gold interdigitated electrodes, *CNx-MWCNTs* nitrogen-doped carbon nanotubes, *GCE* glassy carbon electrode, *GA* glutaraldehyde, *CHIT* chitosan, *PAMAM* amine-terminated polyamidoamine dendrimers, *3DGF* three-dimensional graphene foam sheets, *GNS* graphene nanosheet, *Cu* copper, *MB-Si-CHIT* silicon template/methylene blue/chitosan, *ILL* 1amyl-2,3-dimethylimidazolium hexafluorophosphate, *BSA* bovine serum albumin, *MWCNTs* multiwalled carbon nanotubes, *HRP* horseradish peroxidase, *CNP* carbon nanosphere, *PDA* polydopamine, *ITO* indium tin oxide electrode, *GH@PDA* graphene hydrogel @polydopamine, *MCSs* mesoporous carbon spheres, *SDD–Co (II)* cobalt(II) salicylaldiimine metallodendrimer

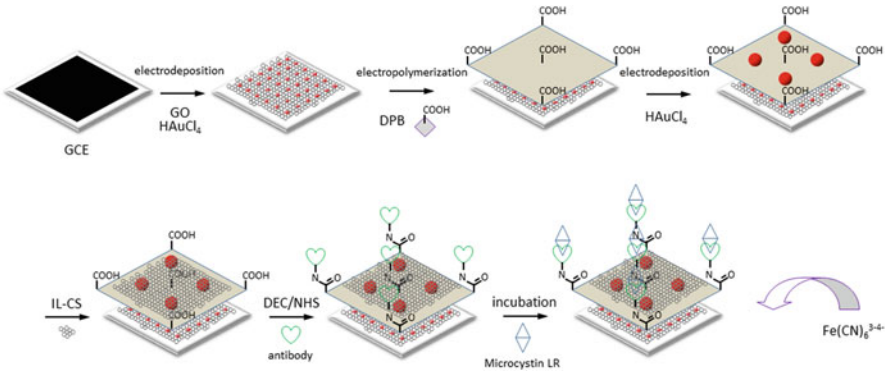

Fig. 9.4 Procedure for preparation of a direct detection immunosensors. Reproduced from Ruiyi et al. (2013). Copyright © 2013 with permission from Elsevier

noise ratio, fast analysis, and shorter detector response time (Ronkainen et al. 2010). Label-free designs enable also reducing the number of testing steps (Tong et al. 2011; Ruiyi et al. 2013; Wang et al. 2009; Fu et al. 2013, 2016; Sun et al. 2010, 2013; Bratakou et al. 2017). It is remarkable that the correct immobilization of the antibodies on the proposed support material presents a significant response improvement in the electrochemical transduction, which can be noted by the low detection limits obtained. For example, Ruiyi et al. (2013) developed a platform comprising a mixture of metallic nanoparticles and conductive polymers to promote the correct immobilization of polyclonal antibodies anti-microcystin-LR (Fig. 9.4), achieving a detection limit of 3.7×10^{-8} μg L^{-1}. However, this type of immunosensors can be affected by nonspecific particle-adsorption effects, generating selectivity problems. For this reason, the use of blocking agents is quite common.

On the other hand, the label-based immunosensors are another subclass of immunosensors, which have been developed to improve the selectivity, sensitivity, and remove interference effects of the sensing. In these immunosensors, labels, like enzymes, catalysts, electrochemically active molecules, fluorophores, and liposomes, are added to the recognition surface to provide signal amplification. The reaction mechanism is commonly based on indirect competitive immunoreaction, such as:

1. The antigen is firstly immobilized on the biosensor surface.
2. The electrode is then incubated in a sample containing a mixture of free antigen plus antibodies.
3. Binding competition is promoted between the immobilized and free antigens by binding to labeled antibodies added to the sample.
4. The antibodies bound to the immobilized antigens on the electrode are immersed in an electrolyte solution containing a specific substrate to enable measurement.

The most common labels are proteins, like horseradish peroxidase (HRP) (Hou et al. 2016a), enzyme–carbon nanospheres (CNS) (Zhao et al. 2013), glucose oxidase (Carter et al. 1993), multi-HRP-(Fe_3O_4@PDA-Au) (Gan et al. 2016a), and

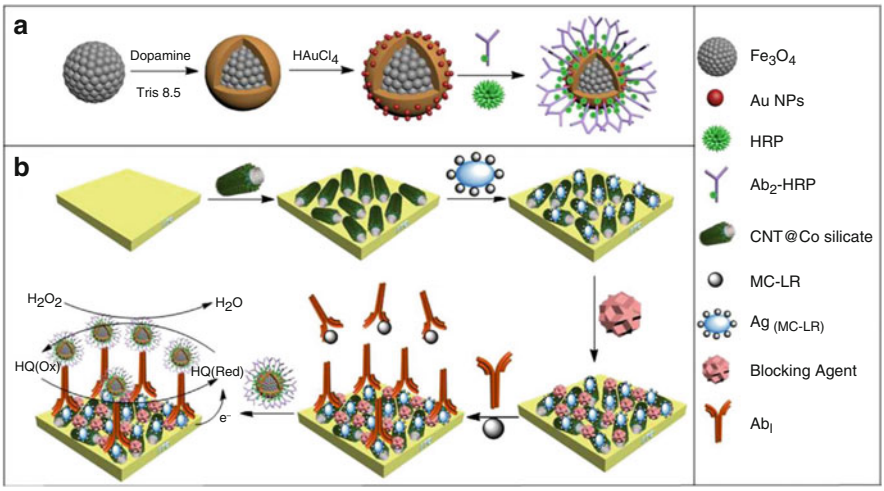

Fig. 9.5 (**a**) Preparation of multi-HRP-(Fe_3O_4@PDA-Au)-Ab_2 conjugate and (**b**) construction of the MC-LR electrochemical immunosensors. Reproduced from Gan et al. (2016a). Copyright © 2016 with permission from Elsevier

multi-HRP-(mesoporous carbon spheres/thionine@AuNPs) (Gan et al. 2016b). It is evident that the HRP enzyme (Table 9.1) has been the preferred route in labeled assays for immunosensors, once is easy to find in commercial suppliers, has high activity, and tolerates wide physicochemical changes, such as pH, pressure, and temperature. In this sense, the presence of enzymes has the function of generating electrocatalytic currents when substrates were used, such as H_2O_2, glucose, methylene blue, hydroquinone, etc., are added. An example is reported by Gan et al. (2016a) who developed an electrode composed of highly conductive materials, through an indirect competitive test between multi-HRP-(Fe3O4@PDA-Au)-Ab2 and standard microcystin-LR (Fig. 9.5). A detection limit of 0.004 μg L^{-1} was obtained attributed to the conductivity signal amplification and to the minimal interference in the presence of its analogues YR, RR, and also nodularin.

In recent years, aptamers have also received a great interest in the development of algal toxin biosensors. Aptamers are single-stranded DNA or RNA oligonucleotides that selectively bind to target molecules, such as nucleic acids, proteins, metal ions, and other small molecules mimicking antibodies. That is why they are usually called synthetic antibodies, because they mimic the affinity and specificities of antibodies for their targets. Considerable aptamers' advantages over antibodies can be indicated, such as structural flexibility, stability, high chemical production reproducibility, chemical stability under damping conditions and complex treatments, and reversible conformation under thermal variations (Radi 2011) and its comparably smaller size (~3 nm) than antibodies (10–15 nm) (Que-Gewirth and Sullenger 2007). Since aptamers can be isolated to bind to almost any molecule understudy, they can be modified in arbitrary positions (specific 3D structures), making this platform technology attractive in the development of biosensors (Zhou et al. 2014).

Biosensors based on the use of aptamers as biorecognition elements are called "aptasensors." Aptamer-based biosensors have generated considerable attention due to their good selectivity, specificity, and sensitivity (Ugo and Moretto 2017). On its development the immobilization process of the aptamer on the electrode surface is the most critical step. The terminal ends of the genetic sequence are modified with terminal moieties, such as disulfide, thiol, ferrocene, and amino groups, to bind to the electrode surface. With this type of biosensors, the typical mechanisms for detecting algae toxins are the sandwich test and the competitive assay. In the simple binding-based assays, the modified aptamer is bonded to the surface of the electrode, and at the other extreme is directly attached to the target molecule or toxin (Lin et al. 2013; Ng et al. 2012; Bilibana et al. 2016; Eissa et al. 2014; Elshafey et al. 2014, 2015; Zhao et al. 2015; Hou et al. 2016b). Then, through redox tests performed before and after exposure to the toxin, the concentration of these is correlated. Also, there is a design with a competitive mechanism, in which the aptasensor has the toxin immobilized; then it is submitted to a solution containing the mixture of free toxin and a fixed amount of aptamer (Eissa et al. 2015). This mechanism is common to different immunosensors, where there is competition between free, immobilized toxins and solution-labeled aptamers, which by means of redox tests correlate the amount of toxin in the sample.

9.5 Conclusions

Human interventions on catchments through the construction of dams, diverting for irrigation endings, the run-offs from manure and agricultural soils enriched by the use of fertilizers and sewages from different industries, and inefficiency of WWTPs (Wastewater Treatment Plants) in treatment of domestic discharges promote sensitive modifications on the silicon, phosphorus, and nitrogen inputs into the oceans. An immediate effect is the change of the natural biogeochemical cycling of these elements, promoting the relative abundance of rapidly growing aquatic organisms in the water column and intensification of aerobic metabolism at the sediments level. In the latter, oxygen becomes additionally scarce due to global warming and with a rise in the average water levels. This increases the risk of hypoxia and irreversible imbalances on the interplay between the benthonic and pelagic biota. Mid-term ecosystem changes, and the occurrence of blooms of toxic flagellates and cyanobacteria are evident ecological events counteracting the societal valorization of the borders between the hydrosphere and the adjacent lithosphere. The result of the emergence of toxins is urgent monitoring of these aquatic environments. Therefore, the contribution of portable sensors to the screening of the most widespread toxins is presented. The objective is to contribute to the strict control and prevention of toxic events and the sustainability of water sources, regardless of their domestic, industrial, or recreational use.

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Chapter 10
Bioinspired Superoleophobic Materials for Oil–Water Separation

Ashwini Patil, Sushant S. Pawar, and Santosh S. Biranje

Contents

Abstract The increasing water contamination generated by human activities is now a serious global environmental concern. Thus, there is an immense demand for the developments of effective, environmentally friendly, and inexpensive approaches for the separation of oil from the water resources. Recently, the superoleophobic surfaces derived from nature are considered an essential solution to the surfaces obtained from synthetic materials due to their numerous fabrication methods and applications. The reusability is another advantage of bioinspired materials, which added one more step towards sustainability. This chapter has discussed the different concepts used in superoleophobic and superhydrophobic surfaces for separation of

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oil from oil/water mixture. The main aim is to provide a summary of different bioinspired superoleophobic materials derived from the plant sources (lotus leaf, rose petals, rice leaf, seaweed, and pitcher plant) and animal sources (skin of springtail, filefish skin, clam's shell, fish skin/scales, sharkskin, and leafhoppers). This review also highlights the recent progress in oil/water separation technologies, such as electrospinning, layer-by-layer technology, spray coating, and lithography. We hope this data will provide essential guidelines for the researchers to design a novel superoleophobic material for oil/water separation with self-cleaning, antifouling, and low adhesion surface properties.

Keywords Bioinspired · Superoleophobic · Wetting theory · Oil–water separation · Electrospinning

10.1 Introduction

Oil pollution is a global problem due to the increasing oily wastewater production from petrochemical (Hu et al. 2013), textile (Kant 2012), and food industries (Kroyer 1995) as well as from frequent oil spill accidents (Galieriková and Materna 2020). The oil-polluted water containing harmful chemicals is a more potential catastrophe to marine environments, people's health, and the ecosystem (Hong et al. 2018). The variety of oil/water separation materials have developed to address the problem associated with oil pollution, but with limited success. The conventional mechanical methods of oil/water separation, such as skimming (Broje and Keller 2007), ultra separation (Li et al. 2018), air flotation (Al-Shamrani et al. 2002), and electrocoalescence (Leary et al. 2020), have limitations of low separation efficiency and are highly energy-consuming. Also, most of the research on oil/water separation relates to superoleophobic surfaces and deals with petroleum products, such as alkanes-fluorinated compounds. In this case, the oil pollution problem is solved indirectly through the chemical method of adding a coagulant to the oil. Also, the in situ burning approach addresses the pollution of the oil spill indirectly. However, solidifying and in situ burning have disadvantages, such as secondary pollution, low separation efficiency, and waste of resources (Darmanin and Guittard 2015; Zhang et al. 2019). The biological treatment also has some drawbacks that limit the application of biodegradation of oils. In biological treatment, floating oil is hardly biodegraded by aquatic microbes; moreover, it requires rigorous conditions for the proliferation of microorganisms. The process variables, such as the optimization of biological activity, accessibility, and inherent biodegradability, limit their potential applications (Zhang et al. 2019). Thus, it is essential to develop an environmentally friendly, facile, commercial, and active oil/water separation material that can purify a large volume of oil/water mixture even in a complex practical environment. One of the possible solutions addressing this issue is the reuse of water through the adoption

of nature-derived superhydrophobic or superoleophobic materials. The continuous growth in research and development in both academia and private industry demands the materials with superhydrophobic or superoleophobic properties for their potential applications, such as in anti-corrosion coatings, liquid-repellent textiles, oil/water separation, nanoparticles assembly, microfluidic devices, printing techniques, optical devices, high-sensitivity sensors, or batteries (Darmanin and Guittard 2015). Recently, the nature-derived underwater superoleophobicity for oil/water separation, such as fish scales, has gained a considerable attention. For example, the fish scales are superhydrophilic materials that can trap abundant water on their rough surfaces. The trapped water significantly weakens the direct contact between oils and materials, which results in "superoleophobic surfaces" with low oil adhesion for the superhydrophilic materials (Chen and Xu 2013).

In this chapter, we have highlighted some nature-derived superoleophobic materials, concepts, and properties for the separation of oil from oil/water mixture. We have also discussed the recent progress in oil/water separation technologies based on superoleophobic material, such as electrospinning, layer-by-layer technology, spray coating, and lithography.

10.2 Superoleophobic and Superhydrophobic Surfaces

10.2.1 Surface Science

Various valuable materials and technologies have developed in surface science for its potential industrial applications in a new scenario. The surface of each material plays a vital role that describes its characteristics and functionality (Somorjai and Li 2011). The surface energy, surface tension, and contact angle are essential characteristics to analyze the surface of any material. The surface tension measures the force required to keep liquid together on the surface of different phases. In contrast, the contact angle explains the angle developed from liquid or vapor on a solid surface (Stammitti-Scarpone and Acosta 2019). The quality of the solid surface, i.e., the wettability of the surface, determines the contact angle. If the contact angle is high, then such surface has low wetting property and vice-versa (Iqbal et al. 2019).

10.2.2 Wetting Theory

The wettability of a surface is due to liquid media, like water, oil, chemicals, etc. In the water medium, the low contact angle is termed a hydrophilic surface with the contact angle (q) less than 90°. The high contact angle is known as a hydrophobic surface with a contact angle (q) more than 90°.

From Fig. 10.1, it is cleared that for hydrophilic surfaces, the $\theta < 90°$ exhibit strong affinities, while hydrophobic surfaces show little affinity for water with

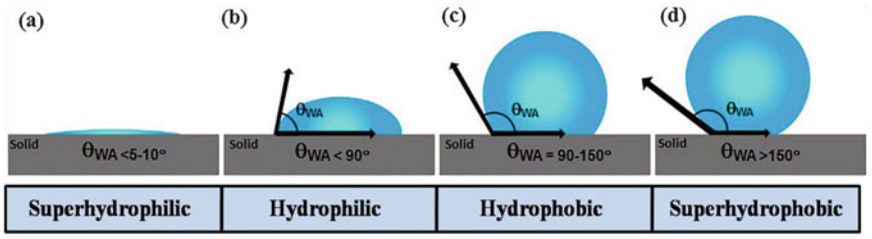

Fig. 10.1 Wetting condition of water droplets onto solid surfaces (**a**) superhydrophilic, (**b**) hydrophilic, (**c**) hydrophobic, and (**d**) superhydrophobic (Zarghami et al. 2019). Copyright © 2019. Reproduced with permission from the Elsevier

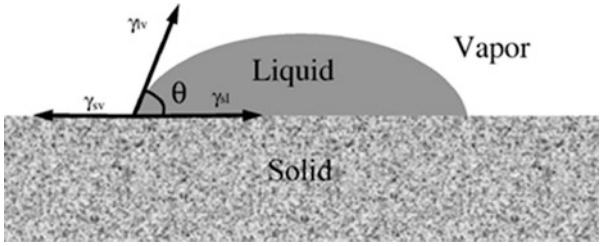

Fig. 10.2 Schematic of a contact angle (θ) of a drop on a solid surface, and the three interfacial tensions (γ_{lv}: liquid–vapor, γ_{sv}: solid–vapor, and γ_{sl}: solid–liquid) (Tavana and Neumann 2007). Copyright © 2006. Reproduced with permission from the Elsevier

$\theta > 90°$ (Commentary 2014). Young's equation determines the contact angle based on thermodynamic equilibrium concerning surface tension between three phases (i.e., liquid, solid, and vapor), as shown in Eq. 10.1 (Rajaram and Laxman 2016) (Fig. 10.2).

$$\gamma SG = \gamma SL + \gamma LG \cos(\theta) \tag{10.1}$$

where γSG = surface tension of solid-vapor phase, γSL = surface tension of solid-liquid phase, γLG = surface tension of liquid-vapor phase, and θ = contact angle.

Also, the wetting of the solid surface is dependent on surface appearance, such as smoothness and roughness or porous. Young's equation is ideal for the smooth surface. Wanzel equation describes the wetting property of the rough surface (Hensel et al. 2013). On a rough surface, contact angles change due to amplification of the solid–liquid interactions. It explains by the Wenzel regime of the wetting equation as given in Eq. 10.2

$$\cos\theta_W = r * \cos\theta_Y \tag{10.2}$$

where r = surface roughness factor, θ_W = contact angle in the Wenzel model, and θ_Y = contact angle in the Young's model.

Table 10.1 Contact angle (θ) of surfaces

Types of surface	Contact angle (θ)
Superhydrophilic	$\theta < 5°$
Hydrophilic	$\theta < 90°$
Hydrophobic	$\theta = 90°–150°$
Superhydrophobic	$\theta = 150°–180°$

Lastly, Cassie-Baxter proposed an advanced model to determine the contact angle of a porous surface where air can trap in the void (Choi et al. 2009; Tran and Webster 2013). The equation is given as follow;

$$\cos\theta_{CB} = f_s\,(\cos\theta_s + 1) - 1 \tag{10.3}$$

where f_s = fractional area of the solid surface with a contact angle of θ_s on the flat solid surface, and θ_{CB} = contact angle in the Cassie–Baxter model.

From Table 10.1, the superhydrophilic surfaces have an excellent affinity towards water, giving the lowest contact angle of 5°. Superhydrophobic phenomenon defines repulsion of water droplets on its surface, indicating the highest contact angle, which is more than 150° and near to 180°.

The wetting states mentioned above (Young, Wenzel, and Cassie-Baxter states) are also valid for an oil droplet on a smooth or rough solid surface. According to these wetting properties of the surface, it can be defined as hydrophilic/oleophilic, hydrophobic/oleophobic, superhydrophilic/superoleophilic, and superhydrophobic/superoleophobic surfaces. The superhydrophobic and superoleophobic surface effects are also known as self-cleaning surfaces (Whyman et al. 2008).

10.2.3 Designing/Fabrication of the Surface

The contact angle, surface tension, and surface interfacial energy play an essential role in the fabrication of such surfaces. Based on this, one must also consider the movement of a droplet on the solid surface and the importance of re-entrant surface curvature. When we talk about the movement of a drop, contact angle hysteresis arises. The contact angle hysteresis is the phenomenon in which the contact angle of a drop is different on the leading edge and trailing edge due to which advanced contact angle (qadv) and receding contact angles (qrec) are developed (Brown and Bhushan 2016). As low is the contact angle hysteresis, droplets easily roll down from solid surface imparting excellent hydrophobicity or oleophobicity. The surface with re-entrant texture is seen in various shapes, like an inverted trapezoid, overhang, mushroom-like or T-shaped microstructures, etc. (i.e., spherical, cylindrical, and oval) (Tuteja et al. 2007). Plenty of reported work is available on superhydrophobic materials, but research work on superoleophobic materials is rare. The surface tension of water is far higher than oil and other chemicals, so it

is difficult to fabricate superoleophobic surfaces than superhydrophobic (Xue et al. 2012).

10.2.4 Superamphiphobic Surface

The surface designed by a combination of superhydrophilicity and superoleophobicity is called superamphiphobic surfaces, which can repel both polar and non-polar liquid media on a solid surface (Yang et al. 2013). Biological surfaces found in nature, like lotus leaves, butterfly wings, gecko feet, mosquito eyes, natural minerals, and biomaterials, can exhibit a fascinating superhydrophobicity (Gao et al. 2007; Neinhuis and Barthlott 1997; Zheng et al. 2007). For example, the natural superoleophobicity found in lotus leaf (lower side), springtails, leafhopper, etc. stabilizes the Cessie-Baxter state using oleophilic materials ($q < 90°$) intrinsically (Darmanin and Guittard 2014).

Recently, the combination of superhydrophobic and superoleophobic materials have been designed to fulfill the given criteria. Literature has proved that superhydrophobic materials are not always superoleophobic, but superoleophobic materials are always superhydrophobic due to low surface tension of oil than water (Bellanger et al. 2014). Coating, electrospinning, grafting, spray casting, layer-by-layer assembly, and lithography are the most versatile methods for the fabrication of such surfaces. These surfaces possess potential applications in the world industry for anti-corrosion coatings, anti-icing coatings, liquid-repellent textiles, oil/water separation, nanoparticle assembly, microfluidic devices, printing techniques, optical devices, high-sensitivity sensors or batteries, self-cleaning, anti-bacteria, and other fields (Darmanin and Guittard 2014).

10.2.5 Importance of Oil/Water Separation

As said earlier, oil pollution is a worldwide concern after water pollution. The oil/water separation is the blooming topic in every industry. Hence, it is mandatory to fabricate a material that will benefit in oil/water separations by lowering the industrial oily sewage discharge and protecting the environment loss. Several physical and chemical techniques have been employed for oil/water mixture separation, but membrane technology has gained attention (Ashaghi et al. 2007; Cheryan and Rajagopalan 1998). This technique is efficiently technical, low cost, and environmentally friendly (Fane et al. 2015). Many researchers have successfully developed a variety of materials for oil/water separation, like foams, textiles, membranes, sorbents, etc. Among which membranes selectively remove one phase from the oil/water mixtures with the unique surface property of materials. For example, in oil-removing materials with superhydrophobicity and superoleophilicity, first oil will be a filter or absorbed from the mixture, and water-removing materials with

superhydrophilicity and superoleophobicity will separate water from the mixture. Materials for membrane fabrication are metals, ceramics, polymers, nanomaterials, or smart materials (inorganic or organic) (Wei et al. 2018).

10.3 Bioinspired Superoleophobic Material

According to weather conditions, plants, insects, animals, and other natural products have numerous inherent multifunctional properties for their survival in the environment (Darmanin and Guittard 2015). The environment and bioinspired materials inspire most researchers and technologies for the separation of oil and water. The different materials, structures, functions, or properties invented based on natural things are known as bioinspired materials. They are also termed as biomimetic materials (Kavalenka et al. 2017; Zhang et al. 2013). The most famous example of bioinspired materials is a flying machine that designed on account of bird structure. However, for the fabrication of bioinspired superoleophobic materials, plants and animals hold the key to this route (Solomon 2013). Fluorinated compounds are mostly researched for the superoleophobicity. The fluorinated compounds easily repel water molecules and also low surface tension liquids, like oil (hydrocarbon component). But nature is not able to produce fluorinated materials. Hence, the invention of superoleophobic surfaces in nature helps find other alternatives to fluorinated compounds with properties. Also, the superoleophobicity can be observed in contact with air or underwater (Darmanin and Guittard 2015).

10.3.1 Superoleophobic Materials Derived from Plants

10.3.1.1 Lotus Leaf

The self-cleaning property of lotus leaf is well-known, and its microscopic surface structure does not allow liquid droplets to rest on it. This surface, i.e., papillae made up of wax in the epidermis of lotus leaf, efficiently minimizes the droplet adhesion and repels the contact on that area (Blossey 2003). It naturally shows hydrophobic property called a lotus effect. The upper part of the leaf is superhydrophobic, whereas the lower part of the leaf shows oil repellency due to its micro-nano composite structure (Barthlott and Neinhuis 1997; Deng et al. 2020). The lower microstructure surface is studied to fabricate superoleophobic materials in underwater (Fig. 10.3).

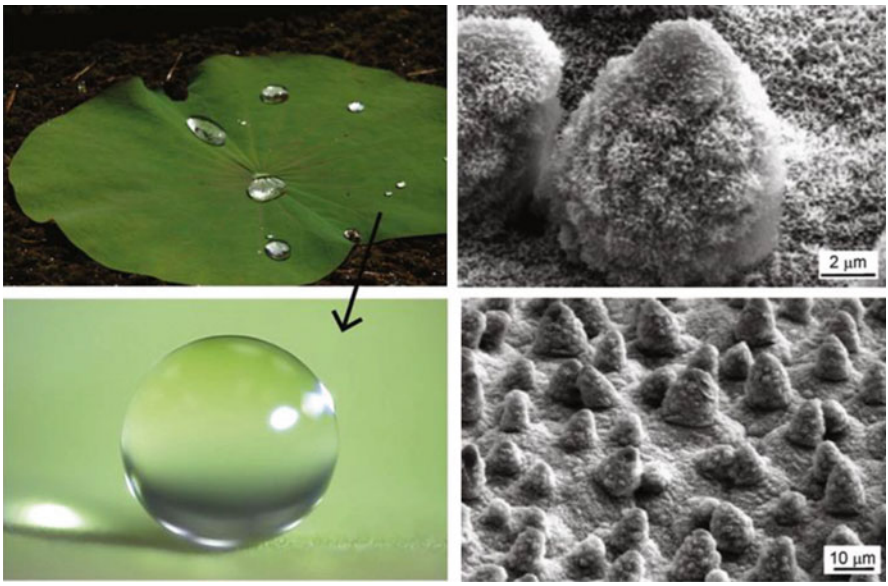

Fig. 10.3 Bioinspiration from lotus leaf for liquid-repellency and low-adhesion properties. A lotus leaf (*Nelumbo nucifera*) is superhydrophobic, repelling water droplets with contact angles of ~164°. SEM (Scanning electron microscope) micrographs consist of a microstructure formed by papillose epidermal cells covered with 3-D (3-Dimentional) epicuticular wax tubules, which create nano-structure (Martin et al. 2017). Copyright © 2017. Reproduced with permission from the Elsevier

10.3.1.2 Rose Petals

The rough surface of a rose petal gives high contact angle to water droplet resulting in high adhesion with water. The shape of water is spherical and cannot roll down even after the petal is turned upside down. This property is known as a rose petal effect or petal effect (Ebert and Bhushan 2012). Based on this effect, artificial biomimetic polymer film has developed to give superoleophobicity in the air (Feng et al. 2008) (Fig. 10.4).

10.3.1.3 Rice Leaf

The rice leaf surface has parallel microgrooves due to which water drops can only roll in a given direction. Still, when the leaves are inclined perpendicular to micro-grooves, the water droplets stay on the leaves and remain motionless. If they tilted to the microgroove's analogous direction, the droplets quickly get roll-off (Deng et al. 2020; Feng et al. 2002). The anisotropic microgrooves-fabricated material was inspired by the rice leaf surface structure which shows oleophobicity in the air (Kang et al. 2013) (Fig. 10.5).

Fig. 10.4 SEM images of the red rose petal surface showing the micropapillae and nano-fold structure. Copyright © 2017 (Szczepanski et al. 2017). Reproduced with permission from the Elsevier

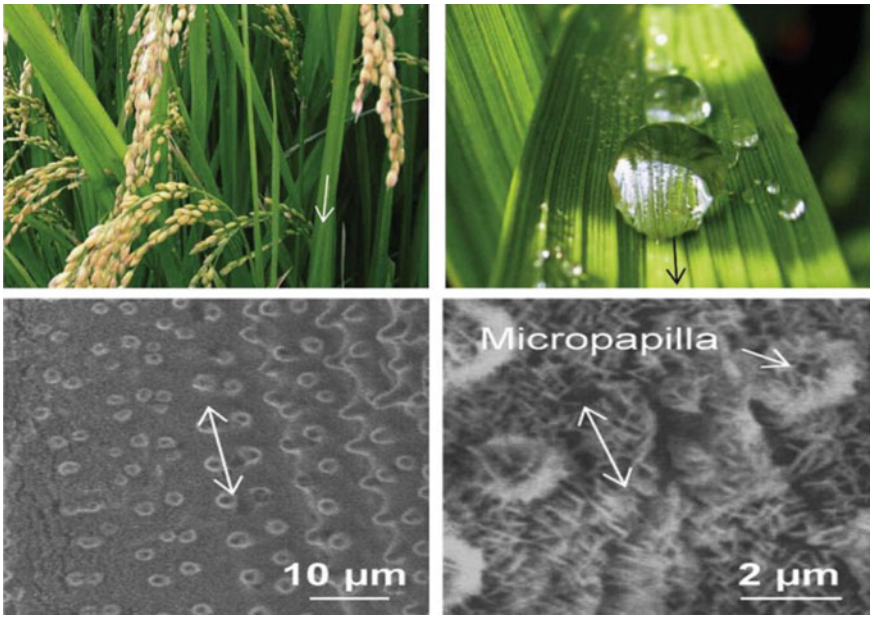

Fig. 10.5 Rice leaves contain longitudinal grooves with a transverse sinusoidal pattern. The water droplets resting atop their superhydrophobic and low adhesion surfaces. Optical profiler height maps of actual rice leaf. Arrows indicate the tendencies of fluid flow in transverse and longitudinal directions (Bixler et al. 2014). Copyright © 2013. Reproduced with permission from the Elsevier

Fig. 10.6 Seaweed underwater and macroscopic view of biomass heterogeneity of *Sargassum muticum* (Laurens et al. 2020). Copyrights © 2020 The Authors. Reproduced with permission from Elsevier Ltd.

10.3.1.4 Seaweed

The surface of the seaweed plant displays underwater superoleophobicity in nature because of the occurrence of porous structure and chemical composition. Chemically seaweeds are composed of polysaccharides with an affinity to water molecules exhibiting hydrophilicity in high-salinity water (Yong et al. 2017). This phenomenon used to develop bioinspired superoleophobic aerogel material for oil/water separation in marine environments (Li et al. 2016a, b) (Fig. 10.6).

10.3.1.5 Pitcher Plant

The pitcher plant from the *Nepenthes* genus, which is carnivorous, can capture insects that come in contact. The plant consists of a pitcher trap with a leaf-like lid on it with the slippery surface (Gorb and Gorb 2006). The bioinspired materials designed by using this concept now called Slippery Liquid-Infused Porous Surfaces (SLIPS). In SLIPS, trapping the prey of pitcher plants has attracted the researcher to design a superoleophobic surface. Here, pitcher catches the prey and slides into digestive juices by repelling oil (Wong et al. 2011). Also, Nepenthes pitcher (NP)-inspired slippery surfaces were successfully developed by immobilizing fluoro liquids on a lotus leaf (LL)-inspired superoleophobic surfaces (Zhang et al. 2014) (Fig. 10.7).

10.3.2 Superoleophobic Materials Derived from Animals

10.3.2.1 Skin of Springtail

The springtails also termed as collembola live in the soil environment. They show superoleophobic surface property because of their outstanding nanostructured

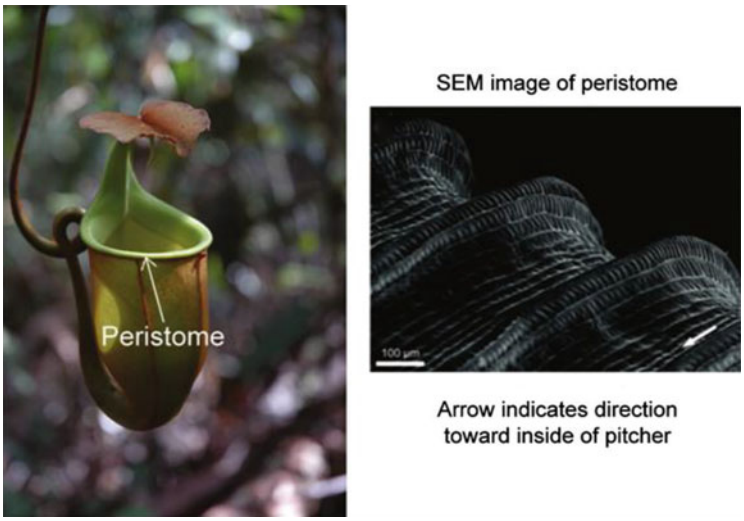

Fig. 10.7 The pitcher plant (*Nepenthes*) has a slippery rim called a peristome that when wet by nectar or rainwater, it creates a thin lubricating film that causes insects to be unable to adhere to the surface (Martin et al. 2017). Copyright © 2017. Reproduced with permission from the Elsevier

cuticle surface. The surfaces of the springtail are capable of repelling some polar and non-polar liquids, such as water, methanol, ethanol, hexadecane, etc., and resistant to bacterial adhesion (Nickerl et al. 2014). The surface of springtails is composed of three layers, and the chemicals present on each layer are responsible for repulsion for polar and non-polar liquids. The outermost layer contains steroids and acids, whereas the middle layer is composed of proteins, and the innermost layer consists of pore channels. The structure of the surface is hexagonal or comb-like. This unique surface of springtails is exhibiting superoleophobic property. One of the most notable examples of natural species with superoleophobic properties was reported by the group of Werner (Helbig et al. 2011; Hensel et al. 2013, 2014; Nickerl et al. 2013). According to this surface property, novel strategies have developed to fabricate bioinspired superoleophobic surfaces without fluorinated compounds (Darmanin and Guittard 2015) (Fig. 10.8).

10.3.2.2 Filefish Skin

The filefish, i.e., *N. Septentrionalis,* can effortlessly swim in oil-polluted sea regions without damage or contamination of its skin. This behavior was observed by Jiang et al., explaining the anisotropic property of fish skin underwater oleophobicity due to the presence of hook-like spines on its surface. Due to this directional hook-like spines and curve on the spine tip, as shown in Fig. 10.9, an oil droplet rolls in a specified direction, which makes this behavior unique. The anisotropic underwater

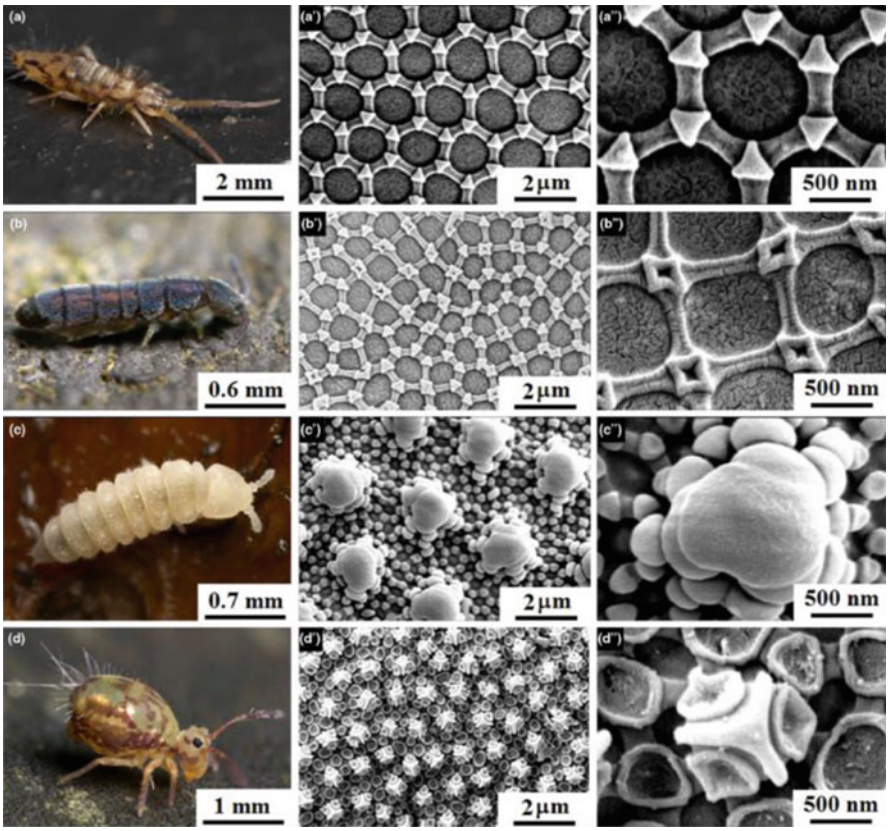

Fig. 10.8 SEM images at different magnifications of various species of springtails (Ghaffari et al. 2019). Copyright © 2019. Reproduced with permission from the Elsevier

Fig. 10.9 Filefish skin with the arrow directing from head to tail and SEM images of hook-like spines with the oriented direction from a side-view (Gao and Guo 2017). Copyright © 2017, Jilin University. Published by Elsevier Limited and Science Press

oleophobicity property derived from rough surface skin of filefish gives self-cleaning ability to survive in oil-spilled seawater (Cai et al. 2014; Darmanin and Guittard 2015).

10.3.2.3 Clam's Shell

The shell has several layers and made of calcium carbonate. The hard layer protects the shell from predators and the environment; the inner and outer surfaces have always observed to be clean in any environmental condition. They naturally present self-cleaning property. The clam's shell has two regions, glossy edge region and rough inside region (Liu et al. 2012). Both regions are similar in chemical composition and different in the structure of the surface. The rough surface can repel oil, showing oleophobicity (Yong et al. 2017). The calcium carbonate layer and coarser structure of its surface create hydrophilic components, giving superoleophobicity underwater.

Besides the clam's shell, the natural columnar nacre from the abalone shell also has excellent underwater superoleophobicity with unique mechanical properties. The inner surface of the natural columnar has an iridescent color that illustrates many convex hexagonal columnar structures. The top surface of hexagonal columns is composed of nanoscale aragonite particle protrusions, as shown in Fig. 10.10b_2. The combination of hierarchical micro/nanometer structure and hydrophilic components incorporates inherent underwater low adhesive superoleophobicity. The contact angle of underwater 1, 2-dichloroethane droplets on the surface of the columnar nacre is as high as $156.8 \pm 0.9°$ (Fig. 10.10b_3) (Yu et al. 2020).

10.3.2.4 Fish Skin/Scales

Mostly several fish skins are distinguished as scales, a rough surface fan-like shape consisting micropapillae all over the surface, as seen in Fig. 10.10a_2. Fish scales are superhydrophilic and superoleophilic, which explains that it can repel water droplets as well as oil droplets from the surface of fish scales (Yong et al. 2017). Under consideration of Cassie Wetting state, the hydrophilic chemical composition and rough microstructures on fish skin give underwater superoleophobicity.

10.3.2.5 Sharkskin

The sharkskin is made up of sharp and pointed scales called denticles due to their tooth-like shape, as shown in Fig. 10.10c_3. This scale contains riblets with longitudinal microgrooves aligned to water flow and reduces drag underwater (Brown and Bhushan 2016; Dubov and Vinogradova 2012). These surfaces are oleophobic in air and superoleophobic underwater. Such property is essential for the hydrophilic coated surface (composed of calcium phosphate skeleton and protein and coated

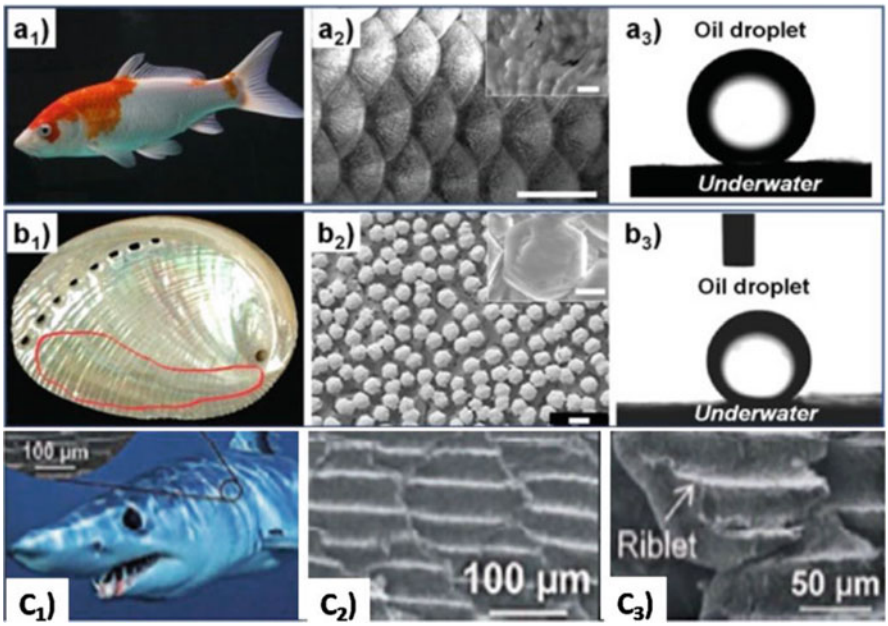

Fig. 10.10 Fish scales showing the superoleophobicity underwater. Optical image of fish in the water ($\mathbf{a}_1$), an optical image of the fish scales and SEM image of fish scale showing the micropapillae ($\mathbf{a}_2$), and oil contact angle on a fish-scale surface ($\mathbf{a}_3$). The inner side of the columnar nacre showing superoleophobicity underwater ($\mathbf{b}_1$), the photograph of the entire abalone shell and top-view SEM images of the inner nacreous layer in the red-marked area showing convex hexagonal microscale columns and nanoscale protrusions ($\mathbf{b}_2$), and the photograph of an underwater oil droplet on the red-marked region ($\mathbf{b}_3$). Sharkskin with low drag and a self-cleaning surface ($\mathbf{c}_1$), SEM images of sharkskin at different magnifications ($\mathbf{c}_2$, $\mathbf{c}_3$) (Yu et al. 2020). Copyrights © 2019 Institution of Chemical Engineers. Published by Elsevier

with mucus layer), which will repel oil and interact with water (Bixler and Bhushan 2013).

10.3.2.6 Leafhoppers

The unique surface structure found on leafhopper tends to show a superoleophobic property, which was reported by Gorb and Rokitov in the twentieth century (Rakitov and Gorb 2013). These insects secrete granules called bronchoscopes that uniformly coat their body surface and protect them from contamination. Bronchosomes are spherical with a hollow core and hexagonal structure, as shown in SEM images of Fig. 10.11. These structures have unique surface morphology named as re-entrant curvatures responsible for superoleophobicity (Bellanger et al. 2014; Zhao et al. 2012). Those granules are chemically made up of proteins, as we know proteins are polar molecules and used to get superoleophobic properties. Hence, it is fascinating

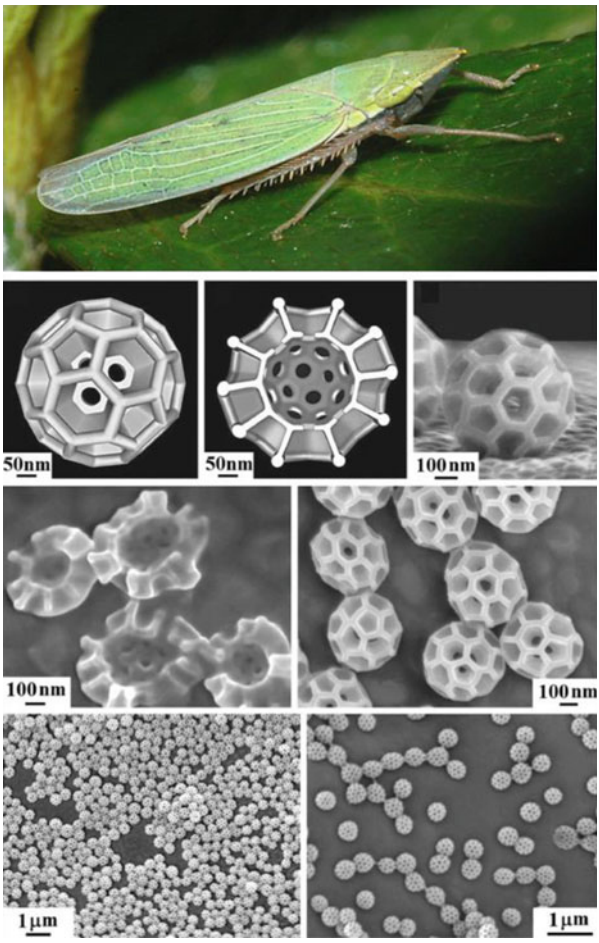

Fig. 10.11 Image of Leafhopper and SEM images at various magnifications of superoleophobic bronchosomes present at the surface of leafhoppers (Ghaffari et al. 2019). Copyright © 2019. Reproduced with permission from the Elsevier

to find protein structures with a polar unit, which can create superoleophobic features (Darmanin and Guittard 2015; Ghaffari et al. 2019).

10.4 Novel Fabrication Techniques for Superoleophobic Materials

Superoleophobic materials can be formed using several techniques, such as electrospinning, layer-by-layer technology, spray coating, lithography, dip coating, chemical vapor deposition method, etc. The related methods/techniques are summarized below.

10.4.1 Electrospinning

Electrospinning is the multipurpose technique used for continuous fibrous membrane production. Superoleophobic materials fabricated from electrospinning has fiber diameter in the range of micrometer to the nanometer and with 30–90% porosity (Ejaz Ahmed et al. 2014; Ghaffari et al. 2019). This method has a potential application for the production of a superhydrophobic surface (Tang et al. 2009; Wan 2008) and superoleophobic surfaces (Cengiz et al. 2012; Pan et al. 2012; Sharma et al. 2013) for oil/water separation. In this process, syringes are filled with a polymer solution or polymer melts. High electric potential is applied at the tip of the needle. Further, charged threads of polymer solutions or polymer melts are drawn from the spinnerets/syringe using electric forces. For the collection of nanofibers, the drum/sheet is placed at the desired distance from the tip of the needle. A 20–25 kV voltage can be applied between the tip of the syringe and collector drum or sheet. This process can be carried out at room temperature to the elevated temperature. Variables of electrospinning, such as viscosity, solvent, electrical conductivity, and surface energy, can be adjusted to control the distance between fibers, fibers' diameter, and surface energy. Superoleophobic and superhydrophobic properties are also improved by using this technique (Cui et al. 2008; Guo et al. 2010). Electrospinning can be carried out as a one-step or two-step process. Fluorinated compounds are insoluble in electrospinning solution (Choi et al. 2010). Due to the limitation of the single-step electrospinning process, this process is carried out in two steps. Initially, in the two-step electrospinning process, electrospinning of conventional polymers is carried out; further a superoleophobic material is deposited on the surface. Further, SiO_2 nanofibers were deposited on the glass using the electrospinning method to impart superoleophobicity. Also, porous silica membranes were applied using a vapor deposition method (Ganesh et al. 2013). Poly (2,2,2-trifluoroethyl methacrylate) (PTFEMA) nanofibers were used to prepare a superoleophobic substrate using the electrospinning method. By using this method, superoleophobic property on the substrate depends on the diameter of the fiber (Choi et al. 2010).

Recently, researchers have focused on the application of nature-based sources for the fabrication of underwater superoleophobic surfaces. Fishes and sea birds are well-known for the protection from contamination by anti-fouling and oil pollution and exhibiting self-cleaning properties. The fish scale contains a thin layer of mucus, protein, and calcium phosphate, which shows superoleophobicity in the water and the air. Fabrication of low adhesive and superoleophobic interface was constructed on the solid surface using oil, water, and a solid three-phase system. This idea was derived from the fish scales that show the anti-wetting behavior on oil (Liu et al. 2009a, b). Similarly, the fabrication of superoleophobic material was carried out by constructing hierarchical macromolecule nano-clay hydrogels. The contact angle of oil droplets can be increased in underwater by increasing the surface roughness (Lin et al. 2010).

10.4.2 Layer-by-Layer Technology

In the layer-by-layer method, the substrate is immersed in the solution having positive- and negative-charged particles to make an oleophobic substrate. This process involves consecutive absorption of chemicals having positive- and negative-charge ions. In this method, multilayer coating can be achieved by applying charged particles on the surface of the substrate/fabric one after another. Due to the use of charged particles for multilayer coatings, layers deposited on the surface of the fabric/substrate are electrostatically cross-linked. This method has been used for various applications, such as superhydrophobic fabrication (Zhai et al. 2004; Zhang et al. 2007) and superoleophobic fabrication (Yang et al. 2012a). The transparent coating was applied with silica and polystyrene nanoparticles using layer-by-layer technology to generate the superoleophobic and superhydrophobic properties. The results obtained from this research were suggesting that the coating thickness, chemical composition, and surface morphology can be controlled to the nanoscale (Cao and Gao 2010). Self-assemble SiO_2 nanoparticles with hierarchical nanostructure and rough surfaces were constructed on sintered stainless steel mesh and stainless steel fiber via layer-by-layer technique. The as-prepared mesh showed efficient hydrophobicity to separate oil-water mixtures (Li et al. 2014). A combination of layer-by-layer technology and fluoro-silane vapor deposition method was used to fabricate superoleophobic and superhydrophobic properties (Brown and Bhushan 2015).

The nacre and short clam's shell inspire the fabrication of underwater low adhesive superoleophobic surface using a novel inorganic high-energy coating (Liu et al. 2012; Xu et al. 2013). Similarly, mussel adhesive and fish skin incite the fabrication of superoleophobic surfaces using several techniques (Cai et al. 2014; Haeshin Lee et al. 2007; Liu et al. 2013).

10.4.3 Spray Coating

The spraying method is also an effective method to prepare a superhydrophobic or superoleophobic material. In this method, a superoleophobic coating/mixture is applied on the surface of the substrate by the spraying method. This process requires very little time for processing. In general, a superoleophobic coating mixture is prepared separately, and then the fabric/substrate has been impregnated in the prepared coating mixture. Further, the coated substrate should be dried and cured. To increase the surface roughness of the substrate/fabric, oleophobic chemicals, solid particles, and adhesives can be added to a superoleophobic coating mixture. The coating method is the most commonly used method to prepare superhydrophobic substrate/fabric/material. Usually, superhydrophobic materials are mixed with organic reagents, and further, the prepared mixture is sprayed onto the surface of the substrate. In the spray-coating method, the fabrication of

superoleophobic surfaces can be carried out using perfluoroalkanoates. Initially, chemical etching of metals is carried out in perfluoroalkanoates and perfluoro carboxylic acid solutions (Meng et al. 2008). Further, a coating is applied to the fabric. This method is a simple method for the fabrication of superoleophobic or superhydrophobic coatings (Li et al. 2016a, b; Steele et al. 2009; Xiong et al. 2014; Yang et al. 2012b). The spray coating process is limited to the specific substrates and requires much time for the processing. Synthesis of copper perfluorooctanoate was carried out using copper acetate and perfluorooctanoic acid in water. Further, superoleophobic finish was obtained by applying/spraying synthesized copper perfluorooctanoate on the substrate using a spray coating method. Uniform coating can be obtained by using this method. A contact angle above 150° and liquids with low surface energy can be achieved using this method (Yang et al. 2011).

10.4.4 Lithography

In this method, precise construction on the surface of the fabric/substrate is possible using the lithography method. Also, different patterns and sizes can be quickly fabricated using this method. The lithography method has some advantages: low process cost, convenient template creation, and multiple uses of the template (Ellinas et al. 2011; Im et al. 2010). Many studies have been carried out on the fabrication of a superoleophobic material using lithography (Deng et al. 2012; Lee et al. 2013; Liu et al. 2009a, b, 2014). Triple-scale hierarchical structure was fabricated on the polymeric substrate of methacrylate (PMMA) to make it oleophobic, superoleophobic, and superhydrophobic surfaces using the lithography method. Polystyrene microparticle colloidal lithography method was used to generate superoleophobic and superhydrophobic surfaces (Ellinas et al. 2011). Also, reverse nanoimprint lithography method was used to prepare superoleophobic and superhydrophobic surfaces (Choi et al. 2013). Zinc oxide was used to generate superoleophobic and superhydrophobic surfaces using ultraviolet nanoimprint lithography method (Jo et al. 2014).

Lotus leaf exhibits underwater superoleophobicity on their lower sides and superhydrophobicity on their upper sides. This effect is called a Janus effect. This type of effect on the materials was fabricated using a template method (Cheng et al. 2011). Short clam shells are well-known for their self-cleaning property and exhibit underwater superoleophobicity. The inner and outer sides of the shell always remain clean. This effect is due to the presence of $CaCO_3$ and a hierarchical structure. With this inspiration, the metal copper sheet was fabricated using high-energy inorganic coating. By using this study, underwater aquatic equipment can be protected from oil contamination (Liu et al. 2012).

10.5 Conclusion

Bioinspired superoleophobic surfaces, combined with biotechnology, material science, and nanotechnology, are promising aspects for various industries due to their innovative solution towards oil/water separation. Oil pollution is a significant concern of the ecology, as it disturbs the aquatic and biotic life. The development of super-wettable surfaces from bio-based sources for their promising application in the oil/water separation is of immense academic and industrial interest. The fabrication of superhydrophobic surfaces is easier than a superoleophobic surface, because oil has lower surface tension compared with water. In this chapter, we tried to elaborate on the importance of superhydrophobic and superoleophobic surfaces for oil/water separation applications. The number of innovations and technologies is inspired by nature, as learning from nature has been a source of humankind. By taking inspiration from nature and adding chemistries, it is easy to achieve oil repellency. For fabrication of bioinspired superhydrophobic and superoleophobic surfaces, nature has provided a range of functional surfaces, including some examples like for superhydrophobic surfaces is the lotus leaf and for oleophobic surfaces underwater is fish scales. Similarly, in this chapter, many other natural sources have been reviewed and discussed that give oleophobicity. Such bioinspired materials are cost-effective and eco-friendly, so they are used for different applications, including self-cleaning, stain-free clothing and drag reduction, etc. From this chapter, it is evident that the progressive development in bioinspired superoleophobic surfaces is a blooming and demanding area which can commercialize for many application in worldwide industries.

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Chapter 11
Biotechnology Applied to Treatments of Agro-industrial Wastes

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Contents

Abstract On a global scale, the agro-industrial sector generates a high volume of waste that generally presents difficulties to be reused, treated, or disposed of in processes that minimize its impact on the environment. Biotechnology emerges as a field that combines the disciplines of life sciences, ecology, and engineering to exploit biological processes (mainly microbial) in environmental applications of waste treatment. This chapter presents alternatives for the treatment of agro-industrial waste, classified according to their origin and composition. Important aspects related to the engineering of waste treatment are discussed, such as the selection criteria for strategies or alternatives, as well as the level of transformation

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achieved by the residual. A topic of relevance in this chapter is the use of enzymes, as biological catalysts, in the treatment of residual effluents. The relevance of this alternative is presented through case studies related to dye removal and wastewater treatment from the coffee production industry. An analysis of the submerged fermentation (SmF) and solid-state fermentation (FES) systems is presented to identify the most suitable configuration for effluent treatment. Finally, an analysis of the scaling stages and the study of the economics of these bioremediation processes are proposed from a simplified perspective.

Keywords Agrowaste · Biological treatment · Dye removal · Biodegradation · Fermentation

11.1 Introduction

Agro-industry promotes global economic, social, and environmental development, as long as it maintains the balance between the activity carried out and the protection of the environment in each of its processes (Corredor and Pérez 2018), from the handling of agricultural raw materials to the distribution and final disposal of by-products or generated waste. According to the Food and Agriculture Organization of the United Nations FAO (Hodder and Hodder 2016), the agro-industries can be classified into two divisions: the first is made up of the food and non-food industries; the second is made up of industries that supply raw materials (such as milling wheat and rice) and consumers of raw materials (such as bread making).

On the one hand, the food industry has an important place in food security, contributing to the transformation and conservation of perishable raw materials. However, the activities of the sector generate waste, both in the production and consumption stages. Livestock and agriculture are two of the activities of greatest interest and are the focus of continuous improvements in the production and control of the waste they generate (Kinyua et al. 2016; Sorathiya et al. 2014). On the other hand, the growing demand for non-food products increases the exploitation of production systems while compromising natural resources and ecosystems from the point of view of environmental health.

Biotechnology is a key factor in the use/treatment of agro-industrial waste. With technological advancement, selection criteria have been defined for waste in order to be used for biotechnological purposes. One of the classifications is made taking into account the sector in which the waste originates. Table 11.1 identifies the groups to which this criterion leads to plant-based waste.

The literature establishes “clean” technologies for the use of agro-industrial waste. The classification of these technologies is oriented to the specific characteristics of the waste: (1) the main component of the waste is a potential substrate for the fermentative production of inputs from industrial processes; (2) the material contains compounds of industrial and commercial interest that are obtained by means of

Table 11.1 Classification of organic waste according to its origin

Type	Description	Examples
Agriculture[a,b,c]	Waste generated after the harvest is completed. They mainly consist of pruning remains, rice husks, wheat straw, gophers (ear of corn), or even the plant when the product of interest extracts the plant from the soil. They are mostly composed of lignocellulose	Wheat, rice, corn, sugarcane, beet, sunflower, coconut crops
Food industry[c]	Wastes characterized by having a known and easily classifiable composition. Also included in this group are biomass that have lost its quality conditions as raw materials that do not enter the process or products that were not properly preserved and transported	Wine and spirits industries, fruit juices, vegetable drinks
Coastal algae and invasive species[d,e,f]	Plant species whose growth in industrialized areas leads to technical, economic, and environmental problems. Its growth rate is high, and its eradication is practically impossible	Macroalgae Water hyacinth
Forests and timber industry[c,g]	It includes green areas that receive periodic maintenance by some public or private organization. It also includes waste from industrial processes whose main raw material is wood	Protected forest areas and parks Paper and wood production
Household[b,c,h]	Waste from household activities, characterized mainly by being heterogeneous waste	Houses, buildings for residential use, and offices

[a]FAO (2002); [b]FAO (2019b); [c]Millati et al. (2019); [d]Ehrenfeld (2010); [e]Villamagna and Murphy (2010); [f]Adhikari et al. (2018); [g]Romaní et al. (2018); [h]Adhikari et al. (2018)

extraction. In any case, an exhaustive characterization of the residual will be required.

11.2 Final Disposal of Agro-Industrial Waste

Agriculture is one of the most widespread socio-economic activities in the world since, together with livestock and fishing, it is a source of food generation. Some crops are the basis of food or are an economic item of interest in many countries, so they are produced in large volumes. Some of these productions are reported in the FAOSTAT database (FAO 2019a). Figure 11.1 relates the contribution of agricultural residues by region, expressed in percentages, to the productions of these crops and the countries that lead them.

In America, the USA stands out for the production of corn and soybeans; and Brazil is the largest producer of sugarcane. According to Manochio et al. (2017), the USA contributes 58% of world ethanol production and Brazil, the second largest producer, with 28%. This is related to the fact that additionally, varieties of these

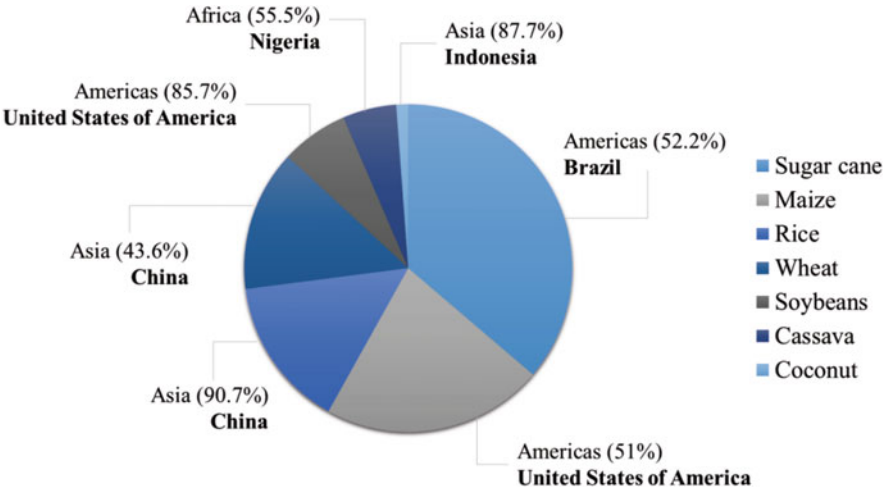

Fig. 11.1 Main agricultural productions and their location. Adapted from (FAO 2019a)

crops are used for the production of biofuels, such as grain, sugar, starch, and oilseed crops (Bordonal et al. 2018).

Asia is the biggest producer of rice and wheat. Inappropriate irrigation practices are associated with these crops, leading to high methane emissions and salinization (Braimoh 2013). According to Kumar et al. (2017) rice production still involves major challenges in terms of generating solid and liquid waste and mitigating its environmental impact. The effluents generated from rice milling contain nutrients and pesticides that cause eutrophication and contamination of groundwater. In China and Japan significant amounts of rice straw and soybean crop residues, respectively, are burned in the open air (Lei et al. 2010; Li et al. 2013). In India, about 92 metric tons of crop residues are burned annually, causing excessive particulate emissions and air pollution (Bhuvaneshwari et al. 2019).

Agriculture contributes to the negative change in the qualities of ecosystems while being a vulnerable sector to the negative impact of this deterioration. In the absence of adequate sustainable management practices, agriculture is a driver of soil and air quality degradation, deforestation, and contamination of surface waters, aquifers, and coastal wetlands (Rohila et al. 2017).

Food waste is a major threat to the environment, as it contributes to climate change (Chiu and Lo 2018), eutrophication and acidification of waters, loss of biodiversity, and destruction of the ozone layer (Brancoli et al. 2017). Beretta et al. (2017) indicate that between 20% and 30% of the environmental impact derived from anthropogenic activities corresponds to the food industry.

The food industry waste can present beneficial or harmful characteristics for their subsequent treatment by means of biotechnology. In general, food industry waste has a high load of organic materials such as proteins, carbohydrates, and lipids, which place them as potential substrates in degradation processes aerobically or

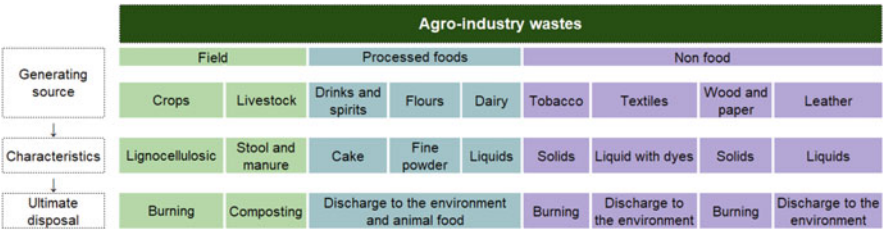

Fig. 11.2 Usual disposal of agro-industrial waste

anaerobically (Singh et al. 2019). This is reflected in the high content of suspended solids, high biochemical oxygen demand (BOD), chemical oxygen demand (COD), as well as the high concentration of nitrogen (Wang et al. 2018b). Furthermore, it is common for these wastes to have characteristics such as a high content of suspended fats or oils (Papadaki et al. 2017), as well as a noticeable variation in pH.

In general, waste from the food industry can be classified into: (a) solid waste and (b) liquid waste. This classification is a criterion for the selection of the technology to be applied for waste treatment. Currently, opportunities have been opened for the use of waste and obtaining products of interest. Figure 11.2 presents the usual disposal of waste from food agro-industry.

Some of the environmental problems are associated with high levels of food loss and inadequate disposal of agricultural residues, which are highly susceptible to rot. Additionally, the collection, processing, and transportation of these carry high costs, making waste management a challenge, especially in developing countries, where access to the necessary technologies is also limited. Vermeulen et al. (2012) state that agricultural production is responsible for 80% of global anthropogenic emissions of greenhouse gases caused by the food system.

11.3 Traditional Approach to Treatment Technologies

Biotechnological nature treatments allow to obtain agronomic, environmental, and economic benefits from the residues of the agro-industry. Traditional biotechnological processes for the treatment of organic waste streams are composting, anaerobic digestion, artificial wetlands, and fungal pre-digestion. Some derived benefits are the efficient synthesis of low-toxicity products, bioenergy, and animal feed, as well as being accessible methods for controlling the sources of environmental contamination.

Composting Composting is a process of aerobic degradation of organic materials. It is a natural process that can be accelerated with little human intervention (FAO 2007). It is important to note that the effectiveness of composting is dependent on moisture, nitrogen source, aeration rate, as well as the particle size of the biomass (Masters and Ela 2014). Külcü and Yaldiz (2014) indicated that lignocellulose

residues are typically low in nitrogen, so mixing with excreta increases the degradation rate. Another alternative is mixing with green leaves and fruit residue. It should be considered that compact waste should be mixed with stiffer and lighter waste in order to facilitate aeration (Masters and Ela 2014). An appropriate mixture of residues ensures the nutritional richness of the substrate, this is achieved through optimization techniques through experimental designs of: (1) Taguchi (Ajmal et al. 2020) or (2) surface response (Sharma et al. 2018). Külcü and Yaldiz (2014) suggest determining the best residue mixture by comparing the area under the curve of the variation of temperature with respect to time, as a measure of the maturation of the compost. Oquab et al. (2014) estimate the degree of maturation from images during the different composting stages, analyzed by neural networks. The results achieved 99% accuracy (Xue et al. 2019).

Fungal Pre-Digestion It is a treatment that is gaining ground in agricultural solid waste treatment alternatives (Xie et al. 2017), especially in the mixed production field where plants are grown and animals are raised. Most of the crop residues are rich in lignocellulose. This structure, despite being rich in polysaccharides, can hardly be digested by animals, resulting in anti-nutritional effects. However, there is a wide variety of fungi capable of growing in lignocellulosic biomass, by exposing cellulose through the secretion of enzymes that break down these polysaccharides (Kirk and Farrell 1987). Among the fungi with the ability to grow in lignocellulosic media, there are fungi that can be consumed as food, which can be inoculated in a process known as solid-state fermentation. As a result of this process, a residue with a higher protein content and less difficulty to be digested by livestock is obtained. Under this criterion, fungal pre-digestion studies have been reported from residues of wheat straw, coffee pulp (caffeine free), corn rachis, tomato pomace (Cohen and Hadar 2001), nettle stalks (Xie et al. 2017), rice husk (Jaybhaye Maya and Bhalerao Satish 2015), among others.

Anaerobic Digestion In this process decomposition of organic materials occurs in an anaerobic environment, the substrates can be solid or liquid waste (Kosseva 2009). The rate of research in anaerobic digestion has not stopped increasing in recent years (Zhang et al. 2018a). This is mainly due to the production of biogas, which has energy potential to supply the needs of the agricultural area. The process is described in four stages: (1) hydrolysis, where the organic matter is broken down into sugars and amino acids; (2) acidogenesis, at this stage the fats/oils are broken down into fatty acids; (3) acetogenesis, where acetic acid is generated; and (4) methanogenesis, the final stage where methane is produced. Additionally, a digested residue is obtained that can be used as compost. Mechanical, thermal, chemical, or biological pretreatments are required to significantly improve the process (Ren et al. 2018). Pre-digestion either enzymatically or biologically has been shown to improve methane production yields by up to 60% (Liew et al. 2020; Zhao et al. 2019). The effectiveness of the process is evaluated in terms of reduction of total solids (TS), volatile solids (VS), volatile suspended solids (VSS), and chemical oxygen demand (COD), as well as through the methane yield (mL/gVS).

Artificial Wetlands Natural wetlands perform water storage and purification functions, as well as the processing of carbon, nitrogen, and other nutrients. Artificial wetlands are created ecosystems, which have the capacity to treat wastewater with a high organic load originating from food, petrochemical, textile, tanning, paper, and gray water industries through their aquatic vegetation (Vymazal 2014). The wastewater processed under this technology can be released into the environment or used for irrigation (Maiga et al. 2017). This technology requires large tracts of land that are adapted based on waterproof tarps, gravel intercalated layers, soil and/or sand, and water. Aquatic plants emerge from the gravel and their roots extend throughout the soil/sand layer. The organic matter in the water decomposes as it passes through the layers due to the action of microorganisms in the ecosystem and deposition at the roots. Consequently, nutrients are released and incorporated into plant metabolism (Martina et al. 2011). The described design corresponds to a vertical flow artificial wetland (Paing et al. 2015). There are also horizontal flow and free surface flow designs. The choice of the appropriate design will depend mainly on the flow of water to be treated, the location, and the extent of available land (Wang et al. 2010). The advantages of technology include the construction of the facility with local materials, the use of native aquatic plants, and does not require chemical inputs. This technology can take advantage of aquatic plant species such as water hyacinth *Eichhornia crassipes* (considered a pest worldwide) for the treatment of wastewater (Ehrenfeld 2010). Pilot scale artificial wetlands with *Eichhornia crassipes* had achieved organic matter removal rates of 2500 mg/L/day (Rangel-Peraza et al. 2017). The application of this technology is limited due to its own disadvantages such as the extension of the terrain, forced inlet flow, and constant aeration with the aim of favoring aerobic environments (Liikanen et al. 2006).

11.3.1 Waste Management of Food Agro-Industries

Food industry waste is produced mainly at two levels: agricultural and industrial production. Agricultural waste considers material from crops, as well as livestock. The waste derived from the industrial production of food mainly consists of currents from the production of beverages, flours, meat, milk, and its derivatives (Kosseva 2009; Millati et al. 2019).

11.3.1.1 Agricultural Waste Treatments

Crop residues are all those obtained during maintenance and post-harvest. They mainly consist of pruning remains, rice husks, wheat straw, gophers (ear of corn), or even the plant when the product of interest extracts the plant from the soil (FAO 2002, 2019b). Most of them are composed of lignocellulose, varying slightly their composition with respect to the concentration of either cellulose, hemicellulose, or lignin (Millati et al. 2019).

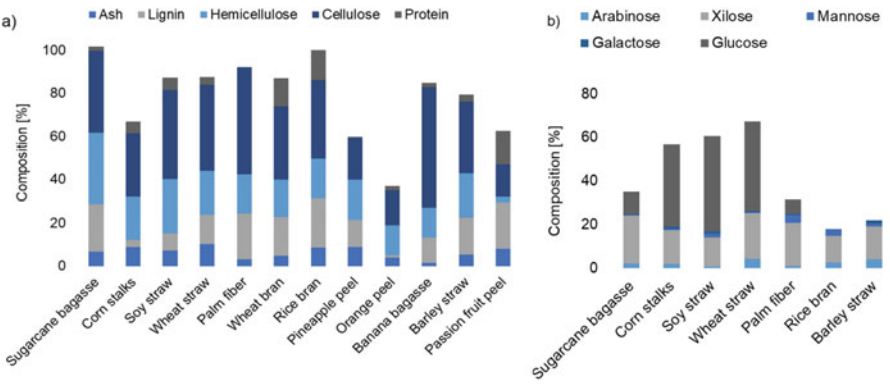

Fig. 11.3 (**a**) Composition of some agro-industrial waste. (**b**) Sugar content. Sugarcane bagasse: (Zhang et al. 2012); (Carroll and Somerville 2009); Corn stalks: (Detroy and Hesseltine 1978); Soy straw: (Detroy and Hesseltine 1978); wheat straw: (Detroy and Hesseltine 1978), (Carroll and Somerville 2009); palm fiber: (Mohammad et al. 2012), (Jafarpour et al. 2010); wheat bran: (Jafarpour et al. 2010); rice bran: (Jafarpour et al. 2010); pineapple peel: (Castro et al. 2011); orange peel: (Castro et al. 2011); banana bagasse: (Meneses et al. 2010); barley straw: (Olvera et al. 2012); passion fruit peel: (Olvera et al. 2012)

Characterization by means of chromatographic tests reveals the presence of various bioactive compounds in agro-industrial residues, such as phytosterols (1666.1–4654.7 ppm), which have hypocholesterolemic activity (Estiasih and Yunianta 2019), as well as phenolic compounds (gallic acid, catechins, carotenoids, caffeic acid, epicatechin, among others) (4.39–49.90 ppm) and tocopherols (14.37–330.80 ppm) in fruit and seed waste (Da Silva and Jorge 2017).

Availability and variety is an important determining factor. Thus, the sources of agro-industrial waste cover a wide range of materials and come in different forms. The forest industry, e.g. generates around 1.7 million tons of waste (Väisänen et al. 2016), which originate from the treatment and use of forest stands (Ginocchio et al. 2016) such as the production of Kraft paper (Grudinin et al. 1984). Agriculture generates considerable amounts of waste. It is estimated that crop residues (leaves and stems) represent 60% of the total, and the rest comes from process waste (seeds, spent pulp, among others) (Beltrán-Ramírez et al. 2019).

The composition of agro-industrial waste is varied, depending on the nature and the process in which it is generated. Figure 11.3 summarizes the compositions of various agro-industrial residuals of plant origin presented.

Biotechnological uses in agro-industrial waste are closely related to their availability and characterization. Four different biotechnological uses have been identified: (1) As a substrate for the generation of bioenergetics, (2) as soil improvers or conditioners obtained by composting, (3) for obtaining food supplements for animals, and to a lesser extent (4) as a substrate for the fermentative production of metabolites of interest.

Regarding the treatment that these residues receive, although burning is the fastest and cheapest method, it can present fire risks, in addition to not being an

option considered by environmental regulations. Furthermore, it has been shown that it negatively affects soil fertility contrary to the beliefs of some farmers. This is due to the fact that the combustion of the residues causes losses of carbon and nitrogen, as well as a negative effect on the microbial population of the soils (Shyamsundar 2019; Zhang et al. 2011).

The waste obtained from the food industry is characterized by being waste of known composition and whose treatment by the generator is usually mandatory in the legislation of almost all countries. The viable biotechnological alternatives for the treatment of these residuals are composting, which returns much of its nutritional richness to the soil; anaerobic digestion, which breaks down waste and generates a combustible gas; fungal pre-digestion, which adapts the waste to be food for farm animals; and the treatment in wetlands, which reduces the organic load of the wastewater, generating biomass in the process.

The pretreatments applied prior to the biological treatment must be simple and low-cost. Thus, chemical pretreatments are based on the solubilization and degradability of the essential components (Pellera and Gidarakos 2018) and the extraction of sugars from agricultural waste, have been the objectives to which these processes are oriented (Chosdu et al. 1993). Numerous acidic or basic reagents and solvents are used (Procentese et al. 2018) to change the structure of lignocellulosic fiber and improve the enzymatic saccharification of cellulose to polysaccharides (Sarip et al. 2016). The application of physical pretreatments, such as mechanical fractionation (centrifugal mill) prior to enzymatic hydrolysis, also show potential efficiency as saccharification methods with yields ranging from 270 mg/g to obtain cellulases to 380 mg/g to obtain xylanases (Licari et al. 2016).

In Brazil, the world's second largest soybean producer, approximately 41 million tons of waste are generated per year (Martins et al. 2018). These wastes are usually destined for the manufacture of livestock feed (Bordonal et al. 2018).

11.3.1.2 Waste from Meat, Poultry, and Fish Processing

Meat industry wastes include feces, urine, blood, grease, carcass washes, floors and utensils, undigested animal feed prior to slaughter, sewage from cooking, curing and pickling meat, and meat condensate. Wastes from the meat industry include feces, urine, blood, fat, carcass washing, floors, utensils, undigested animal feed before slaughter, wastewater from cooking, curing, pickling, meat condensate, offal, and other by-products. In the case of waste from the fish and seafood processing industry, the waste includes meat, shell, bone, cartilage, viscera (Kroyer 1995), as well as process waters.

It is often possible to make significant reductions in the contaminant load by cleaning within the factory or by modifying processes, in particular by separately recovering blood and fat. Most solids can be removed and recovered using advanced biological filtration techniques such as biotrickling filters (Schiavon et al. 2016). However, in some cases even after filtration, the water may contain protein and oil in sufficient quantities to make recovery worthwhile and at the same time to avoid environmental contamination. Waste management alternatives, including dry

cleaning, water conservation, water recycling, and by-product recovery, are effective in reducing environmental burdens.

Slaughterhouse solid waste (SSW) is part of the meat industry waste, which is generated during slaughter. It is the portion of the slaughtered animal that cannot be eaten as meat or used in meat products. Such wastes include bones, tendons, skin, the contents of the gastrointestinal tract, blood, and internal organs (Jayathilakan et al. 2012).

Efficient meat waste management results in economic gains for meat processors. However, its use and elimination are difficult, due to its biological instability, potentially pathogenic nature, high water content, potential for rapid auto-oxidation, and high level of enzymatic activity. Furthermore, SSWs are combined with liquid effluents (LE), mainly blood (Handous et al. 2019). These wastes are rich in protein and fat, the carbon-nitrogen ratio is around 14.4 (Pages Diaz et al. 2011).

Treatments for this type of waste include from filtration processes with hydrophobic or hydrophilic membranes (Mostafa et al. 2017), to separate and reuse organic components, to biological processes of aerobic digestion with enzymes (generally proteases and lipases) (Venugopal 2016), and anaerobic with native inoculant (Malayil et al. 2019) (Fig. 11.4). Digestion carried out by biological agents (microorganisms and enzymes) are versatile when selecting a treatment for these wastes (Yu et al. 2020). Nowadays, the biological digestion processes of these wastes are carried out in two phases, which begins with solid-state fermentation (SSF), with a mixture of SSW, which are generally particles of tissue and bone (Arvanitoyannis and Ladas 2008), with liquid effluents (blood and wash water) (Banks and Wang 2005), in a ratio of 75% SSW with 25% LE, this method attenuates the inhibitory effect of some intermediate products produced, reduces the limitation of the availability of substrates, and improves the bioconversion of the organic fraction of slaughterhouse waste to methane (Handous et al. 2019). However, the processes that use protease and lipase enzymes for the hydrolysis of waste are promising, since they allow recovering a large part of the amino acids present in the structure of the meat fiber present in the effluents (Prasad and Murugadas 2019).

11.3.2 Waste Management of Non-Food Agro-Industries

Non-food agro-industries have a high impact on the development and economic growth of nations. Among the activities that stand out in this sector are those related to tobacco, textiles, paper, rubber, and leather products. The processing and handling operations of these raw materials generate waste streams that require a non-traditional approach to their management. Methods of physical, chemical, biological reprocessing, and combinations thereof are continuously investigated for recycling and final disposal. The selection of the method to be used is conditioned by multiple factors, which include technological and economic criteria, however, the chemical composition and physical characteristics of the residual will be decisive (Demirbas 2011).

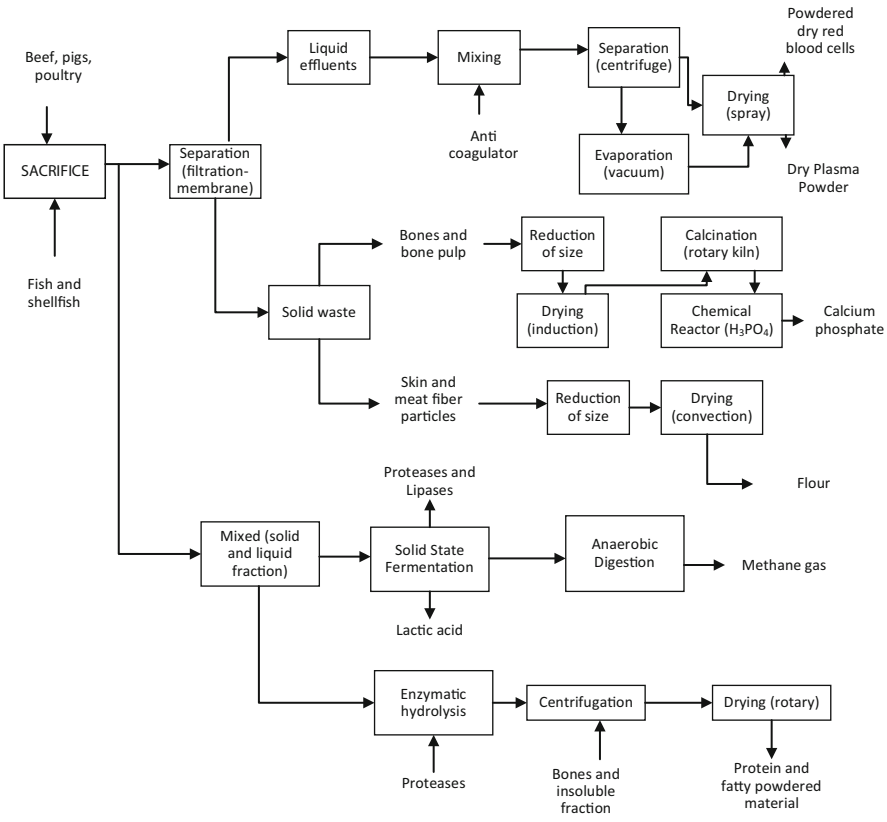

Fig. 11.4 Operations used for the management of waste from the meat industry, products obtained from processes

11.3.2.1 Tobacco

In some countries, the so-called energy tobacco is planted to produce renewable biomass for energy generation by anaerobic digestion (AD) (González-González et al. 2013). Another alternative includes oil extraction from its seeds to produce biofuel (Giannelos et al. 2002) and the resulting cake, in the case of tobacco seeds, has qualities for use as livestock feed (Carvalho et al. 2019; Pesevski et al. 2010). Figure 11.5 presents a diagram of the agro-industrial transformations of tobacco.

The traditional cultivation of the tobacco plant satisfies the tobacco industry's demand for leaves. The stalk is the main waste associated with the harvest (Zhang et al. 2020) (Tuzzin et al. 2016). These wastes are commonly dumped in landfills, incinerated, or piled in the field (Ye et al. 2019). However, tobacco stalk is an excellent microbial culture medium for solid and liquid fermentation. It was possible to produce a bio-organic fertilizer from strains of *Bacillus subtilis, Bacillus mucilaginosus*, and *Paecilomyces lilacinus* (Dai et al. 2020) and synthesize bacterial

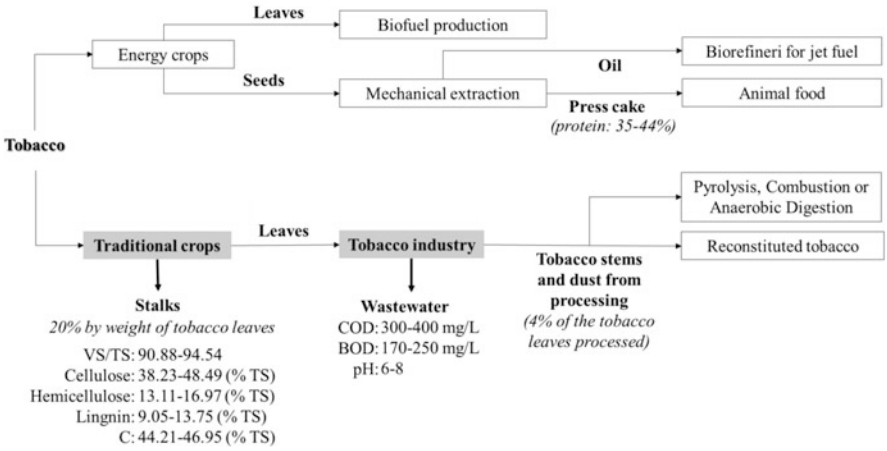

Fig. 11.5 The main sources of waste in the tobacco agro-industry (*VS* volatile solid; *TS* total solid; *COD* chemical oxygen demand; *BOD* biological oxygen demand). (Belan et al. 2019; Carvalho et al. 2019; Zhu et al. 2019)

cellulose using the residual extractor as a low-cost carbon source in the fermentation by bacteria such as *Gluconacetobacter*, *Acetobacter*, *Agrobacterium*, and *Rhizobium* (Ye et al. 2019). These processes use the solid or liquid fraction of the pretreatment of the residual, so they generally have previous stages that include (1) drying, grinding, and sieving and (2) extraction processes. The high VS/TS ratio of these residuals (Fig. 11.4) is a desirable feature in the AD process; however, studies reveal that the expected methane yields for these residuals are relatively low (Liu et al. 2015) (Belan et al. 2019). They are a lignocellulosic residue, aggravated by the high content of nicotine: a bacteriotoxic chemical component that can inhibit the metabolism of methanogenic arches and reduce methane yield.

In the manufacture of tobacco, leaf remains are discarded (tobacco steam or leaves midribs and dust from processing), which are used for the production of energy by thermochemical processes (pyrolysis and co-combustion) (Cong et al. 2019; Wang et al. 2018a; Wu et al. 2015; Zhang et al. 2013; Zhang et al. 2018b) and in the production of reconstituted tobacco leaves. Given the biodegradable nature of wastewater (BOD/COD > 0.5), these waters are potential candidates for biological treatments (Zhang et al. 2014).

11.3.2.2 Leather

In the agro-industry for the processing of leather materials, 80–85% of the raw material that enters the process is converted into residuals whose COD levels reach 875 mg/L and a biodegradable fraction that ranges from 35% to 42%. The residuals derived from leather processing are generated in the different processes: beamhouse

80%; tanning, 19; finishing 1%. These residuals are made up of meats, skin trimmings, and hairs 50–72%; chromium derivatives 35–40% (Kanagaraj et al. 2006; Kumar et al. 2017; Ozgunay et al. 2007). The composition of these residuals will depend on the process from which they come, as well as the origin of the skin. Ozgunay et al. (2007) characterized the sheep, goat, and bovine hides residuals in clothing and shoe industries. This industry generates liquid residuals with polluting potential, Chung et al. (2013) report biological oxygen demand values (BOD) (1439–1914 mg/L), DQO (4379–7124 mg/L), Cr^{3+} (21.127–63.299 mg/L). Eman and Eman (2013) studied the content of metals in tannery waters of leather: Sr^{+2} (320.3±5.1 ppm), Cr^{+6} (154±2.5 ppm), Fe^{+3} (41.7±1.3 ppm), Pb^{+2} (35.8±1.4 ppm), Cu^{+2} (9.7±0.2 ppm), and Mn^{+2} (3.0±0.01 ppm).

Anaerobic digestion of leather residuals has been investigated in order to take advantage of the biodegradable fraction (mainly collagen) whether they have been processed in the tannery or not. Part of the research related to anaerobic digestion is oriented towards obtaining biogas, in some cases with satisfactory results with yields of up to 189 mL/gVS (Priebe et al. 2016) or conversely low yields of 18.8 mL/gVS (Caramiello et al. 2013). Pehlivanoglu-Mantas and Ozturk (2012) evaluated the reduction of VSS (>25%), TOC (>30%) and identified that including pretreatments to leather residuals influences the amount of digested organic matter. Priebe et al. (2016) indicate that the inoculum is a determining factor in the degradation of leather residuals. Ferreira et al. (2010) tested the influence of an acid removal stage, in which it was identified that pretreatment positively influences the biodegradability of solid and liquid residuals while allowing the recovery of up to 50% of chromium.

Pehlivanoglu-Mantas and Ozturk (2012) compared anaerobic digestion of leather residuals against aerobic treatment, in which the reduction of VSS (15%) and total organic carbon (TOC) (50%) was observed. Other investigations reported satisfactory values for the aerobic treatments of the residuals of the leather as the reduction of the COD over 40% from the use of *Thiobacillus ferrooxidans* (Mandal et al. 2010), as well as microorganisms isolated from urban wastewater (Mandal et al. 2012). The pollutant load can be minimized up to 60% by implementing a Fenton reaction as a chemical pretreatment (Mandal et al. 2010, 2012). Factors influencing COD reduction in the process include temperature, pH, and the type of microorganism. Other treatments are used for metal reduction in leather wastewater, specifically Pb^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Cr^{6+}, and Sr^{2+}, by means of *Aspergillus candidus*, *Aspergillus carneus*, *Aspergillus flavipes*, *Aspergillus flavus var columnaris*, *Aspergillus unguis*, *Cephalosporium curtipes*, and *Cylindrophora hoffmannii* (Eman and Eman 2013).

11.3.2.3 Rubber

Gabrian et al. (2019) mention that the treatment of solid rubber residuals is expensive, due to transportation, the added cost to the operation and its low biodegradability, so efforts are directed at integrating them into the development of new materials. Hashem et al. (2019) reported that the elemental composition of the rubber

residuals, this included a high content of carbon (56.43%) and hydrogen (8.23%), low moisture content (4.8%), and high caloric value (8600 kcal/kg), therefore its use as fuel. Likewise, it reported the composition of the oxides of the residuals of the combustion of rubber: SiO_2 (22.7%), Fe_2O_3 (0.51%), Al_2O_3 (5.57%), CaO (33.8), among others, which enhances its use as raw material for cement production (Manoharan et al. 2019; Ullah et al. 2020; Youssf et al. 2019).

In the gaseous emissions of the different stages of the rubber footwear production industry in China, 68 volatile organic compounds (VOCs) have been identified, classified as alkanes, alkenes, acetylene, aromatics, halocarbons, carbon disulfide, and oxygenated compounds (OVOCs), in concentrations that reach 170 mg/m^3 (Li et al. 2019). Among the alternatives for the decrease of VOCs, the literature mentions the biotrickling filters (BTFs) (Baltzis et al. 2001; Li et al. 2008; Li et al. 2012; Luvsanjamba et al. 2007; Mpanias and Baltzis 1998; Sun et al. 2004). BTF is a technology that uses packed columns with countercurrent liquid and gas flows, in which microorganisms are inoculated into the liquid stream for the formation of biofilm (Baltzis et al. 2001; Luvsanjamba et al. 2007; Mpanias and Baltzis 1998). Bio-packing layers are also used as a solid support for microorganisms (Alptekin et al. 2012; Li et al. 2008). It is possible to describe the kinetics of degradation of VOCs and the profile of such degradation with respect to the thickness of the biofilm to determine the effective biofilm thickness (δ), the thickness in which the maximum possible degradation is achieved. Increases in the thickness due to the accumulation of biomass can cause a decrease in the mass transfer area of the material, and the system pressure drop (Baltzis et al. 2001). Luvsanjamba et al. (2007) determined that the pressure drop at 25 °C is 10 times greater than at 52 °C, due to the accumulation of biomass, so it recommends the operation of BTFs in thermophilic temperature regimes to increase the efficiency of the process.

11.3.2.4 Paper

The productive processes of the paper agro-industry as well as the waste streams generated have been studied by various authors, including (Pokhrel and Viraraghavan 2004; Rintala and Puhakka 1994). Figure 11.6 relates the productive processes and residual currents. Vymazal (2014) specified that some components of wastewater from the paper industry are characteristic of wood (tannins, resin acids, stilbenes, and lignin), other compounds are formed during the industrial process (chlorinated lignins, resin acids and phenols, dioxins, and furans). Pokhrel and Viraraghavan (2004) mention that the composition of the waters will depend on the type of wood, as well as on the industrial processes, which is why several biological alternatives have been studied for the treatment of wastewater from paper and highlights processes of activated sludge, aerobic stabilization, aerobic reactors, and anaerobic digestion.

Table 11.2 summarizes the BOD and COD reduction values presented by (Pokhrel and Viraraghavan 2004; Rintala and Puhakka 1994) for various biological

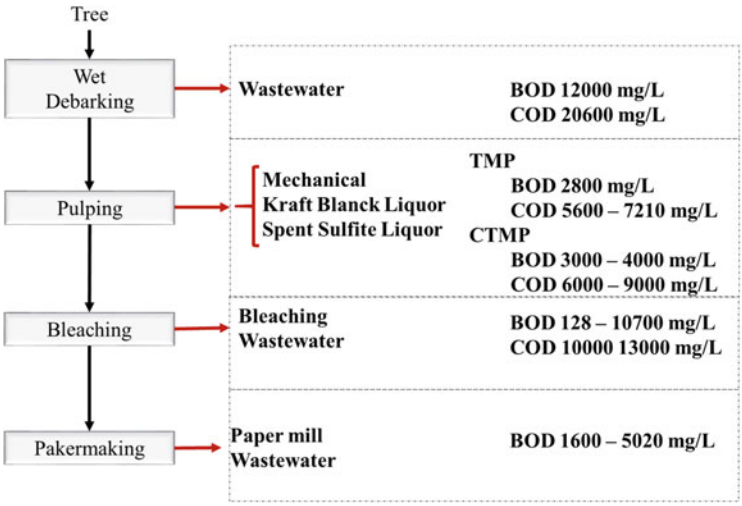

Fig. 11.6 Main wastewaters sources and compositions

Table 11.2 Paper wastewater biological treatment

	Removal (%)			Removal (%)	
Aerobic treatments	BOD	COD	Anaerobic treatments	BOD	COD
Activated sludge			Contact reactor		
Paper mill	94.2	82.4	TMP, groundwood, deink	71	67
Pulp mill	93.8	57.1	Wastepaper alkaline cooked straw	94	66
Kraft mill	98	70	Sulfite effluent condensate	97	85
Pulp and paper mill	96.63	96.8	CTMP	96	77
Paper mill	99	85	CTMP	50	40
Aerobic stabilization			Upflow anaerobic sludge blanket		
Kraft mill	98	73	Tissue	75	60
Kraft mill	–	20–65	Wastepaper	80	80
Biological reactors			Sulfite condensate	90	80
TMP mill	98	79	TMP/CTMP	60	50
MBBR	65–75	85–95	NSSC	80	55
Total plant	99	86	Bleached TMP/CTMP	75	60
SBR	98	85–93	NSSC/CTMP	80	55
Anaerobic (GAC)	–	50	CTMP	85	70
Kraft mill Windsor	69	59	Paperboard	83.3	72.2

CTMP chemithermomechanical pulping, *GAC* granular activated carbon, *MBBR* moving bed biofilm reactor, *NSSC* neutral sulfite semichemical, *TMP* thermomechanical pulping, *SBR* sequencing batch reactor

treatments applied to liquid and solid waste from the paper industry. Pokhrel and Viraraghavan (2004) indicated that aerobic or anaerobic treatments can be an alternative for the reduction of COD and BOD. In addition, physical and chemical

methods can be used as a pretreatment for the reduction of toxic agents, which increases the efficiency of biological processes.

11.4 Enzymes for the Degradation of Pollutants

Industrial development has led to the release of large amounts of highly toxic residual chemical compounds with limited possibility of biodegradation, to natural ecosystems (Dietz et al. 2000), which in some cases has determined its accumulation in the food chain (Krauss et al. 2010). Synthetic dyes are widely used in the textile, paper, cosmetic, fur dyeing, photographic, pharmaceutical, and food industries. Compared to natural dyes, they have a greater variety of colors, firmness, and moderate costs. Depending on the type of dye, it is estimated that 2–50% of these compounds are disposed of in wastewater (Ren et al. 2013) and are considered as persistent pollutants that cannot be removed with conventional water treatment methods (Álvarez et al. 2013), due to the structural complexity they present (Dias et al. 2007). The types of dyes are classified into two main groups; according to its chemical structures and application methods (El Harfi and El Harfi 2017), however, the classification as organic and inorganic dyes and pigments is also used (Gürses et al. 2016).

Treatment of Colored Residuals Traditional physicochemical treatments such as filtration and ion exchange are widely used to treat these contaminants. Currently, adsorption with inorganic supports such as bentonites functionalized by silylation is also included (de Queiroga et al. 2019), treatments with organic supports (Meili et al. 2019), coagulation by chemical reaction or more advanced processes with the application of electric charge (Keyikoglu et al. 2019).

Biological decoloration (aerobic or anaerobic) is another technique used in the treatment of effluents contaminated with dyes and others with similar chemical structures. These biological methods include mechanisms such as biodegradation, generally by oxide reduction (Singh and Gupta 2020) and cell biosorption, which although to a lesser extent plays an important role in the elimination of dyes (Sari and Simarani 2019). Physicochemical alternatives for waste treatment with high dye load have managed to separate these compounds from the effluents with relative efficiency. However, the pollutant only changes matrix or a new colored residual is generated, as in the case of adsorption processes. By contrast, biotech solutions can offer complete dye destruction, with a co-reduction of BOD and COD. However, these two mechanisms can work together and in a complementary way, since white rot fungi are capable of growing in agricultural residues from a previous dye adsorption process (Merino-Restrepo et al. 2020) (Fig. 11.7).

Basidiomycete Fungi for the Treatment of Colored Residuals Basidiomycetes belong to the Basidiomycota division (Lücking et al. 2017). A characteristic that distinguishes them is the production of spores of sexual origin (basidiospores)

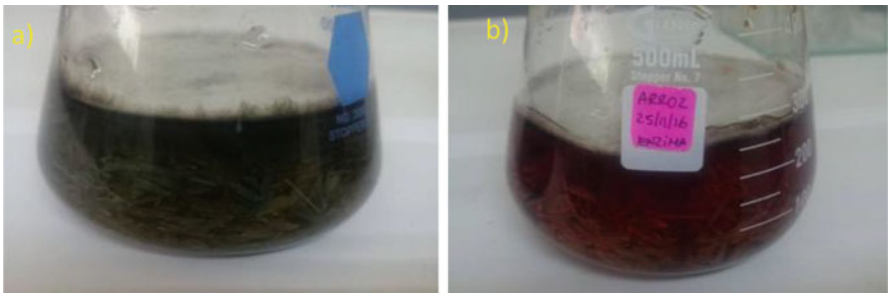

Fig. 11.7 Auricularia auricula growth (Aa-UTM-0332) (white rot fungus), in aqueous medium with dyes: (**a**) blue textile A19 and (**b**) red food R195, on rice husk as a solid support

outside a specialized structure, which is called basidium (Coelho et al. 2017). Basidiomycetes have been classified into two large groups according to the visual pattern of degradation they produce on lignocellulosic material: Fungi of brown rot and fungi of white rot, whose name comes from the brown or whitish appearance of wood after being attacked for these fungi.

Filamentous fungi and basidiomycetes have been extensively studied for the degradation of various persistent organic compounds, including industrial dyes. Of the latter, those that have shown greater efficiency in the degradation of industrial dyes are the white rot basidiomycete fungi (Erkurt et al. 2007), (Asgher et al. 2009).

The ability of white rot fungi to degrade xenobiotic organic pollutants, such as organophosphate herbicides (Gouma et al. 2019), polycyclic aromatic hydrocarbons such as phenanthrene (Harry-asobara and Kamei 2019), explosives like RDX (1,3,5-trinitro-1,3,5-triazine) (Isyaku and Otaiku 2019), and other recalcitrant contaminants such as azo dyes (Patel et al. 2020), is attributed to extracellular lignolytic enzymes, among the most important: manganese peroxidase (MnP), laccase (Lac), and lignin peroxidase (LiP) (Mori and Hirai 2019). Enzymes are synthesized in the metabolism of these fungi in order to transform and transport nutrients to the hyphal apex (Pandya et al. 2020). Since the synthesis of these enzymes is subject to regulatory mechanisms (Periasamy et al. 2019), these are not produced constantly, but rather induced by the appropriate substrate and nutrients. In the particular case of dyes, they can act as an inducer of enzymatic activity, such as carminic acid for *Pycnoporus sanguineus* (Hernández et al. 2017).

Lignolytic Enzymes for Dye Degradation The lignolytic enzymes involved in the degradative processes of phenolic compounds include different types of laccase, peroxidases, and oxidases (Qin et al. 2018). The simultaneous presence of these enzymes is not usual in basidiomycetes and they depend on each species (Elisashvili et al. 2017). Both laccases and peroxidases are capable of catalyzing the oxidation of an electron from the aromatic units of lignin, resulting in various non-enzymatic reactions (Fig. 11.8). Lignin peroxidase (LiP) is a monomeric glycoprotein with two calcium ions, contains a heme group (iron-protoporphyrin IX) as a prosthetic group, is not very specific, and has the highest redox potential, being able to directly

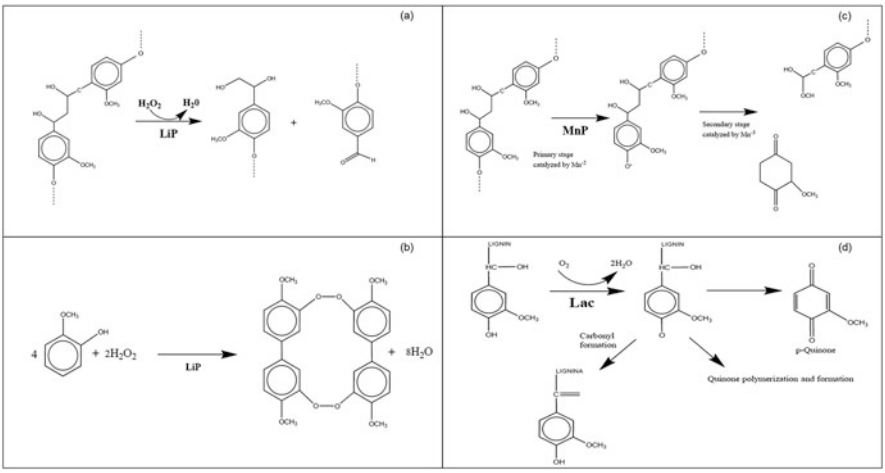

Fig. 11.8 (**a**) Lignin oxidation (non-phenolic structure) by the action of LiP; (**b**) Guaiacol oxidation (phenolic structure) by the action of the enzyme lignin peroxidase; (**c**) Lignin degradation by MnP action, in the presence of Mn^{+2}; (**d**) oxidation of lignin subunits by action of the Lac enzyme

catalyze the oxidation of components non-phenolic in the presence of H_2O_2. Manganese peroxidase (MnP) is a glycoprotein that contains a heme group (iron-protoporphyrin IX), this enzyme requires the presence of H_2O_2 as a co-substrate and the presence of Mn^{2+} (an element naturally present in wood) and catalyzes the oxidation from Mn^{2+} to Mn^{3+} (Ufot et al. 2016). The use of oxygen (non-limiting electron acceptor) by laccase enzymes (Lac) (benzenediol: oxygen oxidoreductase) makes these most suitable for environmental and industrial applications (Chauhan et al. 2017) and (Lettera et al. 2016). The latter are extracellular glycoproteins that generally contain four copper atoms in their active center, but other enzymes have been described with two, three, and up to six atoms of this element. (Dasgupta et al. 2020). Table 11.3 shows some important characteristics of these three lignolytic enzymes.

Strategies to Produce Lignolytic Enzymes Enzyme production is a growing field of biotechnology. Most manufacturers produce enzymes using either submerged fermentation or liquid fermentation (SmF) techniques. However, in recent decades there has been an increasing trend towards the use of the solid-state fermentation technique (SSF) to produce various enzymes of environmental interest.

The SmF strategy has been widely used for the production of enzymes of industrial interest, including the laccase enzyme from white rot fungi (Vantamuri et al. 2019), (Mishra and Kumar 2020). In the SmF system, the process conditions differ from the natural growth characteristics of the basidiomycete fungi (on a solid support); however, the use of a support in which the fungal cells are immobilized,

Table 11.3 Characteristics of ligninolytic enzymes

Enzyme	*T* (°C)	pH	Atomic mass (kDa)	Preferred substrates	Action mode
LiP (EC.1.11.1.14) (diarylpropane: oxygen, H_2O_2, oxidoreductase)	35–38[a]	3.2–4.7[b]	38–47[c]	Non-phenolic aromatics phenolic aromatics (selectively)[d,e]	Subtraction of an electron from the aromatic ring[f]
MnP (EC.1.11.1.13) (Mn (II): H_2O_2 oxidoreductase)	45–55[g]	4.0–5.0[b]	32–62[c]	Non-phenolic aromatics[h]	Catalyzes the oxidation of Mn^{2+} to Mn^{3+}
Lac (EC. 1.10.3.2) (benzenodiol: oxygen oxidoreductase)	40–65	2.0–5.0[i]	50–70[j,k]	Derivatives of the depolymerization of lignin, aldehydes, aromatic acids, and hydroquinones[l]	Uses molecular oxygen as an oxidizing agent and oxidizes phenolic compounds to phenoxy radicals[m]

[a]Mehboob et al. (2011), [b]Periasamy et al. (2019), [c]Wesenberg et al. (2003), [d]Llevot et al. (2016), [e]Sun et al. (2013), [f]Mäkelä et al. (2017), [g]Asgher and Iqbal (2011), [h]Herzog et al. (2019), [i]Jeon et al. (2010), [j]Ravikumar et al. (2012), [k]Quaratino et al. (2007), [l]Bettin et al. (2019), [m]Torres-Farradá et al. (2018)

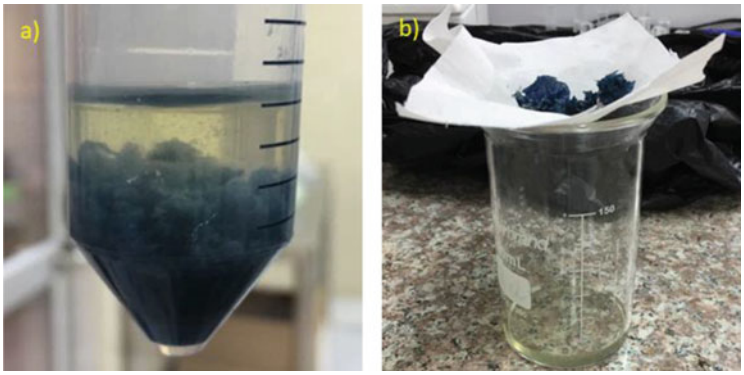

Fig. 11.9 SmF system: (**a**) Pellet formation during the growth of Pleurotus djamor (Pd318) in an enriched liquid medium in the presence of textile dye A19; (**b**) biomass generated after the degradation process

plus the control of the variables as available oxygen and access to nutrients, it ensures the proper growth of fungi (Ofongo et al. 2019).

Another problem in the SmF system is related to the fact that basidiomycete fungi during their reproduction form macro-structures called mycelium (Domingos et al. 2017), which agglomerate and form pellets (Fig. 11.9), this causes the system to become saturated and heterogeneous. This causes problems in the agitation process, so that the transfer of oxygen to the interior of the fungal cells that make up the pellets decreases.

Fig. 11.10 SSF system: (**a**) presence of mycelium during the growth of *Pleurotus djamor* (Pd318) in sugarcane bagasse; (**b**) oxidation of textile dye A19 in a solid medium

The SSF strategy has also been widely used in the production of lignolytic enzymes (Asgher et al. 2016), (Zervakis and Koutrotsios 2017). This system has the advantage that the cultivation conditions are similar to those of the growth of fungi in nature. In comparison, SSF is a simpler and lower cost technique than SmF. Also, the growth and expression rate of metabolites like enzymes is much lower (Díaz-Godínez et al. 2017), but the enzyme concentration obtained is much higher in relation to the cultures with "free" cells in a liquid medium (Rodriguez-Couto 2009). In the SSF system, the fungi are immobilized on a solid support (Fig. 11.10), which is generally the inducer for the expression of lignolytic enzymes (Sooch et al. 2019).

The behavior of the expression of lignolytic enzymes differs in the two systems (SmF and SSF), this is due to the phenomena that govern in a particular way in each one, such as oxygen transfer and physiological state of the fungus. Mass transfer processes are much faster in SmF systems compared to the SSF system, since in the latter there are oxygen transfer gradients (Soccol et al., 2017) (Ashok et al. 2017).

Access to nutrients is another variable to consider, since it is a determinant of enzyme activity, because in SSF and SmF the optimization parameters are notably different (Subramaniyam and Vimala 2012). For the SmF system, the morphology and size of the pellets obtained are determined by mechanical stress during cultivation, so optimization of the system requires a homogeneous distribution of their size Table 11.4 presents characteristics relevant to SSF and SmF.

Regardless of the strategy selected for obtaining lignolytic enzymes for environmental purposes, the process follows a sequence of generic steps that allow obtaining enzyme extracts at the laboratory level (Fig. 11.11), in order to assess the viability and subsequently scale the process to an industrial stage.

Immobilized fungal enzyme extracts (Oliveira et al. 2018) or free in the media (Díaz-Godínez et al. 2017) has the ability to reduce the intensity of the dyes present in industrial effluents by up to 60%, with fungal enzyme extracts obtained in approximately 8 days in a SSF system (Fig. 11.12). However, the use of lignolytic enzymes has a much wider spectrum of application in the recovery of the environment, they cannot only be applied to the degradation of industrial dyes. The

Table 11.4 Characteristics of SSF and SmF systems in the production of enzymes

Solid-state fermentation (SSF)	Fermentation in liquid state (SmF)
• Inert support (natural or artificial), which contains all the components for growth in the form of a solution	• The components used in the culture media are more expensive
• Organisms that require less water for growth such as filamentous fungi are preferred	• The media concentration is much lower compared to the water content
• Less chance of contamination due to low water availability	• Increased water activity becomes the main cause of contamination
• Small size bioreactors can be used	• Large-scale bioreactors are required because the media is highly diluted
• Less energy consumption for aeration and oxygen transfer	• High air pressure consumes more energy and there is little oxygen transfer
• The most important limiting factor in growth is the diffusion of nutrients	• Intensive mixing facilitates the diffusion of nutrients
• Greater complication to measure the amount of biomass generated and other online processes	• Online sensors are available, and sampling is easy for biomass measurement and other parameters
• Down-stream processing is easy, cheaper, and less time consuming	• The water content makes the down-stream process complex and very expensive
• No considerable liquid waste is produced	• A considerable amount of liquid waste is generated, making it difficult to dump

polyphenols present in effluents from the coffee industry have generated problems today and one of the emerging technologies evaluated has been the use of fungal enzymes that degrade caffeine or other components. (Gokulakrishnan et al. 2005). The strategy used to treat this type of environmental pollutants by means of lignolytic enzymes is similar to the process for the treatment of industrial dyes, however, the particularities of the process (times and efficiencies) depend on the characteristic of the residual to be treated (Fig. 11.13). Likewise, it should be considered that the process control parameters are specific to the composition of the medium; for example, in the treatment of residuals obtained from the coffee industry, the degradation rate of caffeine, the initial content of caffeine, the nitrogen source, and pH level are important (Gokulakrishnan et al. 2005).

Scaling Most scaling approaches for lignolytic enzyme production in SmF and SSF systems are based on process attributes such as oxygen transfer (Garcia-Ochoa and Gomez 2009), (Michelin et al. 2019), impeller tip speed and stirring effects (Keng et al. 2008), volumetric power input, mixing time, cutting speed, kinetics, Reynolds number, geometric similarity (Schmidt 2005). The scaling considerations also depend on the selected fermentation system (SSF or SmF), since the configurations and requirements in each system are notably different (Fig. 11.14).

Fermentation involves processes of mass, heat, and moment transfer (Doran 1995) (Casciatori et al. 2016). The methodology used for scaling the production process of lignolytic enzymes in particular does not depart from that applied for conventional processes such as protein production (Carta and Jungbauer 2020). It

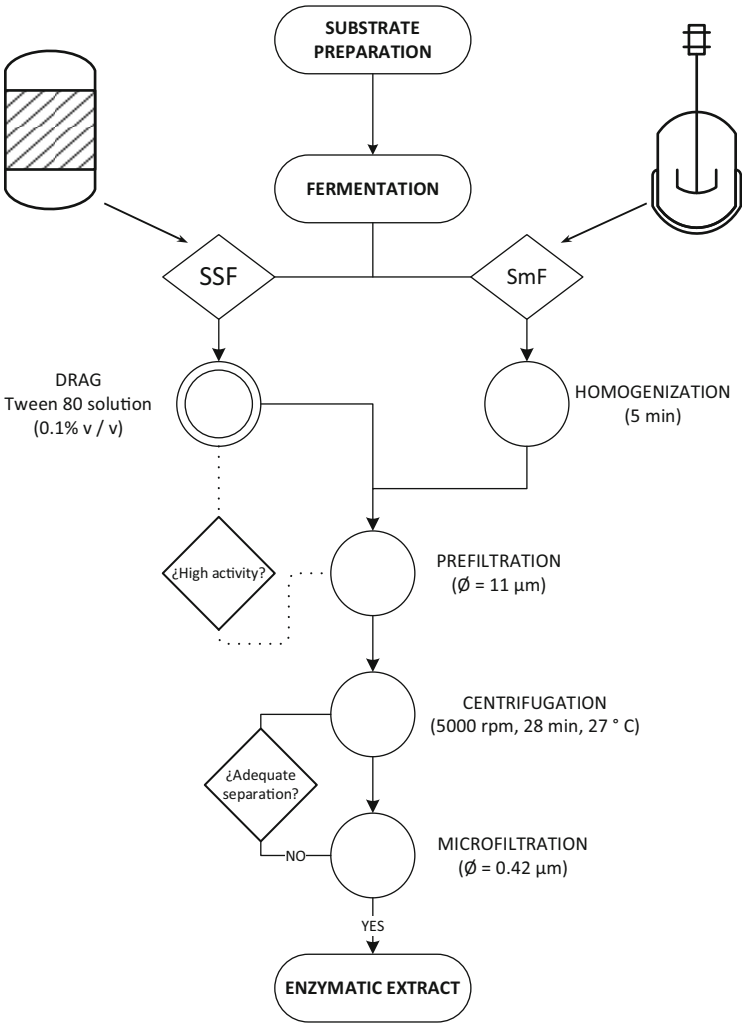

Fig. 11.11 Generic strategy for obtaining enzymatic extracts from basidiomycete fungi

should be borne in mind that there is a certain priority in the ordering of the strategy during its escalation. Some parameters and strategies that are used in the scaling for the production of enzymes are given by the mass transfer coefficients (Bhushan et al. 2019), the air flow supplied and the power consumption required for stirring the fluid or one of its equivalents (Bettin et al. 2019). On the other hand, geometric aspects must be considered, which influence the flow pattern to be used (Ismail et al. 2011). The general strategy most frequently used in the scaling of SmF and SSF processes for the production of lignolytic enzymes is reflected in Fig. 11.15.

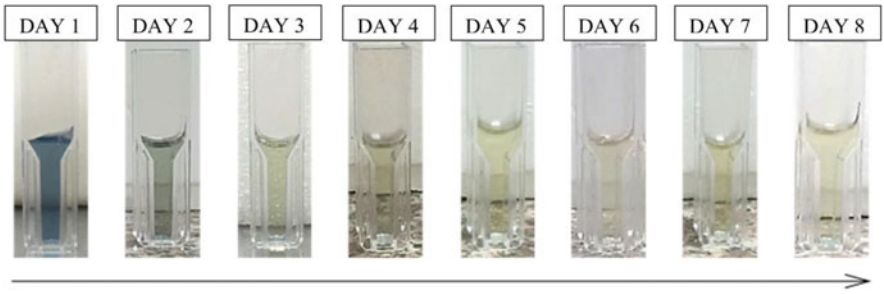

Fig. 11.12 Oxidation of dye A19 with fungal enzyme extracts obtained on different days from SSF of sugarcane bagasse with *Pleurotus djamor* (Pd318)

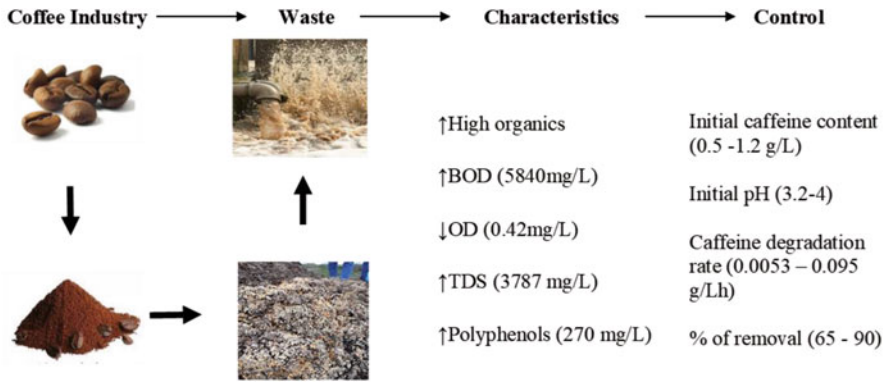

Fig. 11.13 Residuals in the soluble coffee industry, characteristics and control parameters for the degradation of phenolic compounds with lignolytic enzymes

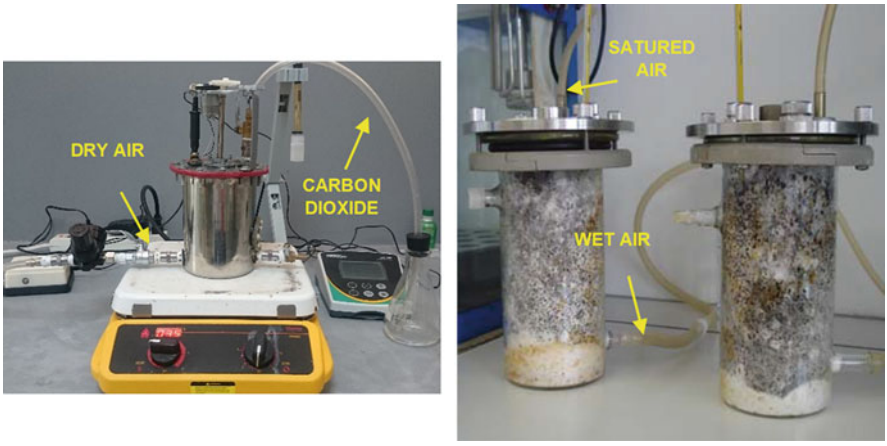

Fig. 11.14 Configuration of the oxygen supply in the pilot scale SmF and SSF systems

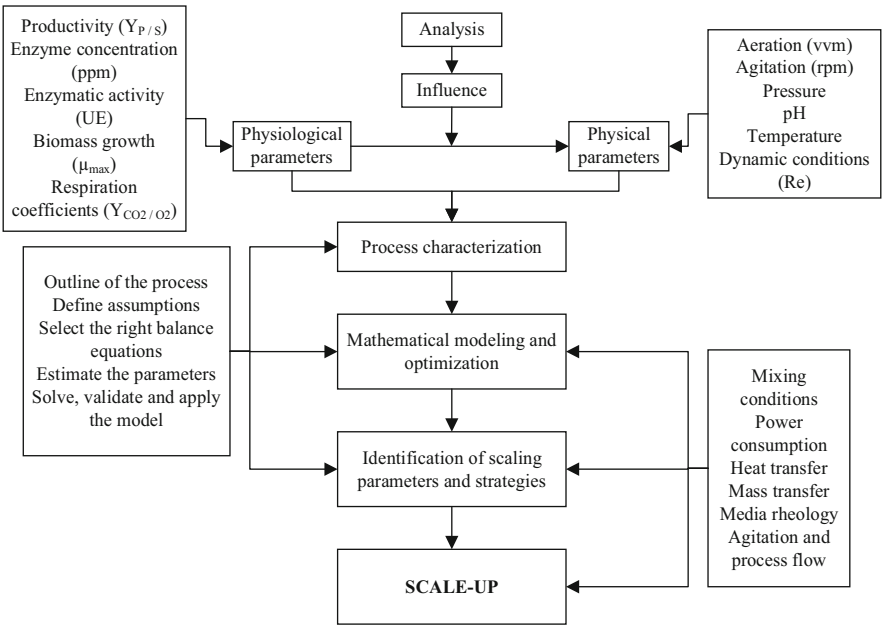

Fig. 11.15 General scaling strategy for the industrial production of lignolytic enzymes

Estimation of Treatment Costs Through SSF and SmF A relatively quick alternative to estimate the cost of enzyme production is from the costs of equipment supplies and energy (Silveira and Tuna 2003). With the data of the annual generation of wastewater from the plant, the quantity of enzymes to be produced is calculated and then the production cost ($C_{\text{production}}$) is estimated by means of the expression (Smith and Mobley 2008):

$$C_{\text{production}} = 1.03 \cdot f \cdot C_{\text{equipment}} + C_{\text{supplies}} + C_{\text{energy}} + C_{\text{maintenance}}$$

where

- f is a discount factor $\left(\frac{1}{\text{year}}\right)$. If the equipment is paid in cash $f = \frac{1}{k}$;
- $C_{\text{equipment}}$ is the equipment acquisition cost (agitated tanks, pumps, filtration units, among others) assuming a 3% maintenance cost (USD);
- C_{supplies} corresponds to the sum of the costs of chemical compounds, water, resins, disposable filters, among others $\left(\frac{\text{USD}}{\text{year}}\right)$;
- C_{energy} is the cost of electricity consumed by equipment and the cost of heating requirements $\left(\frac{\text{USD}}{\text{year}}\right)$;
- $C_{\text{maintenance}}$ corresponds to the cost of equipment maintenance $\left(\frac{\text{USD}}{\text{year}}\right)$. If equipment is new $C_{\text{maintenance}} = 0$.

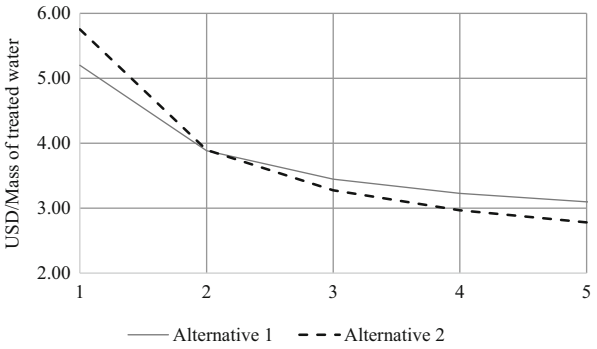

Fig. 11.16 Comparison of costs between treatment alternatives

The factor f is calculated using the following expressions:

$$f = \frac{q^k(q-1)}{q^k - 1}$$

$$q = 1 + \frac{r}{100}$$

where

k represents the time (years) that the equipment will be used in the process;

r is the interest rate (percentage);

q is capital value (dimensionless).

Once the costs are organized in a spreadsheet, the graphical comparison of alternatives can be performed to estimate which one is most convenient over time (Fig. 11.16). It is possible that one alternative is less costly than another in the year of technological acquisition due to the cost of the equipment, but over the years this cost is being cushioned and costs associated with energy or inputs take on greater importance.

11.5 Conclusion

The use of lignolytic enzymes for agro-industrial waste treatment has not yet been fully exploited. Currently, the use of basidiomycete fungi has become a very valuable tool in the bioremediation of contaminated environments, due to its ability to degrade xenobiotic compounds of diverse chemical structure, the utility and versatility they show when undergoing a variety of contaminating compounds such as dyes and alkaloids such as caffeine, demonstrate the potential of these metabolites of microbial origin. This is also due to the fact that most of the enzymatic extracts obtained by SSF or SmF are stable under the conditions of pH, temperature, dye concentration, and salinity characteristic of industrial effluents, which allows their use in the treatment of pollutants from different nature.

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Chapter 12
Biocoagulants as an Alternative for Water Treatment

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Abstract Issues related to water quality are associated with environmental problems caused by humans and industrial growth. In view of all these problems caused by water pollution and shortages, there has been a constant concern to search for new alternatives to existing conventional methods. The coagulation process using natural coagulants is an interesting alternative for the treatment of both surface water and urban and industrial wastewater. The coagulating capacity of several materials has been evaluated, with biocoagulants of vegetable origin being the most studied. Aspects related to the application and production of biocoagulants, from *Moringa oleifera*, in the treatment of surface and wastewaters (urban and industrial) are analyzed. The use of mixtures of synthetic coagulants with biocoagulants as coadjuvants is reported in the literature. However, a less studied case, but of great interest, is the mixture between biocoagulants, presenting the case study of mixtures between *Moringa oleifera* and *Caesalpinia spinosa*. The effects of biocoagulant production process variables, such as the addition of sodium chloride and the drying temperature on turbidity removal, are evaluated. The technical, economic, and environmental challenges in the implementation of biocoagulants in water treatment are analyzed. Aspects related to use of values are highlighted to identify the potentialities of biocoagulant production.

Keywords Natural coagulation · *Moringa oleífera* · *Caesalpinia spinosa* · Total economic value · Biocoagulant mixture

12.1 Introduction

With the rapid growth of population density, access to water becomes more necessary every day. The same must meet the quality parameters to be intended for human consumption and not compromise human health. On the one hand, the demand for water is compromised by environmental problems related to the degradation of water resources and, on the other hand, the pollution generated as a result of population and industrial growth. According to Ang and Mohammad (2020), water scarcity will affect more than 2 billion people, and the quality of drinking water will be compromised by the entry of almost 80% of untreated wastewater into waterways. Runoff, sewage, effluents without adequate treatment, sludge from landfills, among others are the main causes of contamination of surface waters. This contamination is related to color, odor, disinfection products (Caltran et al. 2020), heavy metals,

nanoparticles, derivatives of the oil industry (Suparmaniam et al. 2020), fertilizers, synthetic dyes, among others.

Given the problems caused by pollution and water scarcity, there has been a constant concern for the search for new alternatives to existing conventional methods. Which are classified into:

1. Physical methods (sedimentation, membrane, adsorption, and filtration) (Campos et al. 2018; Dong et al. 2009; Marjani et al. 2020).
2. Chemical methods (coagulation, advanced oxidative processes, electrochemicals, and catalytic disinfection) (do Nascimento et al. 2020; Dong et al. 2009; Rodriguez et al. 2020).
3. Biological methods (anaerobic/aerobic digestion, phytoremediation, and microbial biodegradation) (Chang et al. 2020; Pages-Diaz et al. 2018; Urbaniak et al. 2020).

Although there are great technological advances, mainly in physical and chemical methods, these present several disadvantages, among which stand out are high costs, skilled labor, generation of toxic waste, and the addition of toxic chemical agents.

The commonly used and even the most widely used method today, mainly in rural areas and less-developed countries, is coagulation followed by flocculation, sedimentation, and filtration. In fact, despite the new existing technologies, coagulation processes have never ceased to be in demand, being recognized as one of the simplest and most cost-effective processes for removing suspended impurities in drinking water and wastewater treatments. Coagulation is the process by which impurities that are in fine suspension (colloidal state or solution), such as bacteria, protozoa, and plankton, are transformed into larger particles (flocs) forming a precipitate due to the force of gravity. Coagulation is highly important, not only, in the elimination of colloidal particles and microorganisms attached to these particles (Calabkova et al. 2020) as well as in the removal of heavy metals and organic pollutants (Nkurunziza et al. 2009).

The synthetic coagulating agents are the most used, among them, aluminum salts, iron salts, and calcium hydroxide (Rodriguez et al. 2020; Taiwo et al. 2020). Despite the fact that synthetic coagulants perform with high efficiencies in water treatment, they generate high volumes of residual sludge at the end of the process. The sludge formed presents significant concentrations of the metals used as coagulants (Al and Fe) as well as other contaminants generated in the process. On the other hand, according to Rodriguez et al. (2020), synthetic coagulants contain very little by mass of coagulants, where 1 ton of aluminum sulfate octadecahydrate contains only 81 kg of Al (III), while 1 ton of iron (III) chloride contains 210 kg of Fe (III). Thus showing that, the chemical coagulation process, in addition to handling high volumes of sludge, must handle large amounts of chemical-contaminating agents. In addition, it has been established that exposure to heavy metals can cause a number of affectations to humans, such as anemias, kidney and neurological damage, gastrointestinal inflation (Missaoui et al. 2018; Sousa and Ribau Teixeira 2020, and Alzheimer's disease correlated to the presence of aluminum (Merwad 2018).

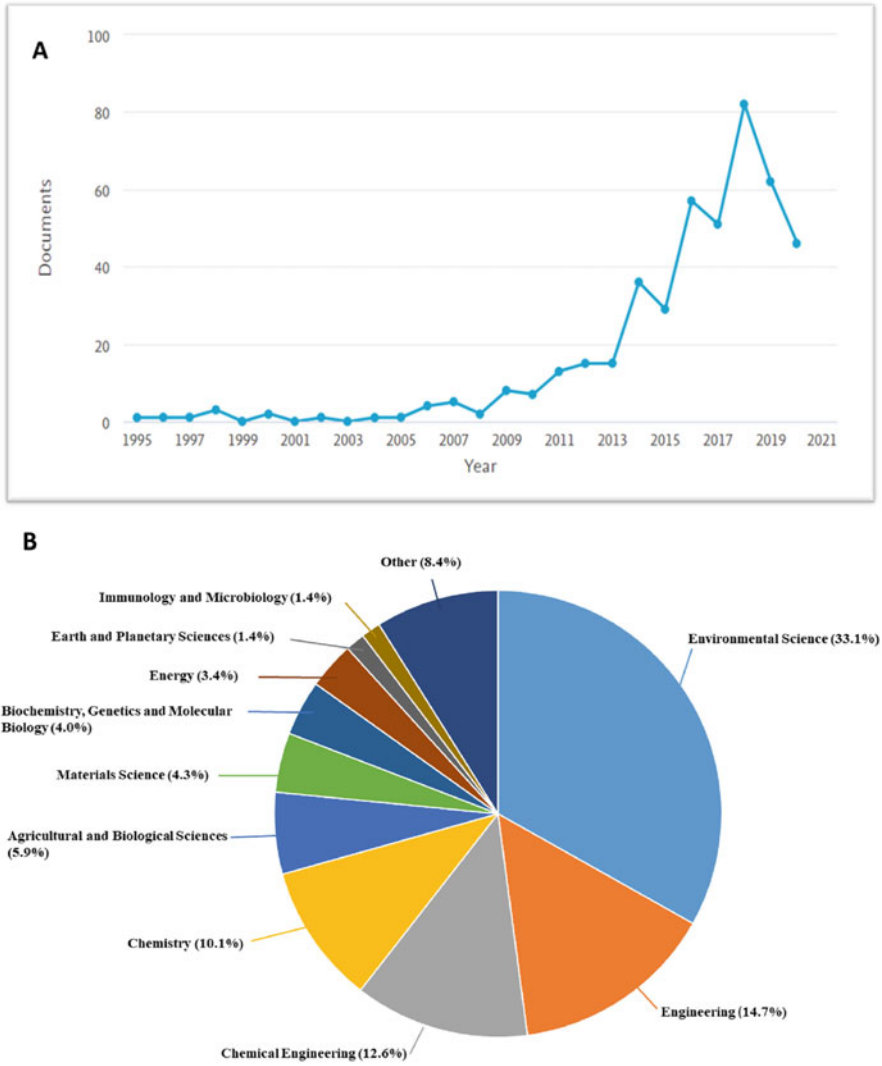

Fig. 12.1 Number of publications on the use of biocoagulants or natural coagulants per year (**a**) and per area (**b**) (source: Web of Science database with searching keywords: biocoagulants OR "natural coagulant" AND "water and wastewater treatment," May 2020)

Although the World Health Organization (WHO) registers the importance of coagulation in water treatment, it recommends several approaches to minimize the application of coagulants of chemical origin in order to avoid damage to the environment (Devesa-Rey et al. 2020). Given the high demand and interest in the application of coagulation processes and the known toxicity of synthetic coagulants, research has been carried out in search of new generations of coagulants.

For the sake of sustainable development and the application of green chemistry concepts, the most recent alternatives studied are coagulants of natural origin or

biocoagulants. Although studies of the use of biocoagulants have increased exponentially in recent years (Fig. 12.1a), they are insufficient in terms of the total number of articles per year. In this way, new lines of research are open in which coagulant properties are not only used in the field of environmental sciences but also other less-studied areas (Fig. 12.1b).

Biocoagulants are obtained from renewable raw materials (biomass) with the advantage of producing biodegradable and ecological sludge (Megersa et al. 2019). According to Ang and Mohammad (2020), biocoagulants can decrease the operational costs of water treatment, once they have been shown to reduce dissolved oxygen levels and organic load in aeration tanks.

There have been several biocoagulants used, including chitosans (Asif et al. 2015; Saleem and Bachmann 2019), bacteria (Xiong et al. 2010), fungi, as well as biocoagulants of animal and plant origin (Saleem and Bachmann 2019). Importance has been given to the use of biocoagulants obtained from residual biomass from agro-industrial processes. These alternatives promote sustainable production cycles by guaranteeing that the raw materials come from renewable resources, in addition to the fact that the residuals generated are biodegradable. These characteristics place natural coagulation as an interesting alternative for the treatment of both surface and urban and industrial wastewater.

12.2 Coagulation

Drinking water as well as wastewater contain a variety of organic and inorganic substances with colloidal structure (Gunaratna et al. 2007). Colloids are polluting particles of small diameter (less than 1μm) that are responsible for the turbidity or the color of the water. The problem of removing colloids present in water lies in its low sedimentation rate and the presence of electrical charges on its surface (Kim et al. 2001; Sincero and Sincero 2002). The formation of particle conglomerates is hampered by the repulsive negative charges that surround them. Consequently, they form a stable suspension (Choy et al. 2015); this makes coagulation an alternative to reduce pollutants in water by charge destabilization.

A coagulant is a substance that can destabilize the colloidal matter dispersed in an aqueous medium, in a process called coagulation (Cissouma et al. 2013; Gunaratna et al. 2007; Teh and Wu 2014). Figure 12.1 presents a simplified diagram of the coagulation–flocculation process.

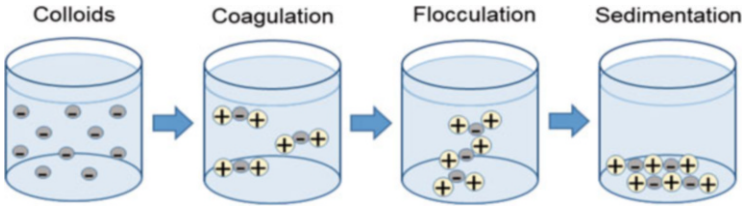

Fig. 12.2 Coagulation–flocculation process

Figure 12.2 shows that the colloidal particle suspension is destabilized on contact with a coagulating agent, due to the cancellation of surface charges. Colloidal neutralization will favor the aggregation of the particles; this will allow the growth of flocs (Jiang 2001; Kumar et al. 2017). As a consequence, these more voluminous and heavier particles can be separated from the water, either through sedimentation or filtration; for this reason, coagulation is one of the first steps in water treatment.

The coagulation–flocculation operation consists of two periods: (1) perikinetic coagulation and (2) orthokinetic flocculation. Perikinetic coagulation disperses the coagulating agent as quickly as possible, under a high-speed agitation regime, in order to destabilize the colloidal matter present in the water. Orthokinetic flocculation occurs under a slow agitation regime, in which the individual flocs collide, and due to the interactions between them, they unite to form larger and denser flocs. The optimization of this process has been of importance in improving water quality due to its ability to remove particles, organic matter, pathogenic microorganisms, inorganic ions, metals, and others (Teh and Wu 2014).

12.2.1 Coagulation Mechanisms

Coagulation of colloidal particles can occur through four mechanisms: (1) compression of the double layer, (2) sweep, (3) adsorption, and (4) charge neutralization, adsorption, and bridge between particles. Some of these mechanisms can occur simultaneously (Renault et al. 2009).

1. The coagulation mechanism through *compression of the double layer* considers that high concentrations of electrolyte in the solution give rise to high concentrations of counter ions in the diffuse layer. Therefore, the double layer surrounding the colloidal particle would compress to some extent where the repulsive energy barrier would also be reduced. This phenomenon will contribute to the binding of the molecules and subsequently the formation of flocs (Duan et al. 2009).
2. Coagulation by the *sweeping mechanism* can occur when a high concentration of the coagulant is added. This could act as a nucleation site to facilitate the formation of precipitation (Sincero and Sincero 2002). As the precipitate forms, the colloidal particles would become entangled in the growing precipitate and would therefore be removed from the colloidal suspension (Duan and Gregory 2003).
3. The *charge neutralization mechanism* involves adsorption of a coagulant with opposite charge on the colloidal surface. Positively charged coagulants are attracted to the colloids, thus neutralizing the surface charge. Colloid–solvent interactions can affect the ability of a coagulant to destabilize colloidal particles. The destabilization of the particles could occur due to a reversal of charges due to an excess of counter ions (Saleem and Bachmann 2019).

4. The *particle bridge mechanism* explains the ability of high molecular weight polymers to destabilize colloidal dispersions. For effective destabilization, the polymer must contain chemical groups in its chains that can interact with the colloidal particles to form a particle–polymer–particle complex, in which the polymer acts as a bridge. Under certain conditions, this bridge can break due to extended shaking (Mer and Healy 1963).

12.3 Biocoagulants

Aluminum-based coagulants, such as aluminum sulfate (alum), are widely used in purification plants as well as in sewage treatment plants (Boisvert et al. 1997; Najm et al. 1998). However, it has been found that it can induce neurological and pathological diseases (Martyn et al. 1989; Rajendran et al. 2015). In general, the use of inorganic coagulants causes environmental and economic problems; the sludge generated is of large volume and is not biodegradable. Sludge composed of inorganic substances must be pretreated before being discharged into the environment, which increases the cost of the process (Ugwu et al. 2017). These drawbacks have prompted the replacement of inorganic coagulants with coagulants of organic origin, called biocoagulants.

Biocoagulants can achieve a performance comparable to that of their inorganic counterpart with additional environmental benefits, such as biodegradable sludge, without toxic effects on human health, in some cases with antimicrobial activity (Ahmed et al. 2010; Teh and Wu 2014) and antifungals (Lim 2012), and capable of removing heavy metals, fluorides, and chlorides (Choy et al. 2015). Biocoagulants are ionic (cationic, anionic, or poly-ionic) and nonionic polymers of varying molecular weights (Saleem and Bachmann 2019) that can be obtained from microorganisms, animals, and plants (Saravanan et al. 2017).

Among the biocoagulants derived from microorganisms, actinobacteria, such as *Cellulomonas* and *Streptomyces*, contain in their structure polysaccharides and proteins with neutral sugars, amino sugars, and uric acid that contribute to its proven coagulant capacity (Nwodo et al. 2014). The most studied biocoagulants of animal origin include chitosan, which is obtained from the exoskeleton of crustaceans, insects, yeasts, and fungi (Bratby 2006). Chitosan is a cationic, non-toxic, biodegradable biopolymer and has the ability to adsorb metal ions due to the amino groups of its structure (Montembault et al. 2005). In the treatment of raw water with high turbidity, the performance of chitosan surpassed that of aluminum sulfate in terms of residual turbidity and the amount of sludge produced (Hu et al. 2013). Its coagulant activity is associated with the presence of functional groups in its structure that interact with the active sites of the contaminating particles, leading to the formation of aggregates (Teh and Wu 2014).

Plant-based biocoagulants can be obtained from *Moringa oleifera* seeds (Okuda et al. 2001a, b), mustard (Bodlund et al. 2014), cereals (corn and rice) (K. Ghebremichael 2007), legumes (beans) (Gunaratna et al. 2007), tubers (cassava) (Kumar et al. 2017), cactus (Zhang et al. 2006), among others. The available sources

of plant-based biocoagulants are the most abundant, so they have gradually gained importance over the years (Choy et al. 2015). Of particular interest are the seeds of *Moringa oleifera*; these contain proteins that promote coagulation (Okuda et al. 2001a, b; Sutherland et al. 1990). Various studies have shown that the use of *Moringa oleifera* is comparable to aluminum sulfate in wastewater treatment (Diaz et al. 1999; Ndabigengesere and Narasiah 1998).

12.3.1 Operating Conditions

The neutralization of charges and the formation of bridges are the mechanisms that have been associated with the activity of biocoagulants (Abidin et al. 2011). It is important to note that the efficiency of these two mechanisms is affected by the pH and the dose of the coagulant used (Teh and Wu 2014). The efficiency of the coagulation process can significantly improve through pH manipulation, because it alters the electrochemical nature of the system (Choy et al. 2015). On the other hand, an adequate dose of coagulant is necessary to achieve maximum removal of colloidal particles at minimal treatment cost (Biggs 2015). Additionally, the bridge mechanism is also favored when coagulants with higher molecular weights are used (Miller et al. 2008), and the space available in the medium is adequate for the coagulant to adsorb the colloidal particles (Bolto and Gregory 2007).

Nharingo et al. (2015) studied the influence of various operating conditions on the decrease of Pb (II) ions, using powder of cactus *Opuntia ficus-indica* as a biocoagulant. The evaluated operating conditions were pH, initial concentration of Pb (II) ions, particle size and dosage of the biocoagulant, contact time, and temperature. The parameters that had a significant effect on the removal of Pb (II) were the pH and the dosage of the biocoagulant, since they are the ones that regulate the coagulation mechanisms.

Among the biocoagulants frequently studied are the seeds of nirmali (*Strychnos potatorum*), *Moringa oleifera*, tannin, and cactus (Sciban et al. 2009; Yin 2010). Seed extracts of *S. potatorum* have been tested in kaolin-based synthetic turbid water. The *S. potatorum* seed polysaccharide mixture contained galactomannans and galactane, capable of reducing turbidity by up to 80% (Adinolfi et al. 1994).

According to Binsi et al. (2017), the stirring speed and the mixing time influence the formation of flocs; this affects the size and cohesion of the flocs to remain united. Wang et al. (2009) explain that excessive mixing can cause breakage of the formed floc particles, resulting in colloidal suspension, thus reducing the effectiveness of solids removal.

According to the results presented by Wan et al. (2007), colloidal stability increases according to the molecular weight of the polymer, so the flocculation efficiency increases. The coagulant components of *M. oleifera* have molecular weights between 3 and 30 kDa, as well as in *C. spinosa* they have a high molecular weight between 50 and 8000 kDa.

Bouyer et al. (2001) indicated that stirring speed is a factor in successfully carrying out the coagulation–flocculation process. At low-speed mixing (60–30 rpm), more

stability occurs in the suspension; this leads to the formation of larger flocs, this favors sedimentation. On the contrary, at high-speed mixing, small flocs formation is favor, which does not have sufficient density to settle.

12.3.2 Prospects for the Use of Biocoagulants

Ang and Mohammad (2020) reflect on the change in the productive vision and how it can influence water treatment by offering "green" alternatives. To achieve this change of vision in water treatment processes from biocoagulants, some relevant aspects must be considered. The type of coagulant will condition its implementation due to:

1. Abundance of the raw material: It implies being able to continuously supply the biocoagulant product without affecting the offer. In this sense, it is required that the production of the raw material is not affected by natural phenomena or by the technical requirements of the process. In this regard, biocoagulants of microbial origin may have the advantage, but species, such as *Moringa oleifera*, ensure that their existence is constant.
2. Technological production process: the technologies implemented vary depending on the type of biocoagulant. But in general, the process consists of three stages: primary processing, extraction, and purification. Plant species stand out at this point, because they can be used at any stage of the production.
3. Environmental impact: This referred to both in production and in the application of biocoagulants. Regarding the impact of production, the use of solvents is an aspect that negatively affects the production of biocoagulants of animal and plant origin; however, in many of the biocoagulants obtained, the raw materials are waste streams that obtain added value. In relation to discharges, no toxic effects or alterations of flora and fauna have been reported, so the environmental impact of the three types of biocoagulants is less than that of inorganic coagulants.
4. Ease of transportation and storage: The distances between raw material collection points, the processing industry, and treatment plants increase production costs. Furthermore, the conditions in which they are stored to ensure that the biocoagulant maintains its properties can be a factor that increases costs. These aspects will be independent of the type of coagulant but will depend on the physical state (solid or liquid) and its degradability.
5. Sludge production and treatment: It is one of the fundamental factors for which the application of biocoagulants is superior to its inorganic counterpart. All the sludge produced may be treated by physicochemical or biotechnological routes for the reduction of chemical oxygen demand (COD) and even for its use in obtaining energy.

Of the biocoagulants studied, *Moringa oleifera* receives much of the attention, because it is a species that meets the previously described aspects. The plant species quickly adapts to the environment and grows and produces seeds in periods of less than a year. That is why the potential of this biocoagulant in water treatment is analyzed.

12.3.3 *Moringa oleifera* for Water Treatment

Moringa oleifera is a plant species; the seeds of which contain up to 40% by weight of edible oil with about 80% unsaturated acids. These seeds have high-protein content, with this component being the coagulating effect that acts effectively in the treatment of wastewater (Broin et al. 2002). *Moringa oleifera* seeds and their extracts have been part of research in the treatment of surface water, urban waste, and industrial waste. The literature reports various investigations related to the uses of *M. oleífera* in the treatment of various types of water, especially municipal and industrial wastewater. The capabilities of this biocoagulant regarding the elimination of various contaminants are interesting. Some research that shows these experiences are mentioned below.

Freitas et al. (2016) experimented with extracts of soluble *Moringa oleifera* lectin and compared the consecutive dosing treatment of aluminum sulfate followed by the extract against the simultaneous feeding of both coagulants. The first treatment turned out to be more effective in reducing turbidity in which 96.8% was obtained, compared with 91.3% obtained in the second treatment.

Basra et al. (2014) obtained the biocoagulants in two ways: (1) aqueous seed extract and (2) seed flour. The tests with the biocoagulants were carried out in municipal wastewater with the aim of reducing heavy metals (Pb and Cr). The results showed that the treatment with aqueous extracts achieved a greater efficiency of elimination of lead and chromium compared with flour. However, *M. oleifera* flour decreased electrical conductivity and maintained better pH stability. They report a reduction of 46% of Pb and 30% of Cr with the application of *Moringa oleifera* seed powder and indicated that it has greater potential than aqueous solutions of the same and even that of alumina.

Awopetu et al. (2011) affected microbial populations in urban wastewater with *M. oleifera*. Microbial populations of *S. dysenteriae*, *S. typhi*, *E. coli*, and *B. cereus* were reduced by 76, 71, 56, and 32%, respectively. Suarez et al. (2002) demonstrated the antimicrobial activity of *M. oleifera* seed extracts from bacteriological evaluations and indicate that the antimicrobial action is due to disruption of the cell membrane by inhibition of essential enzymes.

Besides, Ghebremichael et al. (2005) and Jerri et al. (2012) indicate that there is a positive correlation between the reduction of turbidity and the reduction of helminth eggs in treated water intended for irrigation. This suggests that helminth eggs adhere to the flocs formed in the coagulation of raw water from *Moringa oleifera* and settle simultaneously.

These results demonstrate that the water treatment by means of *M. oleifera* as biocoagulants has the potential to position itself as an alternative to chemical coagulants. Table 12.1 summarizes other results of the application of *M. oleifera* in various aqueous matrices.

Other aspects that can be highlighted from the research related to *M. oleífera* are:

1. Use of salts to increase the coagulant potential: According to Okuda et al. (1999), the coagulant capacity of proteins is increased when inorganic salts are used in its extract. Lo Monaco et al. (2013) reported mixtures of *Moringa oleifera* with

Table 12.1 Results of the application of *M. oleífera* as biocoagulants in various waters

Wastewater	*M. oleifera* dose ($g.L^{-1}$)-($mL.L^{-1}$)	Parameters	Removal (%)	References
Slaughterhouse	7 ($g.L^{-1}$)	COD	64	del Real-Olvera et al. (2015)
Dairy industry	1 ($g.L^{-1}$)	COD	55	Vieira et al. (2009)
		Turbidity	98	
		Color	95	
Tapioca starch	0.11 ($g.L^{-1}$) 0.13 ($g.L^{-1}$) 0.15 ($g.L^{-1}$)	BOD: Biochemical Oxygen Demand	99	Suhartini et al. (2013)
		COD		
		TSS	91	
Coffee industry	10 ($mL.L^{-1}$)	Turbidity	>90	Matos et al. (2007)
	4 ($g.L^{-1}$)	COD	25	Garde et al. (2017)
Palm oil	6 ($g.L^{-1}$)	TSS: Total suspended solids	95	Bhatia et al. (2007)
		COD	52.2	
Distillery	0.5 ($g.L^{-1}$)	Color	97	David et al. (2015)
		COD	90	
		Turbidity	99	
Tannery	50 ($mL.L^{-1}$)	Turbidity	76.2	Mageshkumar and Karthikeyan (2015)
Mixed waters	50 ($mL.L^{-1}$)	Turbidity	45.9	Ndabigengesere and Narasiah (1998)
Textile industry	0.7 ($g.L^{-1}$)	Color	98	Bedekar et al. (2016)
Green waters	0.5 ($g.L^{-1}$)	Turbidity	84	Pritchard et al. (2010)
		E. coli	88	
Superficial water	0.032 ($g.L^{-1}$)	Turbidity	98	Sánchez-Martín et al. (2012)
	0.016 ($g.L^{-1}$)	Coliforms	94–96	
		Streptococcus	99	
	0.025 ($g.L^{-1}$)	Turbidity	87.5	Nkurunziza et al. (2009)
		Color	87.7	
		Dureza	10	
	16 ($g.L^{-1}$)	Turbidity	98	Poumaye et al. (2012)
		E. coli	50	
Household wastewater	0.2 ($g.L^{-1}$)	Turbidity	90	Ugwu et al. (2017)
		BOD	56	
		Coliforms	97.5	
Municipal solid waste leachate	0.1 ($g.L^{-1}$)	COD	84.5	Sivakumar (2013)
		TDS: Total Dissolved Solids	82.6	

NaCl, KCl, and $Ca(OH)_2$, which they obtained maximum turbidity removals of 62, 71, and 85%, respectively. Okuda et al. (2001a, b) investigated the extraction and purification of the active components of *Moringa oleifera* and verified that the active extract with saline solution does not increase the residual organic concentration of the water after coagulation.

2. Use as an adjuvant: The mixture of commercial coagulants with *M. oleifera* is a practice that enhances the results of treatment. Bhuptawat et al. (2007) used a mixture of 100 mg L^{-1} of *M. oleifera* and 10 mg L^{-1} of aluminum sulfate followed by sand filtration, in the treatment of municipal effluents. A 64% decrease in chemical oxygen demand was reported as well as a decrease in the amount of sludge generated during the treatment process.

M. oleifera is a benchmark in terms of its uses as a biocoagulant. Despite the fact that its cultivation has spread to various parts of the world, it is necessary to investigate other species with potential, with the objectives of: (1) diversifying the production of biocoagulants and (2) adapting alternative biocoagulants in regions in which the species mostly researched do not adapt.

12.3.4 Surface and Wastewater Treatment Experiences in the Use of Biocoagulants

The authors evaluated the removal of turbidity from flours of *Moringa oleifera* y *Caesalpinia spinosa* in distilled water. Three types of water were used: (1) kaolin-based simulated turbidity water (1085 NTU), (2) water from the feed channel of a water treatment plant (100 NTU), and (3) urban wastewater (136 NTU).

For the preparation of the biocoagulants, *Moringa oleifera* and *Caesalpinia spinosa* seeds were dried, until a final humidity of 13% was obtained. Subsequently, they were ground and sieved to a particle size of less than 150μm. The flours obtained were mixed with distilled water at 40 °C, in a proportion of 20 g of powder for each liter of distilled water. In a second experiment, 5 g of NaCl were added for each liter of the biocoagulant prepared. The jar test was programmed at 200 rpm for 1 min for the perikinetic phase and 60 rpm for 15 min for the orthokinetic phase and 20 min for sedimentation. The dosage of the coagulants was 15 mL for each liter of water treated; this was applied at the beginning of the perikinetic phase.

Two scenarios were used to evaluate the biocoagulants: (1) the effluent from the treatment by biocoagulants and (2) the effluent from the treatment by biocoagulants and subsequent filtration. In total, 24 experimental runs were performed in triplicate. Table 12.2 presents the processed results of the experimental phase.

It is observed that the results of the 24 applied treatments differ from each other. When applying a multifactorial ANOVA, it is established that only the factors corresponding to the type of water (simulated, superficial, and urban residual) and treatment (BC or BC + F) have a highly significant effect on the process (p-value <0.01).

Table 12.2 Comparison of treatments with Moringa oleifera and Caesalpinia spinosa as biocoagulants, for the removal of turbidity in simulated, superficial, and urban wastewater

Biocoagulant		Moringa oleifera		Caesalpinia spinosa	
NaCl (g L^{-1})		0	5	0	5
Simulated turbidity (1085 NTU)	BC	87.71 ± 3.47	94.38 ± 2.02	90.69 ± 0.9	85.65 ± 10.86
	BC + F	96.8 ± 0.29	98.29 ± 0.3	95.98 ± 0.62	96.82 ± 2.2
Superficial (100 NTU)	BC	75 ± 1.73	45 ± 2.65	64.67 ± 3.21	67.33 ± 4.51
	BC + F	98.67 ± 0.58	96.67 ± 1.53	94.67 ± 2.31	81.67 ± 2.31
Urban wastewater (136 NTU)	BC	40.79 ± 9.31	59.36 ± 11.95	71.86 ± 2.96	72.43 ± 1.3
	BC + F	84.76 ± 4.59	87.11 ± 11.36	74.78 ± 4.23	80.88 ± 3.33

BC: Biocoagulant treatment, *BC + F:* Biocoagulant + Filtration treatment

Multiple range tests indicate that within the water type factor, there are no significant differences in relation to the results between surface water and urban wastewater; it is highlighted that the removal of turbidity was higher in the simulated water with kaolin. Regarding the treatment, the multi-range test indicates that the implementation of a post-coagulation filter increases the removal of turbidity, due to flocs that have not settled and are retained in the filter. Regarding the addition of NaCl in the preparation of the coagulant, the literature indicates that the coagulation efficiency of the crude extract of *M. oleifera* is considerably improved with the use of saline solutions as a solvent due to the increase in the solubility of proteins by the addition of you go out. This mechanism suggests the breakdown of protein–protein or protein–polysaccharide or other existing associations in the *M. oleifera* seed powder, thereby increasing soluble proteins in saline solutions, resulting in increased coagulant activity; the sodium chloride and seawater solutions therefore work better than distilled water (de Souza et al. 2014; Golestanbagh et al. 2011). In research conducted by de Freitas et al. (2003), it is suggested that both protein and tannins are the active components for coagulation–flocculation in surface water. Tannins decrease their ability to form aggregates in solutions with the presence of NaCl. The latter could justify the decrease in the coagulant potential of *Moringa oleifera* in surface waters.

According to Schofield et al. (2001), the tannins and proteins present in *M. oleifera* form a tannin–protein complex through hydrogen bonds, hydrophobic interactions, ionic bonds, and covalent bonds, which causes stability in the solution. Sánchez-Martín et al. (2011) indicated that *Caesalpinia spinosa* contains tannins (in addition to galactomannans), which could be used to enhance the effect of *Moringa oleifera*. From this, a test was carried out with the biocoagulants, in which 15 mL of each were mixed for the treatment of surface water (100 NTU) and urban waste (54 NTU). Turbidity removal was analyzed both with the application of biocoagulants (BC) as well as with biocoagulants and subsequent filtration (BC + F). Table 12.3 presents the most relevant results of the study.

It is interesting to observe how the mixture in the surface water manages to increase the result corresponding to the treatment without filtration, this implies the reduction of costs in the design of processes. In the case of wastewater, the result

Table 12.3 Comparison of treatments with *Moringa oleifera* and *Caesalpinia spinosa* mixtures as biocoagulants, for the removal of turbidity superficial and urban wastewater

Water	Treatment	Turbidity removal (%)
Superficial (100 NTU)	BC	91.26 ± 4.81
	BC + F	98.30 ± 0.34
Wastewater (54 NTU)	BC	10 ± 32.08
	BC + F	83.2 ± 7.88

BC biocoagulant treatment, *BC + F* biocoagulant + filtration treatment

Table 12.4 Evaluation of biocoagulant mixtures in surface water and urban wastewater

	Superficial water			Urban wastewater		
Parameter	Initial value	Final value	Removal (%)	Initial value	Final value	Removal (%)
Turbidity (NTU)	557.20	1.77	99.68	136.00	6.55	95.18
pH	7.14	7.10	–	7.55	7.31	–
Alkalinity (ppm)	204.53	199.98	2.22	331.78	299.97	9.59
Suspended solids (ppm)	1,240	<25	>97.98	136.00	7.00	94.85
Chloride (ppm)	41.85	75.04	−79.31	n.d.	n.d.	n.d.
Hardness (ppm)	322.46	302.00	6.34	256.41	224.36	12.50
H_2S (ppm)	0.74	<0.1	>86.00	0.43	0.15	65.12
Fe (ppm)	2.37	0.15	93.67	1.06	0.34	67.92
Al (ppm)	0.36	<0.1	>72.00	n.d.	n.d.	n.d.
Si (ppm)	36.10	2.00	94.46	38.90	32.30	16.97
COD (mg L^{-1})	100.39	64.45	35.80	360.12	106.88	70.32
Fecal coliform (NMP 100 mL^{-1})	n.d.	n.d.	n.d.	1,600,000	10,000	99.38

differed from the previous experiment due to the initial turbidity of the sample. It was observed how the turbidity of the residual water increased, but from the filtration it was able to remove up to 83.2%. Based on these results, a strategy for optimizing results was proposed, with the aim of identifying whether the mixture of biocoagulants affects the quality of surface and wastewater. A simple centroid mixture design of experiment was proposed. The complete data of the experimental design is not shown in this book. Table 12.4 presents the results obtained from the optimized mixtures of *Moringa oleifera* and *Caesalpinia spinosa* in the dosages 13–27 mL (surface water) and 20–22 mL (urban wastewater).

Based on these results, the potential of biocoagulant mixtures is identified, both in the treatment of surface waters and in urban wastewater. Among the results, the reduction of 70% of the COD in urban wastewater stands out; this indicates a concentration of contamination in the sludge produced.

12.4 Technical, Economic, and Environmental Challenges in the Use of *Moringa oleifera* as Biocoagulant

Moringa oleifera is the plant species that has been studied the most in its applications as a biocoagulant. Gidde et al. (2012) mention some characteristics related to the production of *M. oleifera* seeds: (1) each tree produces between 15,000 and 25,000 seeds/year and (2) each seed weighs approximately 300 mg, of which about 225 mg correspond to the nucleus.

Yin (2010) indicates that there are three stages of *Moringa oleifera* seed processing to obtain biocoagulants:

1. *Primary processing*: corresponds to washing, drying, and spraying operations.
2. *Extraction*: separation of active components by means of organic solvents, water, and even saline solutions.
3. *Purification*: for the removal of impurities in the final product, precipitation, dialysis, lyophilization, or ion exchange operations are carried out.

Yin (2010) indicates that the products of any of the processing stages of plant species can be used as biocoagulants. However, the extraction and purification processes increase the biocoagulant potential and decrease the volume of the final product.

Gidde et al. (2012) present several interesting facts about the processing of *M. oleifera*:

1. The extraction stage reduces the cost of the biocoagulant by 40%, because the efficiency per unit of mass of the product increases.
2. Forty-six trees are required to produce 1 kg of shelled blended seeds of *M. oleifera*, which occupy an area of 5746 m^2.
3. In the extraction process, between 70 and 87% of the shelled blended seeds mass is used as a coagulant. From purification processes, between 19 and 28% of the seed mass is used.

Yin (2010) highlights the existence of anecdotal reports that provide data for the comparison of biocoagulants and also indicates that there are factors that must be considered in these analyses, such as raw material prices in different geographical regions, inflation, and the different precision of the values of costing. Furthermore, it is pointed out that the cost analysis of *M. oleifera* has received priority over other biocoagulants, and this is not surprising due to the well-publicized advantages of the plant.

Despite this scenario, in which the costs associated with the production of biocoagulants are higher than those of its inorganic counterpart, the availability of natural resources should be considered limited, so it must be increased from sources of renewable raw materials. Laurila-Pant et al. (2015) mention the importance of recognizing how natural resources can interact in society and economic activities. Thus, Adu-Dapaah et al. (2017) identify various derivatives of *Moringa oleifera*, among which are hygiene products (soaps, shampoos, and shower gels), foodstuffs

(energy drinks, tea, and infusives), agricultural products (fertilizers and plant grow hormones), and other products. Ali et al. (2012) studied the production process of biocoagulants from *M. oleifera* seeds and indicated that the various streams of by-products obtained can be used to produce activated carbon, oils, and animal feed. In this way, the full use of plant species will be a factor that reduces the cost of biocoagulant production.

In the economic analysis of the production processes, only the internal processes of the industry are considered; production costs are granted from: (1) the use of equipment, (2) purchase of raw materials, (3) payment to workers, and (4) transportation. This approach is unrelated to the origin of the raw materials. The total economic value (TEV) method proposes an analysis of the value of natural resources (Laurila-Pant et al. 2015; Pagiola et al. 2004). The value of a natural resource is divided into two categories: (1) use values and (2) non-use values.

According to Laurila-Pant et al. (2015) and Pagiola et al. (2004), in this perspective, the following aspects are considered: (1) the values associated with the products obtained from the exploitation of leaves, wood, and seeds of plant species (Direct use value); (2) environmental services, such as air and water filtration, temperature decrease (Indirect use value); (3) preservation of a resource for future use (Option value); and (4) future enjoyment of a resource, even if it is never used (Existence value). The gaps in TEV analysis reside in the estimation of indirect, optional, and non-use values. The measurement of the benefits of indirect values depends on estimates, such as the amount of filtered water and carbon stored in the biomass present in the soil. Non-use values tend to be difficult to estimate, due to the personal valoration of natural resources and future value. It is often difficult to distinguish the good or service that any indirect, optional, or non-use values cover for, because there is no such good or service on the market.

Thus, the identification of the potentialities in the use of biocoagulants should highlight the following aspects:

1. The replacement of inorganic coagulants by biocoagulants would promote the reduction of neurological diseases in the long term, with the consequent decrease in economic spending in the social sphere and an increase in the economically active population.
2. Due to the organic nature of biocoagulants, the generated sludge treatments can be carried out biologically.
3. The cultivation of plant species increases the rate of groundwater purification as well as air filtration.
4. The process can adapt to obtain by-products derived from leaves, branches, and seeds extracts of plant species. Besides, closed production cycles, in which the generation of waste is minimum by integrating the production flows into by-products with added value.
5. It is an alternative that does not threaten food security, given that the processes for obtaining biocoagulants from plant species mostly focus on seeds and shells, not on edible fruits.

12.5 Final Considerations

Among the factors that affect the efficiency of biocoagulants are:

1. Processing level: The efficiency of the biocoagulants increases with the extraction and purification processes.
2. Type of water to be treated: The presence of various contaminants can influence the efficiency of the treatment.

In the future, researches should emphasize key aspects such as:

1. Evaluate the potential of biocoagulants in various aqueous matrices.
2. Measure the effect on the environment, both from the production of biocoagulants and from treated waters.
3. Enhance the effect of biocoagulants from mixtures.
4. Optimize production costs.
5. Identify strategies for the use of residual sludge.
6. Express the environmental services provided by crops of species for the production of biocoagulants, in economic terms.

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Chapter 13
Multicriteria Analysis in the Selection of Agro-Industrial Waste for the Production of Biopolymers

María Antonieta Riera and Ricardo Raúl Palma

Contents

Abstract The plastics industry has presented since its inception a disproportionate increase in production, using fossil resources as raw material for its processes. However, environmental concerns, along with the possibility of facing a shortage of these resources, have made global manufacturing trends point to the use of natural resources for the production of materials that traditionally came from the petrochemical industry. Biopolymers are an example of this, which can be obtained from lignocellulosic biomass through bioprocesses perfectly scalable to the industry. One of these biomasses is agro-industrial waste, a carbon and hydrogen reservoir with great potential to be exploited, the selection of which largely depends on a series of variables such as availability, composition, and processing techniques, among others, which may require the use of multicriteria analysis tools to guarantee the

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choice of the best for the expected purpose. This chapter is developed, where the evolution of the plastics industry over time is discussed, presenting its current challenges until reaching biotechnological processes as an alternative solution to the current problems caused by the use of this material and premise of what is expected in the production of biopolymers.

Keywords Agro-industrial waste · Biopolymers · Bioplastic · Biocomposite · Multicriteria decision-making

13.1 Warning: Processing-instruction not allowed here!!! Introduction

Oil exploitation has made possible the production of fuels for power generation, mobility through the use of transportation, as well as the development of materials to make life easier. Among them are polymers, whose widespread use makes it present in almost all activities and sectors. However, given the environmental pressures in recent years, interest has emerged in finding new alternatives for the production of materials in a sustainable manner.

Biopolymers characterized by being biobased and biodegradable provide this solution. It is a niche in full development, ranging from obtaining thermoplastic starches to the synthesis of polymers through biotechnology. There is a combination of methods and materials used to achieve this goal. Among the raw materials used are agro-industrial waste origin, which have among its components: lignin, cellulose, starch, pectin, and collagen, among others, susceptible to be used to obtain biopolymers. To make the production of biopolymers more profitable, we suggest the implementation of multicriteria analysis methodologies and, through the evaluation of different criteria, select the most suitable raw material, processing method, and purpose of use.

13.2 The Plastics Industry and Its Evolution

The first attempts to develop polymers are related to the conversion of natural materials such as rubber into more useful products (Morris 2005). They are mostly macromolecules where plastics, natural and synthetic fibers, rubbers, coatings, adhesives, and sealants, among others, are included. The market for these materials has experienced exponential growth, clearly visible for the last century and a half, and has been led by plastics, followed by fibers and elastomers (Feldman 2008).

When speaking of plastic, reference is made to the physical form of a solid that exhibits plasticity to be molded and deformed without breaking, this being a

characteristic that depends on the degree and nature of the order of molecules within macromolecular solids (Rasmussen 2018). The official appearance of this industry dates back to 1870 with the patent on celluloid, to which Bakelite joined years later (Gillespie 1986) and, thereafter, dozens of thermosets.

Its widespread use occurred after 1945, thanks to the research and development produced by the Second World War. This is when basic plastics began their growth and production on a large scale, starting with polyvinyl chloride and polystyrene, then polyethylenes, polypropylenes, polyesters, and polyurethanes, along with a constant expansion of a number of more specialized products (Deanin and Mead 2007; Geyer et al. 2017).

Plastic has become the revolutionary material of the late twentieth century and went from being a curiosity of chemistry to one of the fastest growing and most developed materials (Crawford and Martin 2020). For this, the work of the scientific community has been required in the development of new polymerization methods, equipment design, polymer modification, and improvements in characterization techniques, making possible the passage from the laboratory to the market with products oriented to satisfy the needs of the users (Grassi 2018).

The world's plastic production is estimated to have gone from 1.5 million tons (MMt) in the 1950s to 335 MMt in 2016, and an increasing market trend is expected to follow. Until 2017, a cumulative world production of 8300 MMt of virgin plastics was calculated, of which a large part was used as packaging material that is usually discarded a few minutes after the purchase (Alimba and Faggio 2019; Geyer et al. 2017; Gironi and Piemonte 2011; Parker 2019).

Most of these materials are manufactured by polymerization of monomers derived from oil or gas by incorporating chemical additives. Its production requires a consumption close to 8% of the world's oil production for raw materials and energy expenses (Thompson et al. 2009a, b). Inventions include the use of lignocellulosic biomass, residues containing starch, protein, or collagen, from the food industry.

Recent research shows the use of spent coffee beans (Karmee 2018), waste from winemaking (Gutiérrez et al. 2018), pineapple peel residue (Vega-Castro et al. 2016), blue crab waste and orange peel (Baron et al. 2017), coconut shell (Amaral et al. 2019), and agro-industrial effluents (Rathika et al. 2019), among others, for the development of biopolymers or as substrates for obtaining fermentable sugars, precursors of some of these.

It is a sustainable concept capable of competing with conventional polymers. It is a bioeconomy model according to the current requirements of the industry, which promotes the use of waste for the maximum use of resources and the development of innovative products in production chains. However, despite the advances existing up to that time, researchers continue to make efforts to develop the properties of these biomaterials, so that they can compete without any disadvantage with conventional synthetics.

13.3 Environmental, Ethical, and Economic Challenges in the Production of Synthetic Polymers

The use of plastics has brought a great advance for humanity. It has made life easier with the incorporation of a cheap, moldable material with wide application in various areas of the industry, but it also represents serious environmental challenges due to the effects it causes on the environment. The first of them is that as it comes from fossil resources, it has a slow degradation rate that can take decades or even centuries (Napper and Thompson 2019). Additonally, there are the environmental damages caused by the activities inherent to the exploitation, refining, and processing of oil, to obtain raw materials used in the plastics industry.

Second is the large amount of waste, which is growing steadily based on the annual plastic production. To date, it is estimated that some 7000 MMt of plastic waste have been generated, of which 10% has been recycled, 14% incinerated and 76% is accumulated in landfills, outdoors or travel long distances to reach the sea. If the same trends in waste production and management continue, it is estimated that by 2050 there will be approximately 20,000 MMt of plastic waste in landfills or open pit (Geyer 2020; Geyer et al. 2017; Jambeck et al. 2015).

Mechanical recycling of plastic waste is a common practice environmental solution, crucial for saving fossil resources, closing the life cycle of materials and minimizing the impact caused by improper waste disposal. However, information on recycling industrialized plastic or recycled materials is limited. It also happens that sometimes virgin plastic can be made of different polymers or additives such as metals, dyes, or others, which must subsequently be removed in waste management and can hinder the recycling process, increase costs, and in the worst case contaminate recycled plastic (Eriksen et al. 2018; Gu et al. 2017).

Of the total plastic waste generated, only a very low percentage can be recovered by mechanical recycling. An aspect that perhaps influences some countries is the culture of society in terms of acquiring the habit of classifying plastic waste at the generation points, which would represent an increase in costs for recycling companies by requiring additional personnel, for the classification and selection of useful waste in the process.

Regarding the burning of plastics, it brings with it the release of gases such as dioxins, furans, mercury, and polychlorinated biphenyls, which, in addition to being toxic to animal and human health, contribute to climate change (Verma et al. 2016). Studies show that these substances are capable of remaining in the adipose tissue of animals and enter the human body through the food chain, causing damage to the endocrine system, reproductive system, and immune system, highlighting their carcinogenic effect (Kanan and Samara 2018; Rathna et al. 2018).

There are also macroplastics (plastics with a size >25 mm), microplastics (plastics with a size between 0.1 μm and 5 mm), and nanoplastics (plastics with a microscopic size of ≤ 100 nm), which represent a risk to the marine ecosystem. These cause damage to some animals due to entanglement, skin lesions or abrasions, feeding restriction, blockage in the intestines due to ingestion, or death. These

hydrophobic plastic pollutants can be absorbed by marine fauna and then pass to humans through the food chain (Alimba and Faggio 2019; Besseling et al. 2019; Li et al. 2016).

Synthetic polymers are the most widespread anthropogenic pollutants in marine and terrestrial ecosystems that threaten wildlife and exert effects on natural food nettings. Plastic pollution is now a global problem that damages ecosystems, destroys biodiversity, and has the potential to affect the planet (Barnes 2019; Krueger et al. 2015). One of the most devastating consequences, and more discussed in recent years, is the accumulation of this material in the oceans. The discovery of new islands but of plastics suggests that if the same rate of consumption continues, it will mean that in the near future there will be more plastic than fish in the sea.

Time to think of a solution to meet current needs without compromising future generations. In the search for alternative solutions, the concept of bioeconomy arises, which seeks to minimize the use of virgin resources in production processes, through the maximum use of raw materials and the reuse of waste generated along the chain of value, for a minimum environmental impact. The production of biodegradable plastics from waste represents a significant saving in CO_2 compared to that of fossil plastics. Its degradation also favors this issue, since it is expected that during this process energy, CO_2 and water will be produced, without harmful by-products (European Bioplastics 2019; Karan et al. 2019).

This commitment can be assumed in part with the implementation of waste biorefineries, which have great potential to produce value-added products—including biopolymers—while providing a solution to the current problem of waste management. Proper implementation could provide environmental and economic benefits, further contributing to the achievement of the UN Sustainable Development Goal (SDG) 12 for the 2030 agenda regarding responsible production and consumption, where governments, the private sector, and civil society are committed to direct actions that ensure the achievement of the goals set.

At present, it is an enterprise little studied in underdeveloped countries, despite the fact that it is an opportunity to produce useful resources. It is an alternative to respond to the problems caused by the improper disposal of fossil-based plastic waste, which has a long useful life. It is also an option for the management of waste generated upstream in other industry sectors, taking advantage of it as raw material in the production of biomaterials.

13.4 Biopolymers, Bioplastics, and Biocomposites

Different authors use the terms biopolymers and bioplastics interchangeably to refer to the same material. However, biopolymers are defined as natural polymers that comply with being biobased and biodegradable, which is why they are also considered both ecological and sustainable. They can be of animal origin (collagen, protein, silk, chitin/chitosan), vegetable (cellulose, starch), or synthesized by microbial agents (polyhydroxyalkanoates, cellulose). They have application as packaging

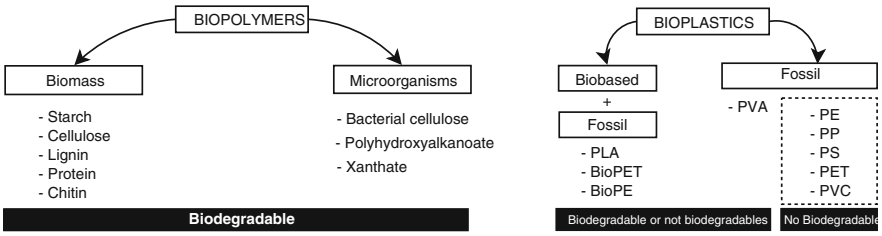

Fig. 13.1 Biopolymers or bioplastics

material in food and drug transport and are quite promising for biomedical applications (Brigham 2017; Kanmani et al. 2017; Sharmila et al. 2020).

Biopolymers include thermoplastic starch (TPS), polyhydroxyalkanoates (PHAs), and other polymeric sources such as lignocellulosic materials, gums, chitosan, and proteins. For its part, polylactic acid (PLA) is a polymer that, although being classified as biobased and biodegradable, is obtained by biological and chemical synthesis.

Bioplastics or biodegradable plastics are a type of polymer that can be biologically based or biodegradable or fulfill both properties (European Bioplastics 2019). They are bio-based when they come from natural polymers, available from renewable sources including those grown microbially or extracted from starch. The non-biobased ones are the synthetic polymers obtained from petroleum resources. Biodegradability describes the ability of the material to degrade by the action of microorganisms in energy, biomass, water, and CO_2 or CH_4 (John Wiley and Sons 2016), depending on the conditions under which this process is carried out (Fig. 13.1).

An example of biobased but not biodegradable polymers is bioPET, which use biomass to produce ethylene glycol later used in the synthesis of polyethylene terephthalate. On the contrary, there is polycaprolactone that is obtained from fossil resources, it is biodegradable but not biologically based. There are also other types of plastics on the market that, depending on their degradation mechanism, are conceived as photodegradable, oxodegradable, oxo-biodegradable, and compostable, among others, but they do not necessarily fall under the classification of biopolymers or bioplastics.

Bioplastics have the advantage over conventional plastics of their reduced carbon footprint, replacing in most cases the resource of fossil origin with biomass. They also favor closed-circuit production that seeks the reuse, recycling, or recovery of resources and incorporation of innovative materials on the market to provide products with better breathability, greater resistance, reduced thickness, and improved optical properties (Ashter 2016; European Bioplastics 2019). By 2019, the bioplastics production represented only 1% of all plastics processed worldwide, with a production of 2.11 MMt, and it is expected to grow to 2.43 MMt by 2024 (European Bioplastics 2019; Payne et al. 2019).

On the other hand, there are compounds, which are materials formed by two or more materials that, when combined, constitute a new material with different

characteristics from the individual ones. Biocomposites are materials composed of biobased polymers, fiber derived from plants (biofiber) and derived from petroleum or biodegradable polymers. Cellulosic fibers have gained great interest in recent years and are classified into flax fibers (flax, hemp, jute, kenaf, and ramie), foliar fibers (abaca, banana, pineapple, and sisal), seed fibers (coconut fiber, cotton, and kapok), as well as all other types (roots and wood). These are added as reinforcement to the polymer matrix to improve the mechanical and barrier properties of the bioplastic (Abdul Khalil et al. 2018; Chan et al. 2018; John and Thomas 2008; Ngo 2018).

Some researchers in the area have worked on the development of sustainable biocomposites, made with recycled conventional plastics and biocarbon from waste from the food industry such as peanut shells (Picard et al. 2020), chicken feathers (Li et al. 2020), grape pomace (Gowman et al. 2018), and walnut shell and rice (Guna et al. 2020). One of the challenges for these materials is their ability to compete with those of synthetic origin, which is why work continues to develop biobased materials capable of meeting the required quality and cost requirements.

13.5 Trend of Biotechnological Processes in the Production of Biopolymers

Industrial biotechnology was initially applied during the First World War for the production of acetone and butanol using *Clostridium*. Although it was surpassed in its beginnings by petrochemical processes, the current environmental pressure has turned its gaze to this technological application to take advantage of the potential of waste streams and by-products of industrial activities, in the production of chemical products and biopolymers through microbial bioconversion in fermentation processes (Koutinas et al. 2014).

White biotechnology provides routes to obtain biopolymers on an industrial scale, by converting plant biomass into glucose, fatty acids or other monomers, into bioplastics through microbial fermentation (Kumar 2020; Van Beilen and Poirier 2007). Bacterial biopolymers can be classified into inorganic polysaccharides, polyamides, polyesters, and polyanhydrides (Rehm 2010). Some of the contributions of biotechnology to the development of biopolymers are obtaining bacterial cellulose, polyhydroxyalkanoates (PHAs), and lactic acid as a precursor of polylactic acid, in addition to the enzymatic modifications that are made to polysaccharides.

Bacterial cellulose is a work of art from nature, which was originally limited to use as an ingredient in a traditional dessert. It consists of pure cellulose nanofibrils synthesized by *Acetobacter xylinum*, which has interesting mechanical properties to be utilized as a reinforcing material. It is a biocompatible and antimicrobial material, so it has been used as a dressing and suture material in medical applications with excellent results, and it is still being studied as a scaffold in tissue engineering (Helenius et al. 2006; Iguchi et al. 2000; Maneerung et al. 2008).

PHAs are water-insoluble biopolyesters that are produced from renewable carbon sources by the action of recombinant microorganisms in fermentation processes. These biopolymers can be short, medium, or hybrid chain, depending on the number of carbon atoms present. They have physical properties similar to synthetic plastics but have the advantage of being biocompatible, thermoprocessable, and completely biodegradable materials (González García et al. 2013; Koller 2017). Given these characteristics, like bacterial cellulose, it is an attractive biomaterial to be used in the transport of drugs, medical devices, dressings, sutures, as well as in applications for tissue engineering (Bonartsev et al. 2019; Chen and Wu 2005; Kalia et al. 2019; Valappil et al. 2006).

Lactic acid is a precursor of polylactic acid (PLA), which is a biodegradable and biocompatible renewable aliphatic polyester, based on a repetitive monomeric unit of lactic acid, obtained by the action of homolactic bacteria on starch-rich biomass and through the microbial fermentation of D-glucose. Its commercial scale production involves the use of a batch fermentation reactor that runs on concentrated lactic acid, typically taking 3–6 days. Polymerization occurs primarily by industrial polycondensation of lactic acid or ring-opening polymerization of lactide (Auras et al. 2004; Castro-Aguirre et al. 2016; Djukić-Vuković et al. 2019), the latter being the route that provides a polymer with a higher molecular weight and therefore better mechanical properties.

PLA, bacterial cellulose, and PHA are considered favorable biomaterials for medical applications because they are biologically based, biodegradable, biocompatible, compostable, and nontoxic. They are commercially available as filaments for the purpose of use in the 3D printing technique. However, its applications are limited by its low resistance to melting, slow crystallization speed, low processability, high brittleness, low toughness, and low service temperature (Matos et al. 2019; Singhvi et al. 2019). To overcome these limitations, other biologically based polymers can be incorporated into PLA blends. An example of this is an invention where PHAs are used to develop compounds from mixtures with PLA and improve the mechanical properties of the thermoformed material (Krishnaswamy 2016; Nofar et al. 2019).

Polysaccharides, on the other hand, are also biopolymers that are sometimes modified to expand their possibilities of use through the improvement of properties such as gelatinization temperature, solubility, hydrophobicity, and retrogradation, among others. Physical and chemical methods are commonly used, but enzymatic bioprocesses have emerged as an alternative, to reduce the environmental impact caused by the chemical and toxic approach of traditional methods.

The growing demand for sustainable processes, orientation to the use of active enzymes such as laccases, peroxidases, lipases, tyrosinases, and transglutaminases, for the synthesis of biomaterials. This is a biotechnological tool for the extraction and fractionation of macromolecular components of polysaccharides into smaller molecules, which serve to obtain products in different sectors of the industry, with characteristics superior to those offered by the market (Karaki et al. 2016; Martínez-Abad et al. 2016; Obro et al. 2018).

The development of biopolymers through biotechnology is a promising field, but it has an economic limitation to overcome. It is a challenge associated with the low conversion of carbon substrates into products, slow growth of microorganisms,

difficulty in downstream separation, and instability in the thermomechanical properties of the obtained product (Chen and Jiang 2017; Kovalcik et al. 2019). In fact, some bacterial exopolysaccharides and polyhydroxyalkanoates (PHAs) are produced commercially, but they do not reach a large-scale production due to the high production costs they have in relation to traditional synthetics (Rehm 2010). That is why the development of new production methods, aimed at improving the economy of the process, is still under study.

One way to reduce these costs is by using cheap and potentially sustainable carbon sources. Among the biologically based raw materials are lignocelluloses, agricultural residues, food residues, and lipid-rich industrial wastewaters that, in addition to being pretreated, require in most cases separation and depolymerization processes. Organic waste represents a use option, but it must be relatively abundant, concentrated, and easily degradable in order to be a viable project (Aslan et al. 2016; Brigham and Riedel 2019; Dietrich et al. 2019; Pratt et al. 2019).

The economically viable production of these biomaterials requires evaluating the availability of the waste, as well as the costs of installation, assembly and operation of the industrial facility, assembly, and operation of the industrial installation (Kumar et al. 2020). New pretreatment methods need to be implemented for lignocellulosic raw materials and less environmentally aggressive and contribute to a better use of the carbohydrates present in the biomass. In a circular economy approach, the vision of integrated biorefineries is proposed. For example, the integration of PHA production in processes such as wastewater treatment plants, hydrogen production, or biodiesel could improve its implementation on an industrial scale (Dietrich et al. 2019; Moretto et al. 2020; Rodriguez-Perez et al. 2018).

The implementation of a biorefinery is especially challenging for underdeveloped countries. However, at the same time, it is an opportunity that promises good results to develop processes through biotechnology, applicable in obtaining biomaterials with a reduced environmental footprint, savings in the use of natural resources, use of waste, and reduction of greenhouse gas emissions, in a context of circular economy.

13.6 Potential Agro-Industrial Waste in the Production of Polymerizable Raw Material

In the food industry, there are three phases of waste generation: production, processing, and distribution. In the production of food in the crop, there are the agricultural residues generated in the harvesting. The processing considers all the waste, both solid and liquid, generated in each of the manufacturing stages. In the distribution are the waste made up of the finished product that contains both food and packaging material (Fig. 13.2). And after consumption, there are the residues of the preparation, food remains, and packaging (Pfaltzgraff et al. 2013).

The waste in food supply chain is an organic material, discarded, lost, or degraded in some of the manufacturing stages, which due to its nature does not compete with

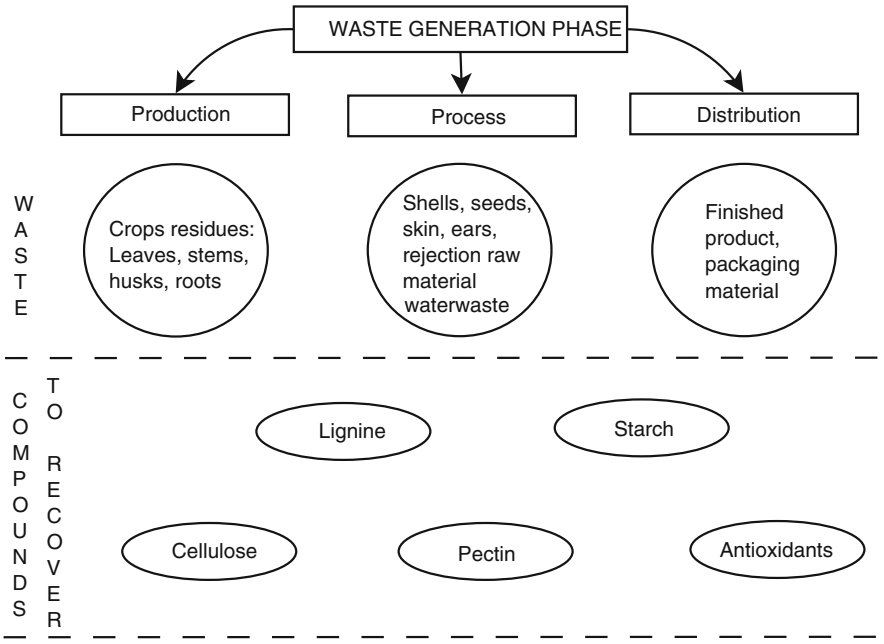

Fig. 13.2 Waste generated in the food industry

the needs of human consumption. It is a reservoir of complex carbohydrates, proteins, lipids, and nutraceuticals, with the potential to be valorized through the production of biofuels, enzymes, bioactive compounds, biopolymers, and nanoparticles, among others (Burgos et al. 2016; Carmona-Cabello et al. 2018; Dahiya et al. 2018; Ravindran and Jaiswal 2016).

From tubers such as potatoes, cassava, and sweet potatoes, the waste of the peel and sometimes the pulp is used, which is discarded due to a change in color or size or noncompliance with quality parameters (Sharmila et al. 2020). From fruits such as banana in their immature state, the peel can be used, while from others such as avocado and mango, the seeds can be used to extract starch and use it in its native or modified form, for the production of thermoplastic starch (Kader Sultan and Wan Johari 2017; Kumar Rana et al. 2018; Lubis et al. 2018a, b).

To modify the crystalline structure of the thermoplastic starch and to facilitate its processing, plasticizers, water, and agents such as heat and pressure are used. Glycerol is one of the most widely used plasticizers. As a filler material to improve the mechanical properties of thermoplastic starch, the use of fibers, wood or sawdust, mixed together with the plasticizer, is reported. In some experiments, mixtures of thermoplastic starch and polylactic acid were made to evaluate its structure and performance as a material (Halley et al. 2007; Li and Huneault 2011; Zhang et al. 2014).

In experiments carried out by the authors to obtain bioplastics, residues of banana peel, avocado seed, and mango seed were used. The process consisted of extracting the starch or flour from the residue, mixing it with the plasticizing agent, and rolling

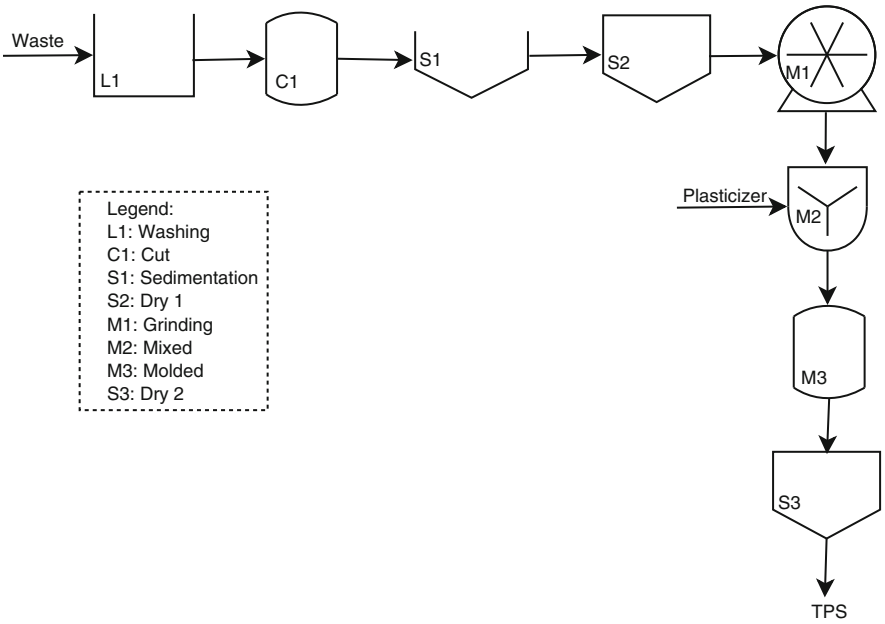

Fig. 13.3 Process to obtain bioplastics

Table 13.1 Mixture used to obtain bioplastics

Waste	Starch (g)	[a]Plasticizer (g)	Polyvinyl alcohol (g)	NaOH 30% (mL)	NaOH 15% (mL)
Avocado	3	2	6	5	–
Banana	5	2	–	–	5

[a]Glycerol, sorbitol

it by the casting and drying method (Fig. 13.3). The amylose content was higher for the starch compared to the flour, which possibly later affected the durability of the thermoplastic material obtained.

A fixed amount of starch, water, and NaOH solution was used in each mixture, but the type and amount of plasticizer was varied (Table 13.1). The best results for the avocado and banana residues were obtained with a starch-plasticizer ratio of 1.5:1 and 2.5:1, respectively (Fig. 13.4). To achieve the formation of the thermoplastic film, we worked at basic pH. Mixtures with acidic pH only took on a lumpy appearance and did not form a film.

The characterization of the TPS allowed to conclude that sorbitol in relation to glycerol, provides greater durability and elasticity to the touch in the film, but this last plasticizer is less hygroscopic, providing better barrier properties for water vapor. The loss of weight of the films in anaerobic conditions was used as a measure of biodegradability, reaching higher percentages for the TPS of the banana (Table 13.2). Although work on the production of thermoplastic starch from

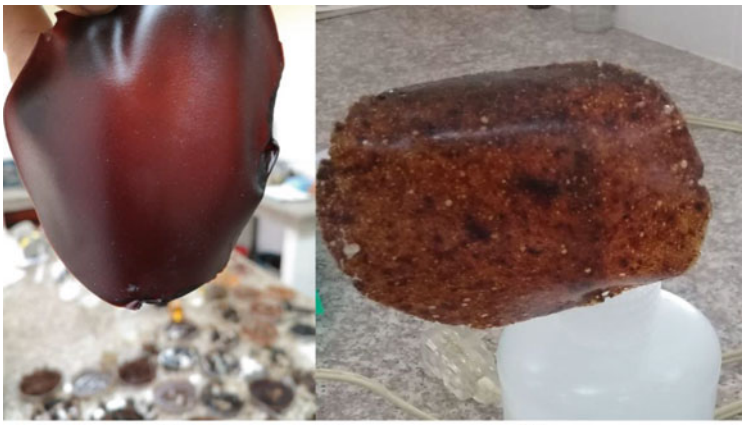

Fig. 13.4 Bioplastic obtained from (**a**) avocado seed and (**b**) banana peel

Table 13.2 Properties of the bioplastics obtained

Waste	Variable	$\bar{x} \pm \sigma$
Avocado	Moisture, %	23.43 ± 1.25
	Solubility, %	34.40 ± 1.86
	Biodegradability (45 days), %	24.53 ± 2.32
Banana	Water absorption, %	83.73 ± 2.26
	Water vapor permeability, 10^{-10} (gm/sm^{2}Pa)	13.6 ± 5.75
	Biodegradability (40 days), %	34.09 ± 1.68

mango seed is underway, preliminary tests indicate that the best durability results are obtained when sorbitol is used as a plasticizer.

Corn cob, rice husk, sugarcane bagasse, pineapple peels and banana waste can be used to produce biopolymers such as PLA or PHA (Jiménez-Bonilla et al. 2012; Miura et al. 2004; Proaños and Piñeros Castro 2014; Sánchez et al. 2012; Torres Jaramillo et al. 2017; Vega-Castro et al. 2016), degrading the lignocellulosic materials present and making a subsequent fermentation or polymerization.

An investigation evaluated the potential of cassava and beekeeping residues was evaluated for the production of fermentable sugars as a substrate for the production of lactic acid, a promoter of polylactic acid (PLA). All residues were subjected to acid or alkaline hydrolysis at concentrations of 3–5% (w/v) before being analyzed. The total reducing sugars (ART) were determined by the DNS method (Bello-Gil et al. 2006), reaching higher concentrations for the starch extracted from the cassava peel when applied an acid treatment with H_2SO_4 at 4% and for the stone in alkaline hydrolysis with NaOH at 5%, with 1.77 g/L and 4.60 g/L respectively (Briones and Riera 2020). Another experiment carried out to determine the amount of total reducing sugars in rice and corn processing residues, revealed that they possess between 0.66–0.87 g/L and 0.18–0.89 g/L respectively of ART.

On the other hand, there are citrus residues of orange, lemon, mango, and grapefruit, which contain pectin which is a natural biopolymer widely recognized in the food industry and in biotechnology for its gelling capacity, in addition to cellulosic fibers that add resistance to the manufactured material (Bátori et al. 2017; Khan et al. 2014). From the shells of crustaceans, the natural biopolymer chitin and its deacetylated product chitosan are obtained, with wide commercial interest (Philibert et al. 2017).

But in addition to solid waste, there is wastewater from agribusiness, from which important components can be recovered in a platform based on mixed microbial cultures to obtain PHAs (Mannina et al. 2020). Research in the area has shown that it is possible to use activated sludge from the treatment of wastewater from the dairy industry (Khardenavis et al. 2007), from olive milling (Ntaikou et al. 2009), from washing in the rice processing (Amini et al. 2020), to isolate poly β-hydroxybutyric (PHB) producing bacteria which is a type of PHAs.

The work carried out shows that waste from the food industry and its by-products have the potential to be used in the production of biopolymers. It remains to be discovered which of them is the most promising and, based on them and their availability, establishes the most appropriate technology to provide a predictable and effective combination of production of these biomaterials, aimed at substantially reducing production costs to satisfy in a sustainable way the needs of the market.

13.7 Multicriteria Analysis Tools Applicable in the Selection of Lignocellulosic Residues for the Formulation of Biopolymers

For biopolymer production to be economically attractive, aspects such as biomass availability, location, associated costs for supply logistics, and processing methodologies, among others, must be taken into account, which in most cases requires the evaluation of different alternatives for decision-making.

Multicriteria decision-making (MCDM) is a technique that is based on operations research models and is used to solve complex problems that depend on multiple criteria or attributes of a qualitative, quantitative type or a mixture of both. It is divided into two types: decision-making with multiple objectives (MODM), which studies decision problems in which the decision space is continuous, and decision-making with multiple attributes (MADM), which focuses on problems with discrete decision spaces and in which the set of decision alternatives for problem solving has been predetermined (Chen and Hwang 1992).

The multicriteria analysis tools have different application areas. For example, it has been used in budget allocation problems, political decisions, and analysis of responses to environmental risks, among others, for the systematic evaluation of different alternatives in complex situations where the best option is expected to be selected (Marttunen et al. 2017; Webster 1999). Among the best known are the

analytic hierarchy process (AHP), Élimination Et Choix Traduisant la Réalité (Elimination et Choice Translating Reality, ELECTRE), PROMETHEE method, and Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS).

The analytical hierarchy process is a theory of measurement through pairwise comparisons and is based on expert judgment to derive priority scales (Saaty 2008). It works by setting preferences. In its first stage, it computes criteria weights. It begins by making paired comparisons between criteria and constructs a square matrix from which the eigenvector is calculated, which is used as a weight vector for the criteria.

The ELECTRE method deals with improvement relationships by using pairwise comparisons between alternatives under each of the criteria separately. To do this, divide the solution game into two areas. One of them is called the nucleus or cluster of more viable solutions and a second involves the least favorable ones. The method places in the nucleus those alternatives that can be the optimal solution for the decision maker and with a high degree of reliability it is stated that those that are outside of this do not have the optimal alternative (Rogers et al. 2000).

The PROMETHEE methodology consists of a preference function associated with each criterion as well as weights that describe their relative importance. It is based on the improvement relationships between the different alternatives compared in pairs, where it is assumed that the decision-maker compares each alternative with another when they are evaluated under different criteria. The criteria can be expressed in different units of measurement or weightings, for which it is necessary to know what is the generalized criterion under which each of these is evaluated (Brans and Mareschal 2005; Munier 2011).

TOPSIS is a multicriteria method to identify solutions from a finite set of alternatives based on the simultaneous minimization of the distance from an ideal point and the maximization of the distance from a node point. So the best alternative must have the shortest distance from the ideal solution and the farthest distance from the negative ideal solution in a geometric sense (Webster 1999).

The application of these tools in the area of biopolymers allows selecting the most suitable raw materials for their manufacture, the plasticizer, or even the best use for the material. In a study, for example, the ELECTRE method was used to analyze four types of biopolymers (thermoplastic starch, polylactic acid, polyhydroxyalkanoates, polyvinyl alcohol) and select among them the best for the manufacture of packaging. They evaluated six criteria: economic benefits achieved by applying the solution, harmonization with legislation, degree of degradation of the biomaterial, cost of project implementation, amount of waste recovery, and energy recovered from waste. These criteria focused on three development indicators: social, economic, and environmental. After evaluation, it was concluded that polyhydroxyalkanoates (PHAs) are economically and environmentally suitable for the manufacture and use of packaging (Comaniță et al. 2015).

In another experience, a lignocellulosic culture used for silage and pasture was evaluated through MCDM tools along with the life cycle for the production of basic products such as ethanol, polylactic acid, or biomaterials such as thermoplastic starch and biocomposites (Chambost et al. 2018). Multicriteria analysis was also

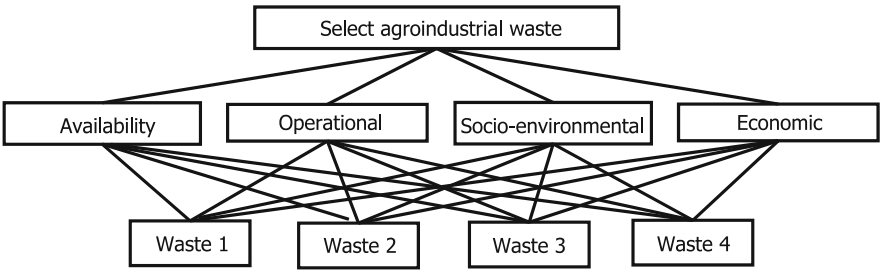

Fig. 13.5 Evaluation criteria to select the best agro-industrial waste for biopolymer obtained

applied to evaluate the use of sugarcane bagasse along with other lignocellulosic waste for three scenarios: co-production of ethanol, lactic acid, and/or electricity (Mandegari et al. 2017). Another work allowed determining the best location for the installation of a biorefinery, capable of processing cocoa crop residues for the generation of bioenergy, with an integrated fuzzy and AHP methodology based on the use of geographic information systems (Rodríguez et al. 2017).

These tools are also applicable in countries where agriculture occupies an important place in the economy. As evaluation criteria for the selection of agro-industrial waste, oriented to the production of biopolymers, four are proposed: availability of the residual, operational, socio-environmental, and economic. The first evaluates the raw material having as attributes the estimation of biomass (current and future), ease of access, and its composition in cellulose or starch content to guarantee its use as a thermoplastic material or source of fermentable sugars for its bioconversion.

The operational one analyzes the technological availability for the residual processing, the technical requirements for the processing methodologies, and the required workforce. The socio-environmental to assess the sustainability of the proposal, risks and environmental impact, as well as assess the benefits for neighboring communities. In addition, the economic one allows to carry out a cost-benefit analysis of the investment and measure the long-term profitability (Fig. 13.5).

The multicriteria analysis for decision-making considers the interaction of multiple criteria, for decision-making in the face of complex problems. Therefore, its application would help to select the best lignocellulosic waste as a biomass source, to guarantee the obtaining of biopolymers, under an economic-environmental scheme, which reduces costs for experimentation and tests, while guaranteeing the production of materials capable of competing with their synthetic relatives, within the framework of the circular economy and the 2030 Sustainable Development Goals.

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Chapter 14
Mathematical Modeling Challenges Associated with Waste Anaerobic Biodegradability

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Abstract Anaerobic digestion (AD) is a biological process, which, due to the multiple stages and microorganisms it involves, is complex to model. The feasibility of AD is highly dependent on the organic matter content, as well as physical and chemical factors that regulate the microbiological activity. Mathematical models are a constant challenge for the simulation and prediction of organic matter degradation and biogas production. This chapter is an overview of part of the great diversity of AD mathematical models from the stoichiometric and kinetic perspectives as well as microbiological and physicochemical points of view. The effect of waste composition and the changes in operational parameters on the AD modeling is analyzed. Stoichiometric, kinetic, and dynamic models are discussed. According to the review, it was confirmed that a wide number of researchers prefer the Buswell model, the first-order model, the modified Gompertz model, and the anaerobic digestion model (ADM) depending on available data and lab infrastructure. The literature related to AD modeling does not present a consensus regarding the use of statistical criteria, being a key factor to reflect the goodness of fit of the models. It was observed that there are still gaps in the co-digestion modeling due to the mixing effects on the kinetics of the anaerobic digestion. Current co-digestion models are derived from the experimental design to prove synergy or antagonism. Nevertheless, there is a need to predict the co-substrate synergy or antagonism with the kinetics, an aspect that is not solved at present using current models. To fill this scientific gap, an additive model is proposed.

Keywords Anaerobic digestion · Co-digestion · First-order model · Gompertz model · Statistical criteria

14.1 Introduction

Anaerobic digestion (AD) is a complex process where facultative and anaerobic bacteria and methanogenic archaea interact to convert organic molecules (e.g., carbohydrates, lipids, proteins) into useful products. The product of greatest interest is a gaseous mixture of methane (CH_4) and carbon dioxide (CO_2), commonly known as biogas. The possibility of efficiently degrading organic matter while simultaneously producing biogas as an energy carrier makes AD suitable for the treatment of solid and liquid organic-rich waste. Today, AD is considered a mature technology (Wang et al. 2020; Zitomer et al. 2008).

The feasibility of AD to degrade organic matter into biogas is highly dependent on the characteristics of the organic matter, as well as physical and chemical factors that regulate the microbiological activity. Among the different factors that influence AD performance, temperature, pH, the presence of inhibitory compounds, and the

lack of micronutrients appear particularly relevant on process performance (González-Suárez et al. 2018; Nguyen et al. 2019).

Mathematical models are tools capable of modeling and predicting variations in process behavior as a result of operational and environmental changes, which allow the prediction of organic matter degradation and biogas production. Additionally, mathematical models are an alternative to lab-based research to improve the understanding of AD, as well as to develop strategies to improve process performance, e.g., act against the presence of inhibitions in the system.

Biomethane potential (B_0) is a critical parameter in anaerobic digestion application since it determines the maximum amount of methane that can be recovered from a substrate (Hafner et al. 2020). Numerous methodologies have been used to quantify the biomethane potential of sole or combined substrates, since this is a necessary step for any AD industrial application (Nguyen et al. 2019). The accuracy of these methodologies is generally high, but they are time-consuming (Amodeo et al. 2020).

There is a great diversity of mathematical models, which approach the AD process from different combinations of stoichiometric and kinetic perspectives as well as microbiological and physicochemical aspects (Echiegu 2015; Pavlostathis and Giraldo-Gomez 1991; Pererva et al. 2020b). Most of the mathematical equations used in the literature provide an explanation of the process by means of kinetic parameters. Finally, another important aspect to take into consideration is the mathematical complexity of the models. Two large groups of models can be distinguished, named as "simple" and "complex" models. Simple models prioritize identifying methane production, using linear and nonlinear algebraic equations. Complex models explain the simultaneous variations of microorganisms, substrates, and methane, generally by means of a set of ordinary differential equations.

14.2 Overview of Waste Biodegradation Under Anaerobic Conditions

14.2.1 Steps of Anaerobic Digestion Process

In early stages, anaerobic digestion was simplified to a two-step process: fermentation (acid formation) and methanization (gas formation), each mediated by communities of bacteria and archaea that are physiologically different. The fermentation step was carried out by microorganisms able to convert carbohydrates, lipids, and proteins into fatty acids through hydrolysis and fermentation, to be later transformed into carbon dioxide and methane, by means of methanogenic archaea (Toerien and Hattingh 1969).

McCarty and Smith (1986) described the AD process using a stoichiometric model that involved three additional steps for the transformation of ethanol, propionate, and butyrate into methane. Each step transformed the organic molecule into

acetate with the release of a hydrogen molecule and a hydrogen cation. Methane was produced from two distinct pathways. On the one hand, the molecular hydrogen reacts with carbon dioxide to form part of the methane produced in the process. On the other hand, acetate ions react to form methane.

Today, the most common AD approach includes four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each step is mediated by a specific group of microorganisms (Batstone et al. 2006; Calusinska et al. 2018; Nakasaki et al. 2019). In hydrolysis, complex particulate biomolecules are degraded to monosaccharides, long-chain fatty acids (LCFA), and amino acids. Hydrolysis is exogenous and occurs from enzymes excreted by hydrolytic-fermentative bacteria. Subsequently, in the acidogenic step, monosaccharides and amino acids are transformed into propionic, butyric, and valeric acids and, to a lesser extent, into glycerol, ethanol, and methanol. In the acetogenic step, monosaccharides, amino acids, LCFA, and volatile fatty acids (VFA) are transformed into acetic acid, carbon dioxide, and hydrogen. In the methane-producing steps, acetic acid is transformed to methane by a group of methanogenic archaea called acetoclastic methanogens, while hydrogenotrophic methanogens produce methane from carbon dioxide and hydrogen (Batstone et al. 2006; Silva and De Bortoli 2020).

14.2.2 Effect of Waste Composition on the Anaerobic Process

Several substrates are used as organic matter source for the anaerobic process. Most of them are waste streams from different anthropogenic activities. Table 14.1 summarizes the main characteristics of different substrates in order to illustrate their diversity. The classification of these wastes depends on their origin and composition. On the one hand, according to Chen et al. (2008), waste can be classified according to their origin, including:

1. Municipal wastes: These are generated in the urban sector from domestic, commercial, and service activities. Anaerobic treatment of these residuals usually includes stages of separation of the inorganic and organic fraction. Two major groups are distinguished: municipal wastewater and organic fraction of municipal solid waste (OFMSW).
2. Agricultural wastes: These are generated in rural sectors; in some cases, the generation points are far from each other, so the most common practices are on-site treatment. They are divided into animal waste and crop residues.
3. Industrial wastes: This group includes waste from agro-industries and chemical industries. Food industry wastes receive the most attention.

On the other hand, the amount and rate of methane production are influenced by the substrate empirical formula (i.e., content of carbon, hydrogen, oxygen, and nitrogen) and the molecular structure of the organic matter. Rasapoor et al. (2020) proposed a classification based on the waste composition:

Table 14.1 Summary of different characterizations of wastes

Waste	COD	TS	VS	Carb.	Lignin	Prot.	Lip.	C/N	Reference
Mango leaves	n.d.	89.85[b]	80.15[d]	n.d.	n.d.	n.d.	n.d.	29.70	Abudi et al. (2020)
Pig manure	n.d.	28.29[b]	19.5[d]	n.d.	n.d.	n.d.	n.d.	12.47	
Paunch	106[a]	117[a]	90.60[b]	55.5[a]	n.d.	10.2[a]	4.5[a]	n.d.	Astals et al. (2014)
Blood	178[a]	187[a]	95.19[b]	3.7[a]	n.d.	129.5[a]	1.5[a]	n.d.	
DAF sludge	353[a]	360[a]	98.06[b]	0.6[a]	n.d.	11.8[a]	265[a]	n.d.	
Zucchini leaves	n.d.	15.31[b]	11.50[d]	5.69[a]	1.76[d]	0.05[a]	n.d.	8.48	Cai et al. (2019)
Snow pea leaves	n.d.	18.01[b]	14.36[d]	7.52[a]	0.74[d]	0.05[a]	n.d.	12.72	
Potato leaves	n.d.	10.50[b]	8.36[d]	3.50[a]	0.82[d]	0.15[a]	n.d.	7.01	
Potato stem	n.d.	5.28[b]	3.68[d]	1.46[a]	0.16[d]	0.03[a]	n.d.	5.05	
Pepper leaves	n.d.	17.55[b]	12.36[d]	5.56[a]	1.18[d]	0.04[a]	n.d.	7.94	
Pepper stem	n.d.	16.39[b]	13.67[d]	11.05[a]	2.56[d]	0.01[a]	n.d.	12.96	
Bell pepper leaves	n.d.	16.59[b]	13.02[d]	5.80[a]	0.84[d]	0.15[a]	n.d.	6.74	
Bell pepper stem	n.d.	12.52[b]	10.07[d]	5.78[a]	0.85[d]	0.05[a]	n.d.	8.58	
Eggplant leaves	n.d.	18.74[b]	14.00[d]	6.89[a]	1.03[d]	0.09[a]	n.d.	8.53	
Eggplant stem	n.d.	14.79[b]	12.46[d]	8.35[a]	1.19[d]	0.15[a]	n.d.	16.23	
Lettuce	n.d.	4.87[b]	3.77[d]	2.07[a]	0.74[d]	0.05[a]	n.d.	8.38	
Spinach	n.d.	8.89[b]	6.81[d]	2.9[a]	0.66[d]	0.05[a]	n.d.	8.04	
Bok choy	n.d.	3.93[b]	2.56[d]	1.22[a]	0.19[d]	0.02[a]	n.d.	5.26	
Chinese cabbage	n.d.	5.67[b]	3.94[d]	1.9[a]	0.37[d]	0.01[a]	n.d.	5.83	
Crown daisy	n.d.	6.40[b]	4.77[d]	2.22[a]	0.78[d]	0.05[a]	n.d.	7.41	
Celery leaves	n.d.	11.41[b]	8.21[d]	3.47[a]	0.64[d]	0.06[a]	n.d.	11.08	
Celery stem	n.d.	3.95[b]	2.71[d]	2.42[a]	0.48[d]	0.04[a]	n.d.	16.46	
Rice husk	n.d.	89.2[b]	77.8[d]	n.d.	18.9[d]	n.d.	n.d.	99	Contreras et al. (2012)
Rice straw	n.d.	87.8[b]	79.6[d]	n.d.	8.6[d]	n.d.	n.d.	43	
Drying process rice	n.d.	89.3[b]	77.5[d]	n.d.	10.4[d]	n.d.	n.d.	33	

(continued)

Table 14.1 (continued)

Waste	COD	TS	VS	Carb.	Lignin	Prot.	Lip.	C/N	Reference
Domestic food	511.5[a]	399.4[a]	73.73[d]	n.d.	n.d.	n.d.	n.d.	n.d.	Dennehy et al. (2016)
Pig manure	80.9[c]	78.1[c]	56.1[c]	n.d.	n.d.	n.d.	n.d.	n.d.	
Sewage sludge	n.d.	6.7[b]	73.9[d]	n.d.	n.d.	n.d.	n.d.	8.54	Güngören Madenoğlu et al. (2019)
Vinasse	299.25[c]	27.87[b]	284.66[c]	268.65[c]	n.d.	9.12[c]	6.89[c]	n.d.	Iqbal Syaichurrozi and Sumardiono (2014)
Glycerol	1056[c]	829[c]	746[c]	n.d.	n.d.	n.d.	n.d.	n.d.	Jensen et al. (2014)
Glycerol	912[c]	933[c]	844[c]	n.d.	n.d.	n.d.	n.d.	n.d.	
Chicken manure	10.74[c]	6.43[c]	6.76[c]	n.d.	n.d.	n.d.	n.d.	12.40	Jijai and Siripatana (2017)
Dairy manure	n.d.	16.91[b]	10.25[d]	7.93[a]	1.58[d]	1.45[a]	0.43[a]	25	Kafle and Chen (2016)
Horse manure	n.d.	24.97[b]	18.61[d]	14.92[a]	4.53[d]	3.03[a]	0.87[a]	23	
Goat manure	n.d.	81.63[b]	64.23[d]	50.19[a]	11.17[d]	12.00[a]	2.72[a]	20	
Chicken manure	n.d.	67.84[b]	47.50[d]	29.30[a]	3.44[d]	18.32[a]	0.69[a]	10	
Swine manure	n.d.	31.02[b]	26.93[d]	15.87[a]	1.18[d]	8.31[a]	2.92[a]	12	
Raw dairy manure	128.9[a]	124[a]	82.34[b]	74.02[a]	14.09[a]	5.82[a]	16.44[a]	n.d.	Labatut et al. (2011)
Cheese whey	64.9[a]	128.3[a]	55.65[b]	57.76[a]	0[a]	9.57[a]	4.07[a]	n.d.	
Plain pasta	934.3[a]	442.6[a]	96.47[b]	342.01[a]	0[a]	70.45[a]	14.52[a]	n.d.	
Meat pasta	562.8[a]	381.8[a]	89.21[b]	227.18[a]	0[a]	65.74[a]	47.68[a]	n.d.	
Used vegetable oil	2880[a]	991[a]	99.78[b]	0[a]	0[a]	0[a]	988.82[a]	n.d.	
Ice cream	266.8[a]	113.8[a]	109.1[b]	65.93[a]	0[a]	10.3[a]	47.92[a]	n.d.	
Snap bean	n.d.	21.08[b]	18.30[c]	15.45[a]	1.08[d]	4.48[a]	0.72[a]	11.65	Li et al. (2018b)
Capsicum	n.d.	20.35[b]	16.41[c]	15.29[a]	1.69[d]	3.08[a]	0.34[a]	15.45	
Cucumber	n.d.	21.87[b]	16.56[c]	16.33[a]	2.46[d]	1.93[a]	0.44[a]	22.76	
Eggplant	n.d.	19.40[b]	16.66[c]	15.13[a]	2.04[d]	2.90[a]	0.69[a]	16.10	
Tomato	n.d.	17.52[b]	14.53[c]	12.66[a]	1.33[d]	3.25[a]	0.43[a]	11.99	
Food waste	n.d.	19.10[b]	93.20[d]	11.80[b]	n.d.	2.50[b]	3.50[b]	14.4	Li et al. (2018a)
	n.d.	12.70[b]	95.40[d]	36.00[b]	n.d.	41.50[b]	18.5[b]	9.71	

Borås-Sweden OFMSW	n.d.	84[b]	4[d]	77[a]	n.d.	26[a]	12[a]	n.d.	Pagés-Díaz et al. (2014)
Mixed manure	n.d.	35[b]	40[d]	180[a]	n.d.	26[a]	180[a]	n.d.	
Slaughterhouse	n.d.	26[b]	95[d]	1[a]	n.d.	130[a]	175[a]	n.d.	
Mixed crop wastes	n.d.	24[b]	90[d]	287[a]	n.d.	21[a]	2[a]	n.d.	
Mixed crop wastes	55[a]	54.0[a]	83[d]	50.1[e]	15.6[e]	12.6[e]	10.0[e]	n.d.	Pagliaccia et al. (2019)
Kitchen waste	325[a]	236[a]	95[d]	79.8[e]	n.q.	13.3[e]	6.0[e]	n.d.	
Treviso-Italy OFMSW	395[a]	334[a]	91[d]	53.2[e]	20[e]	5.2[e]	11.0[e]	n.d.	

Carb carbohydrates, *Prot* proteins, *Lip* lipids
[a][g kg^{-1}ww]
[b][%]
[c][g L^{-1}]
[d][%TS]
[e][%COD]

1. Protein-rich residues include meat, bones, blood waste from slaughterhouse, food industry (Astals et al. 2014; Pagés-Díaz et al. 2014), swine and chicken manure (Kafle and Chen 2016). In anaerobic degradation of proteins, NH_3 is obtained as an intermediate product. Ammonia is used for cell growth, but high concentration can lead to process inhibition (Rasapoor et al. 2020).
2. Carbohydrate-rich wastes such as fruits, vegetables (Cai et al. 2019; Labatut et al. 2011; Pagés-Díaz et al. 2014), food waste (Labatut et al. 2011; Pagés-Díaz et al. 2014; Pagliaccia et al. 2019), and mixed rumen and paunch from slaughterhouse waste (Astals et al. 2014; Pagés-Díaz et al. 2014). Cai et al. (2019) characterized the carbohydrates from residue samples in correspondence of their nature: structural (lignin, cellulose, and hemicellulose) and nonstructural (soluble sugars). Structural carbohydrates are found in cell walls in leaves, stems, some vegetables, and fruits, as well as in manure fibers.
3. Lipid-rich organic matter includes waste from the oil processing industry, food waste (Labatut et al. 2011), dissolved air flotation fat sludge (Astals et al. 2014), and municipal wastewaters Nakasaki et al. (2019). Nakasaki et al. (2019) indicated that microorganisms and their enzymes can actively degrade the water-soluble fraction, but the low solubility of fats limits their degradation. Lipid-rich wastes during the hydrolysis step produce long-chain fatty acids (LCFA) which are inhibitory to AD at different degrees. The principal stages affected by the presence of LCFA are acetogenesis and methanogenesis. LCFA disrupt membrane functionality as they are adsorbed into the cell (Ohemeng-Ntiamoah and Datta 2018). Rodriguez-Mendez et al. (2017) proposed two indicators to prevent inhibition: LCFA dynamics and LCFA/$VS_{biomass}$ ratio. Those control parameters were useful to predict and estimate the process inhibition degree.

It is common to find lipid-rich waste with high content of protein. Microbial behavior is similar due to the complexity of the hydrolysis stage due to the transformation of both components. Nakasaki et al. (2019) found *Methanosaeta* as the most dominant archaea in lipid-rich substrate, while Ning et al. (2018) found *Methanospirillum* as the predominant methanogenic archaea. Studies developed by Zhu et al. (2019) demonstrated that LCFA strongly inhibit acetoclastic methanogens promoting methane formation from the hydrogenotrophic pathway.

Substrate chemical characterization is necessary to evaluate the performance in anaerobic digestion processes. Some basic parameters for substrate characterization are chemical oxygen demand (COD), total solids (TS), and volatile solids (VS), which are used to estimate substrate degradability. More detailed research analyzed the content of carbohydrates, proteins, and lipids to estimate the amount of methane to be produced and elemental analyses to identify imbalances in the supply of carbon and nitrogen in the medium. Some common practices are to present the characterizations based on COD, total solids, or, in the case of liquid residues, the mass concentration.

Factors such as temperature, pH, and acclimatization of the inoculum to the substrate can influence the behavior of the microbial community involved (Chen et al. 2008). According to Astals et al. (2015), in an AD reactor, failures occur due to

(1) inhibitions (reversible effects, cause the decrease in microbiological function) and (2) toxicity (biocidal effect in microbiological communities, generally irreversible but not necessarily fatal). These effects are the product of the accumulation of substances: (1) generated in the biological pathway (LCFA, ammonia, and sulfide), (2) specific to the composition of the substrate (light metals, heavy metals, chlorophenols, halogenated aliphatics, and lignin), and (3) contaminants added to the substrate (nanoparticles, pharmaceuticals and personal care products, surfactants, microplastics, coagulants, and flocculants).

In terms of the process, the substrate composition is crucial for biogas production and rate. Both response variables depend on the relative richness of lipids (yield related) and proteins (rate related) (Lee et al. 2020). In the case of carbohydrate-rich waste, *Firmicutes* and *Proteobacteria* are widely distributed due to the ability to transform macromolecules in acidic media as the findings reported by Satpathy et al. (2015), Zhao et al. (2017), and Li et al. (2019), among others. Mixing several wastes to compensate substrate deficiencies (a process known as anaerobic co-digestion) stands as a feasible option to reach higher methane yields (Pagés-Díaz et al. 2014). The literature mentions that mixtures of substrates can have synergistic or antagonistic effects on methane production (Abudi et al. 2020; Astals et al. 2014; Li et al. 2018a, b; Pagés-Díaz et al. 2014). On the other hand, stimulation from different sources has increased both methane yield and rate in more than 20% as proven by González-Suárez et al. (2018) and Xu et al. (2020). As far as we know, the better environmental conditions might be created to control and optimize the bioprocess. This reinforces the complexity of modeling AD in which minor changes in operational parameters or substrate composition swift the microbial community with relative important changes in response variables.

14.3 Modeling the Anaerobic Biodegradation of Residues

14.3.1 Stoichiometric Models

The literature reflects a great diversity of mathematical models for predicting AD behavior. Symons and Buswell (1933) proposed an empirical oxide-reduction reaction for the DA of carbohydrates in the presence of water, which were transformed to carbon dioxide and methane:

$$C_aH_bO_c + \left(\mathrm{a} - \frac{b}{4} - \frac{c}{2}\right)H_2O \rightarrow \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right)CO_2 + \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right)CH_4 \quad (14.1)$$

This model was an approach to the estimation of methane production with an estimation uncertainty of close to 5% in substrates such as dextrose, lactose, maltose, and sucrose, among others (Buswell and Mueller 1952; Symons and Buswell 1933).

This stoichiometric model involved a two-step reaction, the first to transform organic molecules to organic acids and the second to form methane.

The model did not consider factors such as growth of microorganisms, time, temperature, or inhibitory conditions. Buswell and Mueller (1952) reaffirmed the model (Eq. (14.1)) and defined that the estimates made were valid for the mesophilic and thermophilic regimes, in addition to ensuring that the variations in the experimental and theoretical results were due to causes such as (1) H_2 gas production, (2) the presence of structural carbohydrates such as cellulose and lignocellulose, (3) the variation of pH in the reactors, and (4) concentrations of inhibitory agents. Equation (14.1) adopted the name of Buswell's formula; it was the beginning of the development of other models. From this point on, the mathematical modeling of anaerobic digestion has pursued three objectives: (1) characterizing the steps of AD, (2) evaluating the kinetics of the process, and (3) describing the interactions between the biochemical, chemical, and physical processes.

Labatut et al. (2011) used the Buswell formula to predict the theoretical methane yield ($B_{0\text{-}Theo}$) of food residues, manure, invasive aquatic plants, switchgrass, and various liquid residues, in addition to testing mixtures of excreta with part of the evaluated residuals. Experimental methane yields were estimated as the total volume of methane produced during digestion divided by the amount of volatile solids in the substrate initially added. The results of the comparison showed that the Buswell formula overestimates the methane yield, since the model does not consider the substrate biodegradable fraction. To fit the Buswell's formula, Labatut et al. (2011) defined the biodegradable fraction (f_D) as:

$$f_D = \frac{COD_D}{COD_T} \tag{14.2}$$

This expression considers the degradable chemical oxygen demand (COD_D) from the experimental methane yield and the proposed ratio of 1 g of chemical oxygen demand for each 350 mL of CH_4 produced under standard conditions of pressure and temperature (1 atm and 0 °C). The term COD_T refers to the total chemical oxygen demand of the substrate, which can be determined analytically, or can also be calculated by means of the elemental composition or the macromolecular composition. From the correction of the theoretical yields, the behavior of the results reflected that the Buswell formula reports theoretical performance values of methane close to the theoretical ones, with an error of close to 10%.

The Buswell's formula is widely used in determining the theoretical methane yield. To simplify the stoichiometric model, the expression used is:

$$B_{0Theo}\left[NmL\ CH_4\ gVS^{-1}\right] = \frac{(4a + b - 2c)22400}{(12a + b + 16c)8} \tag{14.3}$$

Achinas and Euverink (2016), Adghim et al. (2020), Contreras et al. (2012), Pagés-Díaz et al. (2014), and Raposo et al. (2011) implemented the Buswell's formula to evaluate the biodegradability of various substrates as starch, cellulose,

gelatin, agricultural residuals, livestock waste, poultry, and piggery. In addition to the modification proposed by Labatut et al. (2011), the literature reports other modifications such as:

1. Modified Buswell's formula: This modification allows the prediction of the methane yield from substrates that contain proteins. The expressions representing the stoichiometric equation and the methane yield are (Contreras et al. 2012; Lubken et al. 2010):

$$C_aH_bO_cN_d + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4}\right)H_2O \rightarrow \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8}\right)CO_2 + \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{4}\right)CH_4 + dNH_3 \quad (14.4)$$

$$B_{0Theo}\left[NmL\ CH_4\ gVS^{-1}\right] = \frac{(4a + b - 2c - 3d)22400}{(12a + b + 16c + 14d)8} \quad (14.5)$$

2. Boyle's equation: This modification includes the estimation of H_2S from the stoichiometric reaction (Deublein and Steinhauser 2008) and the theoretical methane yield (Frigon and Guiot 2010; Raposo et al. 2011):

$$C_aH_bO_cN_dS_e + \frac{(4a - b - 2c + 3d + 2e)}{4}H_2O \rightarrow \frac{(4a + b - 2c - 3d - 2e)}{8}CH_4 + \left[a - \frac{(4a + b - 2c - 3d - 2e)}{8}\right]CO_2 + dNH_3 + eH_2S \quad (14.6)$$

$$B_{0Theo}\left[NmL\ CH_4\ gVS^{-1}\right] = \frac{(4a + b - 2c - 3d - 2e)22400}{(12a + b + 16c + 14d + 32e)\ 8} \quad (14.7)$$

3. Pererva's modification: This modification includes phosphorus and metals in the composition of organic matter. Stoichiometric reaction and performance are (Pererva et al. 2020a):

$$C_aH_bO_cN_dS_eP_fMe_g + \left(\frac{4a - b - 2c + 3d + 2e + 11f + 3g\ v}{4}\right)H_2O \rightarrow \left(\frac{4a + b - 2c - 3d - 2e + 5f + g\ v}{8}\right)CH_4 + \left(\frac{4a - b + 2c + 3d + 2e - 5f - g\ v}{8}\right)CO_2 + dNH_3 + eH_2S + fH_3PO_4 + g\ Me(OH)_v \tag{14.8}$$

$$B_{0_{Theo}}\left[NmL\ CH_4\ gVS^{-1}\right] = \frac{(4a + b - 2c - 3d - 2e + 5f + g\ v)\ 22400}{(12a + b + 16c + 14d + 32e + 30.9f + M(Me)\ g)\ 8} \tag{14.9}$$

14.3.2 Kinetic Models

Kinetic models are characterized by representing DA by means of nonlinear expressions as a function of time. According to Echiegu (2015), the kinetic models can be classified as microbial growth and kinetic models of methane production or cumulative reduction of the organic fraction.

14.3.2.1 Microbial Growth Models

Contois (1959) identified how the specific growth rate of microorganisms in a DA reactor is related to the concentration of nutrients as well as population density. The relationship between the consumption of the limiting nutrient and the population density was represented by means of a linear expression:

$$c(S_0 - S) = (P - P_0) \tag{14.10}$$

The coefficient c is the yield of the process [organisms mL^{-1} mM^{-1}]; S is the concentration of the limiting substrate [mM]; P is the population density; finally, the subscript *0* designates the initial values. For the estimation of the specific growth rate (R) [h^{-1}], Contois used a non-steady-state mass balance of population density:

$$\frac{dP}{dt} = RP - DP \tag{14.11}$$

where D is the dilution factor in the medium [h^{-1}]. Contois (1959) evaluated expression Eq. (14.11) for a continuous reactor under steady-state conditions and

deduced from the Monod equation a relationship between the growth rate and the concentration of the limiting nutrient:

$$D = R = \frac{R_m S_e}{A + S_e} \tag{14.12}$$

where R_m [h^{-1}], A [mM], and S_e [mM] are the maximum growth rate, the concentration of the limiting nutrient when R is ½ R_m, and the concentration of the limiting nutrient in equilibrium, respectively. McCarty and Mosey (1991) present the Contois model as:

$$-\frac{dS}{dt} = \frac{kXS}{(aS_0 + S)} = \frac{kXS}{(K_s + S)} \tag{14.13}$$

As observed in Eq. (14.13), aS_0 is related to the Monod kinetic constant (K_s) [mM], k is the growth rate [h^{-1}], and X is the concentration of the microorganism [organisms mL^{-1}]. In addition, the Monod performance equation is implemented in two ways. The first is for the growth of microorganisms, in which the maintenance constant (m) [h^{-1}] is presented:

$$\frac{dX}{dt} = Y\frac{dS}{dt} - mX \tag{14.14}$$

where Y is the growth yield coefficient [mM mM^{-1}]. The second is for the cell decay stage, with its respective constant (b) [h^{-1}]:

$$\frac{dX}{dt} = Y\frac{dS}{dt} - bX \tag{14.15}$$

Both cases present equations with similar structures; these differ in the interpretation of b and m. These constants in high-load reactors are ignored, as well as for methanogens, which have low maintenance and decay coefficients. Lawrence and McCarty (1969) implemented the model for steady-state reactors and concluded that the reciprocal of biological solids retention time (θ_c) [h] relates to the specific net rate of growth (μ) [h^{-1}]:

$$\frac{1}{\theta_c} = \frac{1}{X}\frac{dX}{dt} = \mu = \frac{akS}{K_s + S} - b \tag{14.16}$$

The behaviors of microbial communities in anaerobic reactors are diverse, for which other models have been developed to describe the system. Some models have been presented to evaluate the microbial growth rate (μ), the substrate variation $\left(\frac{dS}{dt}\right)$, and the concentration of the substrate (S). Echiegu (2015), Öktem (2019), and Pavlostathis and Giraldo-Gomez (1991) summarize some models of microbial growth kinetics and substrate consumption:

1. First-order model:

$$\mu = \frac{kS}{S_0 - S} - b \tag{14.17}$$

$$-\frac{dS}{dt} = kS \tag{14.18}$$

$$S = \frac{S_0}{1 + k\theta_c} \tag{14.19}$$

2. Monod model:

$$\mu = \frac{\mu_{max} S}{K_s + S} - b \tag{14.20}$$

$$-\frac{dS}{dt} = \frac{\mu_{max} XS}{Y(K_s + S)} \tag{14.21}$$

$$S = \frac{K_s(1 + b\theta_c)}{\theta_c(\mu_{max} - b) - 1} \tag{14.22}$$

where μ_{max} is the maximum rate of microbial growth [h^{-1}].

3. Contois model:

$$\mu = \frac{\mu_m S}{BX + S} - b \tag{14.23}$$

$$-\frac{dS}{dt} = \frac{\mu_m XS}{Y(BX + S)} \tag{14.24}$$

$$S = \frac{B\ Y\ S_0(1 + b\theta_c)}{B\ Y\ (1 + b\theta_c) + \theta_c(\mu_m - b) - 1} \tag{14.25}$$

4. Grau model:

$$\mu = \frac{\mu_{max} S}{S_0} - b \tag{14.26}$$

$$-\frac{dS}{dt} = \frac{\mu_{max} XS}{YS_0} \tag{14.27}$$

$$S = \frac{S_0(1 + b\theta_c)}{\mu_{max}\theta_c} \tag{14.28}$$

5. Chen and Hashimoto model:

$$\mu = \frac{\mu_{max} S}{K S_0 + (1 - K) S} - b \tag{14.29}$$

$$-\frac{dS}{dt} = \frac{\mu_{max} XS}{KX + YS} \tag{14.30}$$

$$S = \frac{K\, S_0 (1 + b\theta_c)}{(K - 1)(1 + b\theta_c) + \mu_{max}\theta_c} \tag{14.31}$$

Microbial activity can be affected; in this sense, models have been developed to study factors such as temperature as well as inhibitions. Lawrence and McCarty (1969) related *Ks* to the fermentation temperature, with an expression similar to the Arrhenius equation:

$$\log \frac{(K_s)_2}{(K_s)_1} = 6980 \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \tag{14.32}$$

Echiegu (2015) indicated that a more accepted alternative to estimate the effect of temperature on reaction speed is the van't Hoff relationship:

$$r_T = r_o\, T_a^{(T - T_0)} \tag{14.33}$$

where r_T can be either μ or k, at an operating temperature (T); r_0 is defined at a reference temperature (T_0), usually of 20 °C; and T_a is the temperature activity coefficient.

McCarty and Mosey (1991) studied the influence of pH on AD and proposed an inhibition factor (K_i) and a pH inhibition function (i_{pH}):

$$-\frac{dS}{dt} = \frac{k\, K_i\, XS}{(aS_0 + S)} \tag{14.34}$$

$$\frac{dX}{dt} = Y \frac{dS}{dt} - i_{pH} bX \tag{14.35}$$

The inhibition factor (K_i) has been implemented in various expressions to estimate the microbial growth rate in the presence of recalcitrant agents. One of the most recurrent expressions is the Haldane equation, which is a direct modification of the Monod equation. Haldane equation and other modifications are presented by (Kul and Nuhoğlu 2020; Li et al. 2020; Pishgar 2011; Priya et al. 2018):

1. Haldane equation:

$$\mu = \frac{\mu_{max}\, S}{K_s + S + \left(\frac{S^2}{K_i}\right)} \tag{14.36}$$

2. Aiba equation:

$$\mu = \frac{\mu_{max}\, S \exp\left(-\frac{S}{K_i}\right)}{K_s + S} \tag{14.37}$$

3. Yano and Koga equation:

$$\mu = \left(\frac{\mu_{max}\, S}{K_s + S + \frac{S^2}{K_i}}\right) - \left(\frac{S^3}{K_i\, K_{YK}}\right) \tag{14.38}$$

4. Tseng equation:

$$\mu = \left(\mu_{max}\left(\frac{S}{K_s} + S\right)\right) - (K_i(S - S_m)) \tag{14.39}$$

where S_m is the concentration of the recalcitrant agent for which the system has no inhibition [mg L^{-1}] and K_{YK} is an adjustment positive constant of the Yano and Koga equation. Table 14.2 presents expressions for pH inhibition (i_{pH}) (Astals et al. 2014; Batstone et al. 2002; Rosén and Jeppsson 2006):

Table 14.2 Inhibition expressions

Noncompetitive inhibition	Hydrogen, free ammonia, propionate, butyrate, valerate, LCFA
$i = \frac{1}{1+\frac{S_I}{K_I}}$ (14.40)	
Empirical	Any pH range Low pH range
$i_{pH} = \frac{1 + 2x10^{0.5(pH_{LL} - pH_{UL})}}{1 + 10^{(pH - pH_{UL})} + 10^{(pH_{LL} - pH)}}$ $i_{pH} = e^{\left(-3\left(\frac{pH - pH_{UL}}{pH_{UL} - pH_{LL}}\right)^2\right)}$ (14.41)	
Hill inhibition function	Amino acids, acetic acid, hydrogen
$i = \frac{K_{pH}^{n_i}}{S_{H^+}^{n_i} + K_{pH}^{n_i}}$ $K^n = 10^{-\frac{pH_{LL} + pH_{UL}}{2}}$ (14.42) $n_i = \frac{3}{pH_{UL,i} - pH_{LL,i}}$	

14.3.2.2 Production, Yield, and Cumulative Reduction Kinetics of the Organic Fraction

According to Husain (1998), the complete mathematical treatment of the DA process requires the simultaneous solution of material balance equations for each individual substrate and microbiological population. Due to the complexity of microbiological processes, part of the efforts has focused on the study of methane production as well as the reduction of organic components. Brulé et al. (2014) describe the organic matter consumption by means of a first-order reaction model:

$$\left(\frac{dS_t}{dt}\right) = -k\, S_t \tag{14.43}$$

where k is the first-order kinetic constant [d^{-1}] and S_t is the organic substrate concentration over the time [g L^{-1}]. The negative sign stands for the consumption of organic matter. When applying the separation of variables, the integral expression is:

$$\int_{S_0}^{S_t} \frac{dS_t}{S_t} = -k \int_{t_0=0}^{t_f=t} dt \tag{14.44}$$

The solution of the definite integral is:

$$S_t = S\, e^{-kt} \tag{14.45}$$

Brulé et al. (2014) explain that by rearranging terms Eq. (14.45) can be expressed in relation to the products formed (B):

$$B = B_0 \left(1 - e^{-kt}\right) \tag{14.46}$$

where B represents the variable on which the system is evaluated; this can be (1) methane yield [NmL gVS^{-1}] or [NmL $gCOD^{-1}$]; (2) methane production [mL] or [L]; or cumulative reduction of organic compounds (e.g., volatile solids, lipid, carbohydrates, and protein reduction) over the time [d]; B_0 is the maximum value of B. The literature reports the use of the first-order kinetic model and other expressions such as modified Gompertz model, Cone model, Fitzhugh model, and transfer function. Table 14.3 presents these models of great relevance in the kinetic study.where k is the first-order kinetic constant of the process [d^{-1}], λ is the lag time phase [d^{-1}], and n is a shape factor. The literature explores other models that are used much less frequently. Some models are based on the mathematical structure of microbial growth rate models such as Monod as well as Chen and Hashimoto. Another part of these models are empirical modifications to the first-order model.

Table 14.3 Most frequently used models to describe methane yield kinetics

Modified Gompertz model $B = B_0\, e(-e^{-\frac{e\ \mu_m(t-\lambda)}{B_0}+1})$ (14.47)	Abudi et al. (2020), Andriamanohiarisoamanana et al. (2017), Astals et al. (2014), Bedoić et al. (2020), Benabdallah El Hadj et al. (2009), Bohutskyi et al. (2018), Buendía et al. (2009), Cai et al. (2019), Chatterjee et al. (2017), Das Ghatak and Mahanta (2017), Dennehy et al. (2016), Dumitrel et al. (2017), Gallipoli et al. (2020), Güngören Madenoğlu et al. (2019), Iqbal Syaichurrozi and Sumardiono (2014), Jijai and Siripatana (2017), Kafle and Chen (2016), Koch et al. (2019), Li et al. (2018a, b), Maamri and Amrani (2019), and Zhen et al. (2016)
Cone model $B = \frac{B_0}{1+(kt)^{-n}}$ (14.48)	Abudi et al. (2020), Achinas and Euverink (2019), Bedoić et al. (2020), Cai et al. (2019), Güngören Madenoğlu et al. (2019), Li et al. (2018a), and Zhen et al. (2016)
Fitzhugh model $B = B_0(1 - e^{-kt})^n$ (14.49)	Cai et al. (2019), Contreras et al. (2012), and Li et al. (2018a)
Transfer function $B = B_0(1 - e^{-\frac{\mu_m(t-\lambda)}{Y_{max}}})$ (14.50)	Abudi et al. (2020), Bohutskyi et al. (2018), Gallipoli et al. (2020), and Li et al. (2018a)

Pererva et al. (2020b), who reviewed existing empirical kinetic models, identified 19 kinetic models (including the models in Table 14.3).

The kinetic study of an AD system allows predicting the behavior of an anaerobic digester against variations in the concentration of substrates, organic loads, variations in pH, temperature, and other operating conditions. Therefore, the proper selection of the model is a rigorous task. The various investigations report statisticians for the selection of a model, among which are coefficient of determination (R^2), model efficiency (ME), sum of absolute errors (SAE), sum of squared errors (SSE), root-mean-square error (RMSE), average relative error (ARE), hybrid fractional error function (HYBRID), Marquardt's percentage standard deviation (MPSD), Akaike's information criterion (AIC), and corrected Akaike's information criterion (AICc). These error function can be classified in four groups: (1) percentual or fractional indicators of approach to the trends of the experimental data, (2) model errors, (3) error deviation, and (4) model comparison criteria. Table 14.4 shows the relationship between the groups of statisticians and the objectives pursued by the optimization of each statistician.where $B_{model,\ i}$ is the model evaluated in the *i*th point, B_{exp} is the *i*th experimental value, *N* is the number of data points, and p is the number of adjusted parameters in the model. Table 14.4 shows that the objective to which each model is oriented is different. R^2 and ME present values between 0 and 1. A value close to 1 is an indicator of the best fit of the model. SAE, SSE, and ARE explain the differences between models by quantifying the absolute distance between each point of the model and the experimental data. The values of these error functions are positive, and their proximity to zero is the indicator of a smaller error and, therefore, a better fit of the model. SAE, SSE, and ARE explain the differences between models by quantifying the absolute distance between each point of the model and the experimental data. The values of these statisticians are positive,

Table 14.4 Goodness of fit parameters

Group	Expression	Objective
(i)	$R^2 = \frac{\sum_{i=1}^{N}(B_{model,i}-B_{\exp,i})^2}{\sum_{i=1}^{N}[(B_{exp,i}-\bar{B_{exp}})^2+(B_{model,i}-\bar{B_{exp}})^2]}$ (14.51)	$R^2 \approx 1$
	$ME = \frac{\sum_{i=1}^{N}(B_{\exp,i}-\bar{B_{exp}})^2-\sum_{i=1}^{N}(B_{\mathrm{mod},i}-B_{\exp,i})^2}{\sum_{i=1}^{N}(B_{\exp,i}-\bar{B_{exp}})^2}$ (14.52)	ME ≈ 1
(ii)	$SAE = \sum_{i=1}^{N}\left\lvert B_{model}-B_{exp}\right\rvert_i$ (14.53)	SAE ≈ 0
	$SSE = \sum_{i=1}^{N}\left(B_{model}-B_{exp}\right)_i^2$ (14.54)	SSE ≈ 0
	$ARE = \frac{100}{N}\sum_{i=1}^{N}\lvert\frac{(B_{model,i}-B_{exp,i})^2}{B_{exp,i}}\rvert$ (14.55)	ARE ≈ 0
(iii)	$RMSE = \sqrt{\frac{\sum_{i=1}^{N}\left(B_{model,i}-B_{exp,i}\right)^2}{N}}$ (14.56)	RMSE ≈ 0
	$MPSD = 100\sqrt{\frac{1}{N-p}\sum_{i=1}^{N}\left[\frac{(B_{model,i}-B_{exp,i})}{B_{exp,i}}\right]^2}$ (14.57)	MPSD ≈ 0
	$HYBRID = \frac{100}{N-p}\sum_{i=1}^{N}\left[\frac{(B_{model,i}-B_{exp,i})^2}{B_{exp,i}}\right]$ (14.58)	HYBRID ≈ 0
(iv)	$AIC = N\ln\left(\frac{\sum_{i=1}^{N}(B_{model,i}-B_{exp,i})^2}{N}\right)+2(p+1)$ (14.59)	Minimization
	$AIC_c = N\ln\left(\frac{\sum_{i=1}^{N}(B_{model,i}-B_{\exp,i})^2}{N}\right)+2(p+1)+\frac{2(p+1)(p+2)}{N-p}$ (14.60)	Minimization

and their proximity to zero is the indicator of a smaller error and therefore a better fit of the model. RMSE, MPSD, and HYBRID quantify the dispersion of the errors and, as in the previous case, values close to zero indicate a good fit. Finally, AIC and AICc are alternative methods used for model comparison, in which the change of goodness of fit of the model is balanced by the number of model parameters, in addition to comparing nested and non-nested models. According to Donoso-Bravo et al. (2011), model selection is a delicate task due to the parameter identification dependence on experimental information. The literature related to AD does not present a consensus regarding the use of statistical criteria. Table 14.5 identifies the expressions used in various investigations, relates the applied models, and identifies the statistics used.

Table 14.5 indicates that the first-order kinetic model and the modified Gompertz model are the most used expressions to describe these processes. The first-order kinetic model is the simplest model to interpret, adjust, and apply to any chemical, biological, or physical process. On the other hand, the modified Gompertz model is

Table 14.5 Statistical criteria used in model evaluation. First-order kinetic (FO), modified Gompertz (MG), Cone (CM), Fitzhugh (FM), transfer function (TF)

Reference	FO	MG	CM	FM	TF	Statistical criteria
Abudi et al. (2020)	x	x	x		x	RMSE; R^2; AIC
Achinas and Euverink (2019)	x		x			RMSE; R^2
Astals et al. (2014)	x					SSE
Andriamanohiarisoamanana et al. (2017)	x	x				R^2
Bedoić et al. (2020)	x	x	x			RMSE
Bohutskyi et al. (2018)	x	x			x	RMSE; R^2
Buendía et al. (2009)		x				R^2
Cai et al. (2019)	x	x	x	x		R^2
Contreras et al. (2012)	x			x		R^2
Chatterjee et al. (2017)	x	x				SSE; SAE; ARE; HYBRID; MPSD; R^2
Da Silva et al. (2018)	x					R^2
Das Ghatak and Mahanta (2017)		x				R^2
Dennehy et al. (2016)	x	x				RMSE; R^2
Du et al. (2019)	x					R^2
Dumitrel et al. (2017)		x				RMSE; R^2
Gallipoli et al. (2020)	x	x			x	R^2
Güngören Madenoğlu et al. (2019)		x	x			R^2
Iqbal Syaichurrozi and Sumardiono (2014)	x	x				R^2
Jijai and Siripatana (2017)		x				R^2
Kafle and Chen (2016)	x	x				RMSE; R^2
Koch et al. (2019)	x	x				ME
Li et al. (2018b)	x	x				RMSE; R^2
Li et al. (2018a)	x	x	x	x	x	RMSE; R^2; AICc
Maamri and Amrani (2019)		x				SE; R^2
Pagés Diaz et al. (2011)	x					R^2
Zhen et al. (2016)	x	x	x			RSS; RMSE; R^2; AIC

used in conditions for which the inoculum supplied to the medium presents a delay due to substrate adaptation.

It is also observed that R^2 is commonly used to describe goodness of fit in the kinetic models. Spiess and Neumeyer (2010) indicate that R^2 is the measure in which the variance of the data is explained by a linear fit, so its use as a measure of the goodness of fit in nonlinear models is a common error.

Nash and Sutcliffe (1970) developed the ME error function, which is a derivation from R^2, but the interval in which is bigger ($-\infty$ to 1). In addition, the interpretation is different: (1) a value of 1 indicates the best fit and (2) a value of zero indicates that the model is as good as using the mean of the observations. Values above 0.8 ensure the fit of the models.

The SAE, SEE, ARE, and RMSE criteria do not evaluate the number of parameters in the models, so two or more models could coincide; in this case, the selection will depend on the variability of the errors. HYBRID and MPSD consider in the denominators a term for the *i*th observation, so in the case of measurements at the initial time for which the values are zero, the model is indeterminate.

Akaike (1974) indicates that the AIC logarithmic probability term decreases the gaps between the experimental data and the models. Abudi et al. (2020) and Pererva et al. (2020b) consider the use of AIC pertinent, given that the probability that the selected model is the best increases as the AIC values are lower.

A common practice in fitting kinetic models is the use of only one or two statisticians in the analysis. To increase reliability in model assessment is necessary to implement combined goodness of fit criteria. Within the model selection strategy, the graphic analysis of the residuals must be considered since this allows us to easily identify if the model generates random errors or if, on the contrary, they describe trends.

14.3.3 Dynamic Models

A dynamic model considers changes in one or more dependent variables in relation to time. The general formulation of these models considers macroscopic mass balance:

$$\frac{dm_i}{dt} = -\Delta(\rho vs) + W_i^m + r_i V_{tot} \tag{14.61}$$

where dm_i/dt is the accumulation of an i component, in a defined control volume; $-\Delta(\rho\ v\ s)$ is the mass flow differential between control volume limits; W_i^m represents mass transfer processes; r_i is the kinetic law; and V_{tot} is the reactant system volume. These models can represent processes in steady or non-steady states, in which the boundary limits correspond to the inputs and outputs of the reacting system. The terms for reactive processes in Eq. (14.61) are supported by the expressions of kinetic models.

In these models, the interactions between various components and processes are related. One of the dynamic models that stands out the most is the anaerobic digestion model 1 (ADM1) created by Batstone et al. (2000), which served as a baseline for other dynamic models. The ADM1 is a macroscopic mass balance model, which takes into account biochemical conversion processes (kinetics of microbial growth and digestion), as well as physicochemical conversion of mass transfer processes. According to Batstone et al. (2000), for the creation of ADM1, kinetic models were taken into account for the degradation of substrates that consider the substrate and biomass concentrations, as well as including inhibitions by hydrogen and pH. Enzymatic conversion processes are considered, which are carried out by means of soluble enzymes produced by the bacterial group that directly uses the

Table 14.6 Biological, physical, and chemical processes in ADM1

Process (number of species involved)	Kinetic model
Disintegration of complex material and hydrolysis (4)	$r_i = k_{dis,\ i}\ X_i$ (14.62)
Acidogenesis (3)	$r_i = k_{m,i} \frac{S_i}{K_{S,i}+S_i}\ X_i\ I_i$ (14.63)
Acetogenesis (2)	$r_i = k_{m,product} \frac{S_i}{K_{S,product}+S_i}\ X_{product} \frac{S_i}{S_j+S_i+1e-6}\ I_i$ (14.64)
Methanogenesis (3)	$r_i = k_{m,i} \frac{S_i}{K_{S,i}+S_i}\ X_i\ I_i$ (14.65)
Cell decay (7)	$r_i = k_{dec,X_i}\ X_i$ (14.66)
Acid base dissociation (6)	$r_{A,\ i} = k_{AB,\ i}(S_{i-}(K_{a,\ i} + S_{H+}) - K_{a,\ i}S_i)$ (14.67)
Gas transfer (3)	$r_{T,\ i} = k_L a(S_i - K_{H,\ i}P_{gas,\ i})$ (14.68)

associated substrate. Enzyme production is directly related to the growth rate of the specific bacterial group and can be inhibited by the concentration of soluble substrate. The kinetic equations implemented in ADM1 are divided according to the process. Table 14.6 presents the general expressions for biological, physical, and chemical processes.

ADM1 has been widely implemented for the prediction of AD substrate, product, and by-product concentrations. Jeong et al. (2005) implemented genetic algorithms to estimate the model parameters. Thus demonstrating that the results of methane concentration by simulation fit the experimental data, although the concentrations of acetic and propionic acid presented deviations between the calculated and experimental data. Rosén and Jeppsson (2006) implemented the ADM1 model, with 110 equations and complex characterization of inocula and substrates required to model the digesters' start-up. Modifications have been made to the kinetic equations of ADM1 for simulation with various types of substrates including municipal, agricultural, and excreta solid waste (Zhao et al. 2018).

Batstone et al. (2006) indicated that ADM1 is a model that can be adapted to the study conditions of anaerobic digestion reactors. Some modifications have been made to ADM1, among which are:

1. Trace elements: Frunzo et al. (2019) evaluated the addition of trace elements in the AD. They considered dissolution processes for metal ions, sulfur, and phosphorus that influence biological processes due to consumption and the inhibition or stimulation effects of the process. Other physical and chemical processes considered were precipitation, complexation, metal sorption, and hydrogen sulfide mass transfer.
2. Phosphorus, sulfur, and iron: Flores-Alsina et al. (2016) modeled three extensions that considered processes related to phosphorus, sulfur, and iron. In the first extension, they added terms related to phosphorus consumption and its impact on the production of valerate, butyrate, propionate, and acetate. In the second extension, they evaluated the reduction of sulfate to sulfide from two pathways. The first pathway considered a single group of reducing sulfate microorganisms with H_2 consumption as electron donor. In the second pathway, multiple electron

donors were considered (H_2, valerate, butyrate, propionate, and acetate). The third extension evaluated the transformation of Fe(III) to Fe(II), using H_2 and sulfide as electron donors; this also estimates the precipitation rate of iron sulfide (FeS) as well as phosphates of iron ($FePO_4$ and $Fe_3(PO_4)_2$).

3. Propionate degradation pathway: Uhlenhut et al. (2018) used ADM1 as a basis for comparison against a previously established extension ($ADM1_{xp}$). This modification considered the fraction of inert decay products, through the evaluation of a cell death factor. From this comparison, the authors proposed an original extension called ADM1xpro. In this extension, the term referring to the propionate-oxidizing microorganisms was divided into three terms for a tri-culture media. Also, the researchers tested two propionate bioconversion paths. The first path considered the transformation of propionate into butyrate, and later into H_2 and acetate. The second path proposed the direct reaction of propionate into H_2, acetate, CH_4, and CO_2.
4. Free ammonia inhibition: Bai et al. (2017) implemented ADM1 and compared three free ammonia inhibition models: simple inhibition, Monod, and non-inhibition forms as established in Table 14.2.

According to Li et al. (2014), the most common parameters for the characterization of DA processes are pH, volatile fatty acids, alkalinity, biogas production, as well as the variation of total and volatile solids. Another modifications to ADM1 that allow working the model with kinetic parameters related to conventional characterizations in total, volatile, and suspended solids, in addition to carbohydrates, lipids, proteins, inerts, and elemental analysis (Zhao et al. 2018). The results obtained in this modification are adjusted to the experimental data (R^2 between 0.991 and 0.993), despite the fact that they only adjusted 5 of the 18 kinetic parameters proposed by Batstone et al. (2000).

Kythreotou et al. (2014) and Aceves-Lara et al. (2005) agree that the predictive capabilities of a model, such as ADM1, will be reduced due to parameters that have not been correctly estimated, even when the model's structure is relevant. Wichern et al. (2008) indicate that the results obtained using ADM1 may vary due to the fact that there are parameters such as the fractions of particulate materials and carbohydrates that have a strong impact on model outputs.

14.4 Co-digestion

Estimating methane production in anaerobic co-digestion represents a challenge due to the synergistic and antagonistic effects from substrate interaction. The usual practices reported in the literature for the evaluation of co-digestion mixtures include:

1. Comparison of the experimental methane yields of the co-substrates against the substrates individually

Table 14.7 Substrate mixture experiment designs

No.	*ML*		*PM*		*Experimental methane yield (mL gVS^{-1})*	Abudi et al. (2020)
1	75		25		340	
2	50		50		375	
3	25		75		465	
No.	*SW*	*MM*	*CR*	*OFMSW*	*Experimental methane yield (mL gVS^{-1})*	Pagés-Díaz et al. (2014)
4	50	50	0	0	613	
5	0	50	50	0	432	
6	0	0	50	50	470	
7	50	0	50	0	461	
8	0	50	0	50	461	
9	50	0	0	50	647	
10	33.33	33.33	33.33	0	622	
11	0	33.33	33.33	33.33	535	
12	33.33	0	33.33	33.33	621	
13	33.33	33.33	0	33.33	617	
14	25	25	25	25	641	
No.	*M*	*PP*		*CW*	*Experimental methane yield (mL gVS^{-1})*	Labatut et al. (2011)
15	75	25		0	353,5	
16	90	10		0	285,6	
17	75	0		25	252,4	
18	90	0		10	237,6	

2. Analysis of the variation of the kinetic constants of the co-substrates with respect to the mono-substrates

To evaluate co-substrate methane yield, it is necessary to apply a mixture design of experiments. The complexity of the design of experiments depends on the objective of the study and the complexity of the substrate. To estimate whether or not the co-substrates have synergistic or antagonistic effects, a single mixture would suffice. However, a larger number is typically required to build up evidence. In the case of optimization, efforts should be made to identify every possible interaction between the substrates.

Below are the experimental designs from three experiences reported in the literature. Abudi et al. (2020) evaluated the anaerobic digestion of mango leaves and pig manure, which reported yields of 157 and 281 mL gVS^{-1}, respectively, subsequently performed binary mixtures in volatile solids ratios of 75:25, 50:50, 25:75. Pagés-Díaz et al. (2014) report methane yield from slaughterhouse waste (SW) (609 mL gVS^{-1}), mixed manure (MM) (384 mL gVS^{-1}), mixed crop residues (CR) (422 mL gVS^{-1}), and OFMSW (533 mL gVS^{-1}), from which they consider the TS and make binary mixtures (50:50), ternary (33.33:33.33:33.33), and

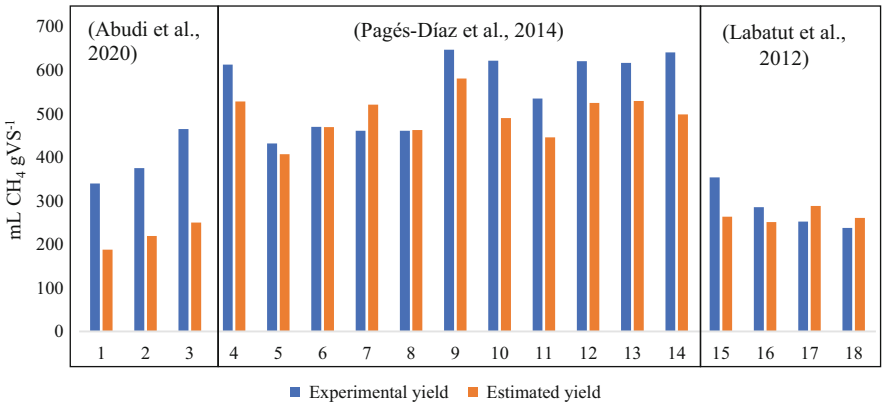

Fig. 14.1 Comparison between experimental and estimated methane yields

quaternary (25:25:25:25). Labatut et al. (2011) evaluated the effects of binary mixtures of manure (242.7 mL gVS^{-1}) with various substrates like cheese whey (423.6 mL gVS^{-1}) and plain pasta (326.1 mL gVS^{-1}) in VS ratios of 75:25 and 90:10 with respect to manure. Table 14.7 presents the mixtures made in the different investigations as well as experimental methane yields.

The literature evaluates the results of anaerobic co-digestion by means of statistical analysis. Pagés-Díaz et al. (2014) evaluated co-substrate effects by means of the polynomial:

$$B = \sum_i \beta_i X_i + \sum_i \sum_j \beta_{i,j} X_i X_j + \sum_i \sum_j \sum_k \beta_{i,j,k} X_i X_j X_k \tag{14.69}$$

where X_i, X_j, and Xk are the fractions of each substrate, β_i refers to the maximum mono-substrate yield, and $\beta_{i,j}$ and $\beta_{i,j,k}$ are related to the synergistic and antagonistic effects of binary and ternary mixtures, respectively.

Labatut et al. (2011) applied Buswell's formula to identify substrate and co-substrate theoretical methane yields. They established the biodegradability of substrates and co-substrates from expression (14.2) and used it as a measure of synergistic and antagonistic effects.

Abudi et al. (2020) applied a simpler approach to evaluate methane yield based on the VS mixture proportion. The expression by which they estimate the methane yield of a mixture that does not present synergistic or antagonistic effects is:

$$B_0 = \sum_i VS_i B_{0,i} \tag{14.70}$$

Expression Eq. (14.70) refers to the *i* component of a mixture. For its simplicity, this expression will be used to analyze the synergistic and antagonistic effects of the

Table 14.8 Kinetic data of selected cases

Mango leaves to pig manure ratio		*100:0*	*75:25*	*50:50*	*25:75*	*0:100*	Abudi et al. (2020)
B_0	mL CH_4 gVS^{-1}	155.1	336	368	454	278	
k	day^{-1}	0.1645	0.0694	0.0707	0.2245	0.1505	
n	–	1.2669	0.776	0.6954	1.1409	0.9957	
Microalgae to food waste ratio		*100:0*	*38.15:61.85*	*14.9:85.1*	*5.98:94.02*	*0:100*	Du et al. (2019)
B_0	mL CH_4 gVS^{-1}	292	329.8	385.4	415	383.9	
k	day^{-1}	0.16	0.46	0.35	0.32	0.31	
Microalgae to sewage sludge		*100:0*	*66.83:33.17*	*50.01:49.99*	*33.34:66.66*	*0:100*	
B_0	mL CH_4 gVS^{-1}	292	324	314	295	278.7	
k	day^{-1}	0.16	0.16	0.16	0.16	0.15	

mixtures reported in Table 14.7. In the case of Pagés-Díaz et al. (2014), VS data reported in Table 14.1 were used. Figure 14.1 presents the comparison between the experimental and estimated results.

Figure 14.1 shows that the estimates are, in some cases, less than the experimental results. The increased methane yield is an indication of the synergistic effects experienced in co-substrate. Mixtures 1–4 and 9–15 exhibit synergistic effects during co-digestion. Mixtures 6 and 8 do not present effects. Mixtures 5 and 16 cannot be defined as synergistic due to minimal differences between experimental and estimated values. This approach can be applied to the treatment of kinetic data, for which expression Eq. (14.70) is set as:

$$B = \sum_i VS_i B_i \tag{14.71}$$

The difference between Eqs. (14.70) and (14.71) is that the first one only evaluates the maximum methane yield and the second one evaluates the kinetic behavior of the methane yield of each experimental run. Table 14.8 presents the adjusted kinetic for mono- and co-substrate presented by Abudi et al. (2020) and Du et al. (2019) that used Cone and first-order models, respectively.

From the information presented in Table 14.8, Eq. (14.71) was evaluated. Figure 14.2 presents the kinetics from the experimental data and from Eq. (14.71).

According to Abudi et al. (2020), Du et al. (2019), Labatut et al. (2011), and Pagés-Díaz et al. (2014), mixing waste tends to improve methane yield. With both expressions, the results obtained differ from what was experimentally proposed. It should be noted that both expressions only consider the additive contribution of each mono-substrate component to the co-digestion methane yield.

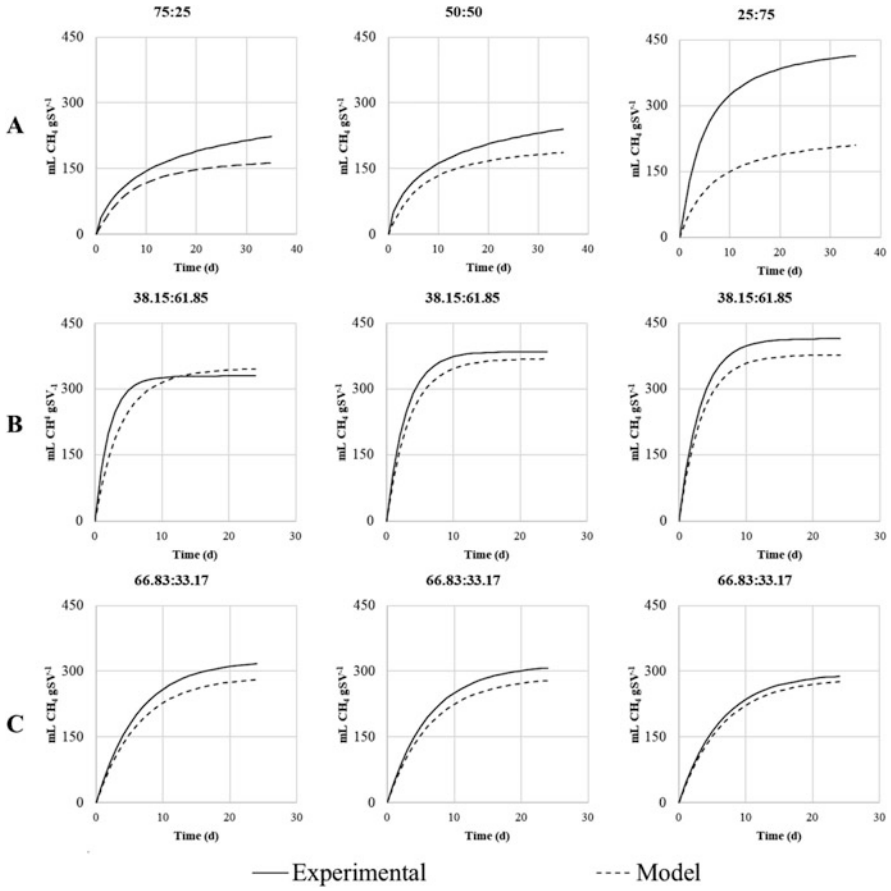

Fig. 14.2 Comparison between experimental kinetics and modeled kinetics in co-digestion. (**a**) Mango leaves—pig manure. (**b**) Microalgae—food waste. (**c**) Microalgae—sewage sludge

The importance of Eq. (14.71) lies in the possibility of establishing the extent to which the methane yield of the substrates varies, which is of interest to establish a mixture optimization route. On the other hand, it should be noted that the conventional analysis of the mixture effects is based on mono-substrate kinetics (first-order, modified Gompertz, Cone models, and others), which is convenient in terms of making a comparison, but does not allow to estimate the performance of methane or kinetic constants without having an experimental design. Astals et al. (2014) indicate that mixture effects, both synergies and antagonisms, affect the kinetics of the process. Therefore, a mathematical expression can be established to consider the mixture effect on methane yield as well as on the kinetic constants of the process.

14.5 To Model or Not to Model: Where Is Really the Opportunity?

14.5.1 Trends in Anaerobic Digestion Modeling

To identify research trends, an analysis of titles, keywords, and abstracts of the publications made in Scopus between 2010 and 2020 was carried out. The search terms focused on:

1. First-order kinetic model to describe methane performance
2. Modified Gompertz kinetic model to describe methane performance
3. Cone kinetic model to describe methane performance
4. Fitzhugh kinetic model to describe methane performance
5. Transfer function to describe methane performance
6. Anaerobic digestion model 1

In total, 853 journal articles between 2010 and 2020s first semester refer to terms related to kinetic models to describe methane performance. Figure 14.3 shows the proportion of the research items that evaluate the proposed models. The analysis of Fig. 14.3 did not rule out publications using more than two models.

It is observed that the first-order model is the most widely used expression to describe the methane yield kinetics. This analysis is in accordance with what is proposed in Tables 14.3 and 14.5, which highlights the wide application of the first-order model.

In addition, the comparison of the application of the first-order model and the ADM1 was made; this was done from two perspectives:

1. Countries with the highest number of publications (Fig. 14.4)
2. Number of publications from 2010 to 2020 (Fig. 14.5)

It is observed that the country with the highest contribution in terms of methane yield modeling is China, with just over 100 publications in the period evaluated, followed by the USA with around 60 publications. Regarding ADM1, a total of

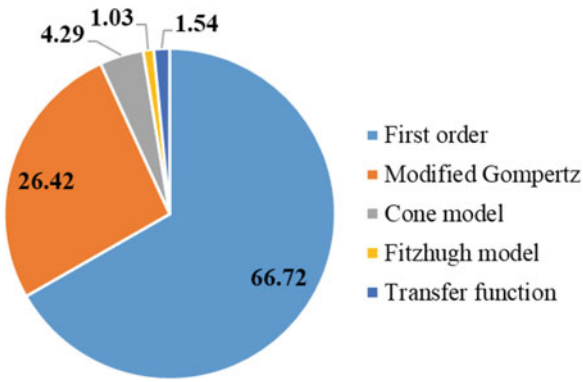

Fig. 14.3 Percentage of publications between 2010 and 2020 related to the methane yield kinetics

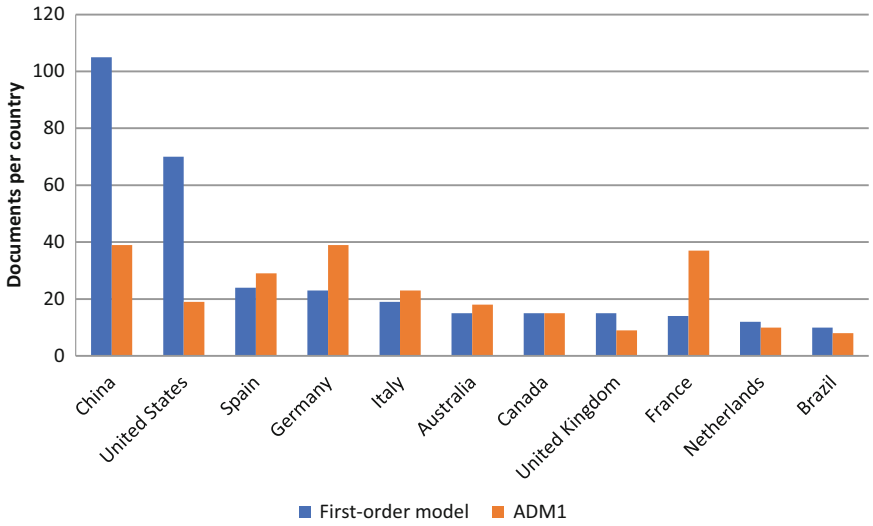

Fig. 14.4 Number of publications per year in the period 2010–2020

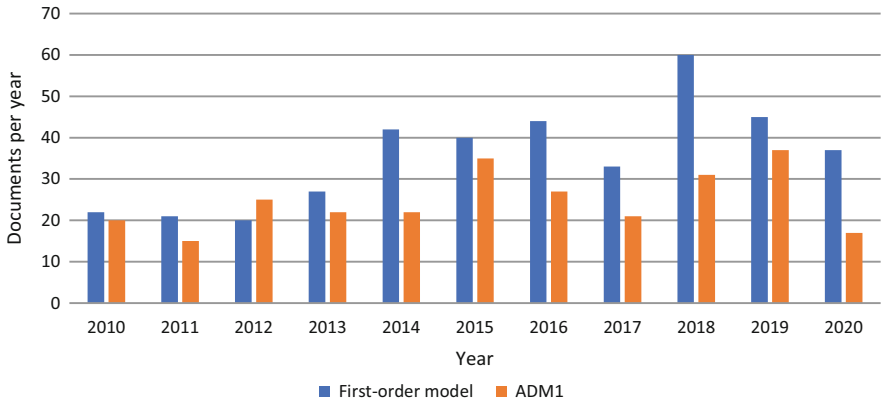

Fig. 14.5 Number of publications in the last 10 years

381 publications were registered at the date of the analysis (until June 2020). Figure 14.4 shows that the countries that concentrate more than 30% of the publications are Germany, China, and France. In Fig. 14.5, it is notable how the publications referring to ADM1 increase but still do not exceed those related to the first-order model.

14.5.2 Feasibility of Applying the Models

Mathematical modeling is a tool to broaden the understanding of processes in general. In the field of anaerobic degradation of residues, mathematical modeling allows estimating:

1. Methane production (methane yields, accumulated volume)
2. Composition of sludge and gas streams (TS, VS, particulate and soluble material)
3. Interactions between substrates in co-digestion (synergies and antagonisms)
4. Effects on the system (temperature, pH, toxic substances)

Different approaches to mathematical modeling allow its application in industrial and agricultural sectors. To this is added that the models that have been developed present different mathematical structures (algebraic equations and ordinary differentials) developed from the study of the phenomenon.

Models in algebraic equations are characterized by their relative simplicity for studying the degradation of a substrate; they allow to identify constants of production and consumption. These can be implemented from complete characterizations of the substrates (C, H, O, N, metals, carbohydrates, lipids, proteins, and others) or from basic parameters (TS and VS), as well as measurements of volumes of the methane generated. The resolution of these models is relatively simple; the software used does not require high performance and can even be solved without the need for processors. This facilitates the evaluation of proposals for the application of AD in areas where economic resources are limited.

Dynamic models stand out due to the application of these in the design, control, and optimization of processes. In addition, they allow the study of the degradation of organic residues, variation of microbial populations and interactions between substrates, as well as the effect of factors such as temperature and pH. These models require specialized software for the simultaneous resolution of differential equations. The implementation of control systems requires the collection of data from monitoring systems and the sending of analog or digital signals to control systems.

14.6 Remarks

1. Anaerobic digestion is a biological process, which due to the multiple stages and microorganisms it involves is complex to model. Other factors that influence the modeling of the process are related to the sensitivity of microorganisms to variations in environmental conditions such as temperature, pH, and concentration of inhibitory substances. Furthermore, the model is subject to the complexity of the composition of the substrate.
2. For the determination of reaction kinetics, a discontinuous study is recommended. For this task, the most widely accepted models are first order and Gompertz modified. The goodness of fit of the models must be performed by

comparing statisticians that reflect the least error as well as the least variability of these.
3. The selection of the model is linked to the possibilities of characterizing the reacting system (substrates, inocula, sludge, biogas, among others), according to the available economic and technological resources.
4. There are still gaps in the modeling of mixing effects on the methane yield kinetics. From the mono-substrate kinetics, the aim is to study the co-substrate kinetics, which cannot be done using current models.

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Chapter 15
ANAMMOX in Wastewater Treatment

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Contents

Abstract Water is a universal solvent that is used both for domestic and commercial purposes. Used water is referred to as wastewater which is released in a varying quantity of volumes to the environment. Wastewater could be point source or non-point source. This water consists of wastes, solid, liquid and gaseous. Ammonia, also known as NH_3, is a colourless gas with a discrete odour and a compound of nitrogen and hydrogen, but when the compressed liquid of anhydrous ammonia gets

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into the atmosphere, it turns into a dangerous gas. In order to minimize these effects, both biological and physico-chemical technologies have been applied in the elimination of ammonium from wastewaters for a long period; however, these methods are not very effective in the removal of this ammonium, in accordance to the stringent discharge standards; hence, more effective technologies are called out, and one of these effective and efficient technologies is referred to as anaerobic ammonium oxidation (ANAMMOX). ANAMMOX process is an economical and energy-saving biotechnology that encompasses a great potential in the treatment of ammonium-rich wastewaters, especially after its successful case in the treatment of sludge digest liquids. This chapter is therefore written to focus on ANAMMOX organisms, their applications in wastewater treatment and their advantages and disadvantages.

Keywords Wastewater · Environment · Ammonia · ANAMMOX · Technology

15.1 Introduction

The indiscriminate discharge of wastewaters has impacted negatively on the environment and aquatic lives; this is as a result of the high content of ammonia present in the water, which is toxic to living organisms and could lead to eutrophication in water bodies. In order to minimize the effects, both biological and physico-chemical technologies have been applied in the elimination of ammonium from wastewaters for a long period (Bonmatí and Flotats 2002; Sugiyama et al. 2005); however, these methods are not very effective in the removal of this ammonium, in accordance to the stringent discharge standards; hence, more effective technologies are called out, and one of these effective and efficient technologies is referred to as anaerobic ammonium oxidation (ANAMMOX).

ANAMMOX process was clearly observed in a denitrifying fluidized bed reactor in 1994, where two pollutants of ammonium and nitrite are removed simultaneously (Mulder et al. 1995). The application of the ANAMMOX process in wastewater systems has resulted in lower energy requirements and high rates of nitrogen removal than those for conventional nitrogen removal (Laureni et al. 2016). This treatment system needs the supply of oxygen for the production of nitrite. This oxygen may inhibit ANAMMOX; therefore, careful regulation of the oxygen supply is very important for the success of the application of ANAMMOX in wastewater treatment (van Kessel et al. 2018).

15.2 ANAMMOX Bacteria (Species Diversity)

The microorganisms competent of ANAMMOX are broadly classified into three groups of chemolithoautotrophic bacteria; they include the aerobic ammonium oxidizers, aerobic nitrite oxidizers and anaerobic ammonia oxidizers (Eq. (15.4)) often referred to as ANAMMOX bacteria. All the organisms derive energy for carbon fixation and microbial growth from the oxidation of inorganic nitrogen compound (Zhang et al. 2008; Yang et al. 2020).

15.2.1 The Aerobic Ammonium Oxidizer

The ammonium-oxidizing bacteria (AOB) form two monophyletic groups: the beta-ammonia oxidizers, which comprised of the genera *Nitrosomonas* and *Nitrosospira*, and the gamma-proteobacteria (ammonia oxidizers), which include the genus *Nitrosococcus* with exception of *Nitrosococcus mobilis*, which is related to *Nitrosomonas* (Sepehri and Sarrafzadeh 2019). Different members of these genera have been found to dominate different wastewater treatment plants and are very crucial in the first step of conversion of ammonia oxidation to nitrite (Cho et al. 2019). The most common and dominant AOB in the wastewater system is *Nitrosomonas eutropha*, one of the most effective and efficient denitrifiers among nitrifiers and was found to control the nitrifier denitrification (NOx) process with NO_2 as electron acceptor under anoxic conditions (Schmidt and Bock 1997; Zhang et al. 2008).

The proteobacterial ammonia oxidizers obtain their energy for growth from both aerobic (Eq. (15.1)) and anaerobic (Eq. (15.2)) ammonia oxidation, and the substrate for the oxidation process is strictly ammonia (NH_3) which yields nitrite and dinitrogen, nitrite and nitric oxide under oxic and anoxic conditions, respectively. This is initiated by the enzyme ammonia monooxygenase (AMO) that oxidizes ammonia to hydroxylamine using oxygen and dinitrogen tetroxide (a dimer of NO_2) as the electron acceptors.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \left[\Delta G^{o'} - 275 \text{ kJ mol}^{-1}\right] \quad (15.1)$$

$$NH_4^+ + N_2O_2 \rightarrow 0.33NO_2^- + 1.33H^+ + 0.33N_2 + 2NO + 1.33\ H_2O \left[\Delta G^{o'} - 295 \text{ kJ mol}^{-1}\right] \quad (15.2)$$

$$NO_2^- + 0.50O_2 \rightarrow NO_3^- \left[\Delta G^{o'} - 74 \text{ kJ mol}^{-1}\right] \quad (15.3)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O \left[\Delta G^{o'} - 357 \text{ kJ mol}^{-1}\right] \quad (15.4)$$

15.2.2 The Aerobic Nitrite Oxidizers

Nitrite oxidizers are organisms involved in the second step of nitrification, the oxidation of nitrite to nitrate, and it is accomplished by nitrite-oxidizing bacteria, such as members of the genera *Nitrobacter* and *Nitrococcus* which belong to the alpha-proteobacteria and *Nitrospira* with a separate division and unrelated phylogenetically to any cultivated species (Ehrich et al. 1995; Daims et al. 2015; van Kessel et al. 2015; Kits et al. 2017). A recent study has revealed that *Nitrospira*, a member of nitrite-oxidizing bacteria (NOB), can completely oxidize ammonia and therefore assigned the name complete ammonia oxidizer (comammox) (Yang et al. 2020).

The key enzyme of nitrite-oxidizing bacteria is the membrane-bound nitrite oxidoreductase which oxidizes nitrite with water as the source of oxygen to form nitrate. The electrons released from this reaction are transferred via a- and c-type cytochromes to a cytochrome oxidase of the aa_3 type (Yao and Peng 2017).

15.2.3 Anaerobic Ammonia Oxidizers

There are many species of ANAMMOX bacteria, mainly belonging to the genus of *Brocadia*, *Kuenenia*, *Jettenia*, *Anammoxoglobus*, *Scalindua* and *Anammoximicrobium* (Table 15.1).

The first ANAMMOX bacteria studied were isolated through density gradient centrifugation; they possess distinctive phenotypic characteristics, which included budding production, red colour, crateriform structure on the cell surface, intracellular compartment "anammoxosome" and intracytoplasmic membrane containing ladderane lipid (van de Graaf et al. 1996; Lindsay et al. 2001; Sinninghe et al. 2002).

Table 15.1 ANAMMOX bacteria discovered and isolated from wastewater sources

Genus	Species	References
Brocadia	*Candidatus Brocadia anammoxidans*	(Strous et al. 1999)
	Candidatus Brocadia fulgida	(Kartal et al. 2004a, b)
Kuenenia	*Candidatus Kuenenia stuttgartiensis*	(Penton et al. 2006)
Scalindua	*Candidatus Scalindua brodae*	(Schmid et al. 2003)
	Candidatus Scalindua wagneri	(Schmid et al. 2003)
Candidatus	*Candidatus Jettenia asiatica*	(Tsushima et al. 2007)
	Anammoxoglobus propionicus	(Kartal et al. 2007)

The anammoxosome is a specialized organelle in the cell, with three basic functions: (1) site for catabolism, (2) generating energy for ATP synthesis through proton motive force across the anammoxosome membrane and (3) protecting the bacteria from the proton diffusion and intermediate toxicity due to their impermeable membranes (Lindsay et al. 2001). This membrane-bound organelle also converts ammonium and nitrite to dinitrogen gas using a process involving the production of the toxic and extremely energy-rich hydrazine intermediate (Kartal et al. 2010a, b; Cho et al. 2019; Sunja et al. 2020).

Generally, ANAMMOX bacteria preferred a low nitrogen concentration for faster activation (Jung et al. 2007; Awata et al. 2015). The abundant availability of nitrite and nitrate (higher than 0.07 g/L) at an early stage can interrupt ANAMMOX growth, whereas a lower nitrite concentration (up to 0.04 g/L) can enhance ANAMMOX growth and specific ANAMMOX activity (Roller et al. 2017). The growth rate of ANAMMOX bacteria is quite slow; unlike that of other bacteria, it has an exponential phase between 10 and 22 days (Kartal et al. 2013; Ma et al. 2018) or within 10–12 days when cultured at 35 °C; therefore, its application is limited to the operation of continuous wastewater treatment processes containing high concentrations of ammonium or other organic matter.

15.3 ANAMMOX-Involved Processes

The processes involved in the removal of nitrogen from wastewater take various steps, which included partial nitritation-ANAMMOX, ANAMMOX and completely autotrophic nitrogen removal over nitrite (CANON).

15.3.1 Partial Nitritation-ANAMMOX

Partial nitritation-ANAMMOX technique is based totally on two biotechnologies. Firstly, ammonium is in part nitrified to nitrite in the partial nitritation stage, after which the produced nitrite is denitrified with the residual ammonium in the ANAMMOX. The nitritation of ammonium is carried out by the usage of aerobic ammonium-oxidizing bacteria (AOB) that differentiate substantially in physiology from nitrite-oxidizing bacteria (NOB). Therefore, selective retention of AOB is important inside the partial nitritation stage. In practice, certain factors such as temperature, pH, sludge retention time (SRT), dissolved oxygen and influent alkalinity are considered as important priorities in the partial nitritation-ANAMMOX process (Matthieu et al. 2020).

15.3.1.1 Temperature and Sludge Residence Time (SRT)

The two groups of bacteria involved in the process are very sensitive to temperature. A rise in temperature facilitates AOB to outcompete NOB (Fig. 15.1). A stable nitritation process has been obtained at temperature within 35 °C, although AOB and NOB have an optimal temperature of 38 °C and 35 °C, respectively (Li et al. 2018; Hoekstra et al. 2018). A nitritation process had been successfully started up and maintained between 15 °C and 30 °C (Hoekstra et al. 2018). However, the system performance deteriorated dramatically below 15 °C, which agreed well with the theoretical value in Fig. 15.1. However, the doubling time of AOB is shorter than that of NOB at higher temperature as stated above; thus, SRT should be properly mediated in a limited range that enables retention of AOB but knocks out NOB. Full-scale experience in Utrecht and Rotterdam wastewater treatment plants suggested that SRT between 1 and 2.5 days was acceptable (van Kempen et al. 2001; Cheng et al. 2020). Nevertheless, SRT as long as 5 days in an SBR (sequential batch reactor) also created a favourable environment for AOB to outgrow NOB (Galí et al. 2007; Yandong et al. 2017). A recent study by Anja et al. (2019) has suggested that the partial nitritation process should not be carried out below 30 °C as it limits the performance of the organisms responsible for the process.

15.3.1.2 Influent Alkalinity/Ammonium and pH

Another factor to be considered is the inlet total alkalinity/ammonium ratio (TA/-N) since it will determine the pH value inside the reactor and, therefore, the concentrations of free nitrous acid (FNA) and/or FA (Durán et al. 2014; Belmonte et al. 2017; Cheng et al. 2020); this is important because ammonium oxidation is an alkali-consuming reaction. Therefore, for a proper and assured NH_4^+/NO_2^- reaction ratio to take place, one mole (1 mol) alkali per mol ammonium must be used (Eq. (15.5)).

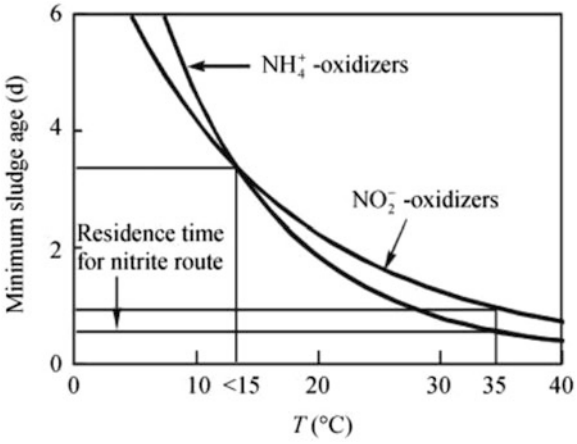

Fig. 15.1 Minimum residence time for ammonium and nitrite oxidizers at different temperatures (Hellinga et al. 1998)

Table 15.2 Characterization of the different operational stages of the SBR

Parameter	Unit	Stage I		Stage II		Stage III	
		Influent	Effluent	Influent	Effluent	Influent	Effluent
Operation time	d	0–75		76–190		191–270	
ALRs	g NH_4^+-N/L-d	0.06		0.09		0.15	
Total alkalinity/NH_4^+-N	g/g	9.4 ± 0.0	—	7.5 ± 0.0	—	4.1 ± 0.0*	—
pH		7.5 ± 0.1	7.4 ± 1.3	7.5 ± 0.1	6.8 ± 0.9	7.5 ± 0.1	7.2 ± 1.3
COD_s	mg/L	734 ± 85	415 ± 28	801 ± 100	363 ± 189	1907 ± 319	293 ± 58
NH_4^+-N	mg/L	350 ± 26	82 ± 25	550 ± 67	128 ± 77	880 ± 100	102 ± 60
NO_2^--N	mg/L	<1.0	174 ± 32	<1.0	165 ± 86*	<1.0	2 ± 2
NO_3^--N	mg/L	<1.0	45 ± 5	<1.0	42 ± 17*	<1.0	293 ± 138
NO_2^--N/NH_4^+-N	g/g	—	2.1 ± 0.5	—	1.3 ± 0.5	—	<0.1 ± <0.1
NIT	%	79 ± 5		76 ± 5		86 ± 11	
NAR	%	78 ± 2		83 ± 6**		1 ± <0.1	
$N_{removed}$	%	21 ± 13		30 ± 7		45 ± 19	
$COD_{removed}$	%	39 ± 9		54 ± 11		85 ± 3	
$COD_{removed}/N_{removed}$	g/g	2.8 ± 1		2.6 ± 0.4		3.5 ± 0.5	

Both lab experiments and engineering practices have proved that an alkalinity/ammonium ratio around one was suitable for the partial nitritation-ANAMMOX (van Dongen et al. 2001; Fux et al. 2002; Cheng et al. 2020).

$$NH_4^+ + HCO_3^- + 0.75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + CO_2 + 1.5H_2O \quad (15.5)$$

In an experiment carried out by Belmonte et al. (2017), it shows that the decrease of the inlet total alkalinity concentrations increased the efficiency of the system in accumulating nitrite. This can be attributed to the decrease of pH value inside the reactor, which promoted the inhibitory effect of FNA on NOB (Table 15.2). Prior to the ANAMMOX, pH plays several roles in the partial nitritation process. Firstly, it straightforwardly impacts the rates of growth of the two groups of bacteria. The growth rate of NOB at pH 7 is eight times that at pH 8, whereas the variation of AOB is negligible (Yandong et al. 2017; Belmonte et al. 2017). Secondly, pH is closely related to the available forms of substrate (NH_3 and HNO_2). Wastewaters with pH around eight created an environment containing more NH_3 and less HNO_2, which obviously promotes AOB but suppresses NOB (Belmonte et al. 2017). Therefore, the partial nitritation process is recommended to operate in a weak alkaline condition. Thirdly, pH is a simple indicator for automatic control of HNO_2/NH_3 in a fixed alkalinity/ammonium ratio (Zhang et al. 2008).

15.3.1.3 Dissolved Oxygen (DO)

DO strategy for nitritation process control is based on different affinities of AOB and NOB. The K_s (half saturation constant) value of AOB (0.3 mg/L) is lower than that of NOB (1.1 mg/L), which means that AOB will outcompete under DO limitation condition (Wang and Yang 2004). Some researchers have taken DO concentration

Table 15.3 Control strategies for the partial nitritation phase in the partial nitritation-ANAMMOX process

Reactor type	pH	T (°C)	DO (mg/L)	SRT (d)	NH_4^+-N/NO_2^--N[a]
CSTR		30–40	–	1.0	1.09
		30	–	1.2	1.10
	7.23		Cascade control	–	NR
SBR	–		3–4	1	0.60
Swim-bed	–	15–30	–	0.5–0.25	0.61

CSTR continuous stirred tank reactor, *SBR* sequential batch reactor, *NR* not reported
[a]NH_4^+-N/NO_2^--N after the partial nitritation phase (Alba et al. 2019)

around 1.0 mg/L as suitable concentration for nitritation (Ciudad et al. 2005; Ruiz et al. 2006). This value can be quite a specific case due to variation of the oxygen mass transfer efficiency in reactors (Ciudad et al. 2005).

Orthogonal experiment suggested that pH, temperature and DO concentration greatly influence the performance of nitritations (Alba et al. 2019). Furthermore, other factors such as aeration pattern, operation mode, operating costs and reactor configurations should be extensively considered. For example, it was feasible to run the nitritation process at lower temperature (around 15 °C) with an extended SRT. Besides, if the DO concentration was limited, the process would remain stable with SRT as long as 24 days. Both the SBR and the chemostat with the nitrogen conversion rates of 1.1 kg N/(d m^3) and 0.35 kg N/(d m^3), respectively, were reliable reactors for the nitritation phase, whereas the chemostat was more stable. In engineering, cascade O_2 control plus pH control was superior to other strategies based on the economic cost analysis. Table 15.3 lists several SHARON practices with different control strategies.

15.3.2 Completely Autotrophic Nitrogen Removal Over Nitrite (CANON)

The prototype of the CANON process was derived from conversion of ammonium to dinitrogen gas in a microaerobic or a combined aerobic-anoxic environment. For the lack of knowledge about the microbial reactions in the reactors, the process was named as "aerobic/anoxic deammonification". It was not until 2000 that the concept of CANON was proposed for the treatment of low C/N wastewaters (Strous 2000). Two groups of autotrophic bacteria are involved; these included the AOB and the ANAMMOX bacteria. The AOB take advantage of oxygen to oxidize ammonium to nitrite. After the depletion of oxygen, nitrite with the remaining ammonium is converted to gaseous nitrogen. The overall reaction is as follows:

$$NH_4^+ + 0.85O_2 \rightarrow 0.435\,N_2 + 0.13NO_3^- + 1.4H^+ + 1.3H_2O \quad (15.6)$$

As a result, the CANON process saves 63% oxygen and 100% carbon sources in comparison with the traditional nitrification-denitrification process (Peng et al. 2013) known as "OLAND" (oxygen-limited autotrophic nitrification-denitrification). Unlike the co-existence of AOB and the ANAMMOX bacteria, nitrifying bacteria such as *Nitrosomonas eutropha* conduct aerobic ammonium oxidation and anaerobic ammonium oxidation simultaneously.

The CANON process is an integration of partial nitritation-ANAMMOX into one single reactor. Although the mentioned factors in partial nitritation-ANAMMOX control are applicable to the CANON system theoretically, with attention more on the manipulation of influent ammonium loading rate and DO concentration, which greatly influence the microbial composition in the CANON system. Appropriate ammonium and DO concentration enable the consumption of oxygen by AOB to an extent in which DO concentration is not over the threshold toxic to the ANAMMOX bacteria and inadequate for the growth of NOB. Subsequently, the produced nitrite, an inhibitor to AOB, is used as electron acceptor by the ANAMMOX bacteria. Thus, a symbiosis between AOB and the ANAMMOX bacteria is finely established as indicated by fluorescence in situ hybridization (Nielsen et al. 2005; Peng et al. 2013). For ammonium-limited or DO-excess influent, ammonium is exhausted by the AOB rapidly. The accumulated nitrite and relatively excess oxygen in bulk liquid stimulate the outgrowth of NOB over the ANAMMOX bacteria and thereby destroy perfect balance among the three bacteria. Lower limit of an ammonium loading rate of 0.12 kg N/(d m^3) for steady nitrogen removal was reported by some investigators (Cho et al. 2019). The nitrogen elimination efficiency resulted from limited ammonium decreased by 35% in both SBR and chemostat when the DO concentration was kept around 0.24 mg/L. According to model simulation, the result indicated that the maximum rate of removal of nitrogen was achieved only when the DO concentration kept pace with the ammonium surface load (Liang et al. 2015). Thus, the stoichiometry of ammonium and oxygen is the key control parameter in the CANON process. For fluctuating ammonium loading rates in engineering, DO can be regulated through online feedback control (Nielsen et al. 2005; Liang et al. 2015; Cho et al. 2019).

By and by, lab-scale CANON has been led in biofilm reactors, SBRs and gas-lift reactors (Table 15.4). The effective maintenance of biomass in SBR empowers itself an incredible asset for gradually developing microbes, yet its exhibition in the CANON procedure is not extraordinary. The most elevated nitrogen expulsion pace of 1.5 kg N/(d m^3) was gotten in a gas-lift reactor (Sliekers et al. 2003). In a consistent oxygen-restricted reactor, AOB and the ANAMMOX microscopic organisms collaborate at the same time and cause most extreme change rates. For something else, it appears that oxygen mass exchange is the rate-restricting advance. The transference of oxygen to bulk liquid is dramatically elevated in the gas-lift

Table 15.4 CANON process in different reactors

Reactor type	NRR (kg N/(m^3 d))	Sludge form	Aeration type
SBR	0.160	Suspended	Continuous
	0.120	Suspended	Continuous
	0.080	Suspended	Intermittent
Gas-lift reactor	1.500	Suspended	Continuous
RBC	7.390	Biofilm	Continuous
GSBR	0.057	Granular	External aeration

NRR nitrogen removal rate, *SBR* sequential batch reactor, *RBC* rotating biological contactor, *GSBR* granular sludge bed reactor (Zhang et al. 2010; Ma et al. 2018; Sunja et al. 2020)

reactor due to its suspended flocs. The compact granular sludge bed reactor and the rotating biological contactor (RBC) with biofilm are unfavourable for the oxygen to approach the active microbes. The thicker the biofilm, the more DO is needed to get the maximum nitrogen elimination (Hao et al. 2001). Thickness of the biofilm or size of the granular sludge influences bacteria composition as well. Large aggregates (>500 μm) account for 68% of the ANAMMOX bacteria, while small aggregates (<500 μm) only account for 35% (Nielsen et al. 2005). The reason is obvious. Large granular sludge is not so permeable to oxygen as small one, and a relatively large anoxic sphere is created to facilitate growth of the anaerobic bacteria.

The CANON procedure has an extraordinary bit of leeway over the incomplete nitritation-ANAMMOX in venture. In any case, challenges in DO guideline in huge reactors and inadequate nitrogen evacuation of high nitrogen loads keep them from application to wastewaters with high ammonium fixation. For wastewaters with higher ammonium, it merits giving up moderately high speculation for a halfway nitritation-ANAMMOX practice. The costs will be repaid by lower operational expenses and effective nitrogen expulsion execution (Hao et al. 2001; Nielsen et al. 2005; Yao et al. 2013; Liang et al. 2015).

15.4 ANAMMOX Application to Different Wastewaters

The ANAMMOX and relevant processes mentioned above with a merit of low operational costs have attracted much attention since their inception. A number of researches have been conducted on various ammonium-rich wastewaters as summarized in Table 15.5 below.

1. Sludge reject water: Sludge reject water (sludge liquor) was the first primary wastewater treated with the partial nitritation-ANAMMOX process as a result of its characteristics which ranges from low organics and appropriate NH_4^+/alkalinity (van Dongen et al. 2001). The 10 L lab experimentation was straightforwardly scaled up to an engineering practice. This full-scale ANAMMOX was initiated in Rotterdam on July 1, 2002. The start-up phase took almost 3.5 years, and the

Table 15.5 ANAMMOX application to different wastewaters

Wastewater	Process	NRR (kg N/(m^3·d))	Start-up (d)	Scale	References
Domestic sewage	Partial nitritation-ANAMMOX		200	5.5 L	Yuan et al. (2020)
Municipal/industrial	Separated and combined ANAMMOX/partial denitrification coupling			193 m^3	Qing-Guo et al. (2020)
Sludge liquor	Partial nitritation-ANAMMOX	0.710	110	10 L	van Dongen et al. (2001)
Sludge supernatant	Partial nitritation-ANAMMOX	2.400	150	2.5 m^3	Fux et al. (2002)
Partially nitrified sludge digestate	ANAMMOX	3.500		3.5 L	Fux et al. (2004)
Sludge digestate	Partial nitritation-ANAMMOX	9.500	1250	70 m^3	van der Star et al. (2007)
Slaughterhouse wastewater	Nitrification-denitrification	0.031		790 ml + 745 ml	Reginatto et al. (2005)
Piggery wastewater	ANAMMOX	0.600		1.5 L	Ahn and Kim (2004)
	Partial nitritation-ANAMMOX	1.360	~60	1 L	Hwang et al. (2005)
Synthetic coke-oven wastewater	ANAMMOX	0.062	~465	1 L	Toh and Ashbolt (2002)
Monosodium glutamate wastewater	ANAMMOX	0.460	71	5 L	Chen et al. (2007)

quantitative PCR (polymerase chain reaction) portrayed exponential development of the ANAMMOX bacteria.

2. Wastewaters from anaerobic treatment of animal waste had been likewise attempted (Ahn and Kim 2004; Hwang et al. 2005; Waki et al. 2007). These wastewaters are acknowledged for high organic nitrogen content. During anaerobic assimilation, ammonium raised impressively due to protein deterioration. The ANAMMOX is therefore developing with the denitrification (Dong and Tollner 2003). Most investigations portrayed that denitrifiers contributed more than the ANAMMOX microbes. NO_2^--N to NH_4^+-N proportions ranged between 1.48 and 1.79 (Ahn and Kim 2004). This was firmly recognized with the usually higher COD (chemical oxygen demand) of 600–25,700 mg/L (Ahn and Kim

2004; Hwang et al. 2005). The COD was later observed to be 800–1800 mg/L in sludge digest liquids (Hellinga et al. 1998). It has been accounted for that if COD was reduced to less than 135 mg/L, the NO_2^--N/NH_4^+-N ratio was close to 1:1 (Waki et al. 2007).

3. Coke-oven wastewater: Application in coke-oven wastewater was first proposed by Toh et al. (2002). The supposition was considered as major breakthrough for the ANAMMOX idea, in light of the fact that the coke-oven wastewater contains not only high concentration of organics (COD: 2000–2500 mg/L) but also some toxic chemicals such as phenols (300–800 mg/L), cyanides (10–90 mg/L) and thiocyanates (300–500 mg/L) (Toh and Ashbolt 2002; Toh et al. 2002). Despite the fact that the underlying endeavour to improve the bacteria from industrial coke-oven wastewater sludge fizzled, the acclimation of the ANAMMOX consortium (from municipal wastewater sludge) to synthetic coke-oven wastewater was successfully established. Phenol was added to the influent from 50 ± 10 to 500 ± 10 mg/L stepwise. After a culture of 15 months, the ammonium removal rate crested to 0.062 kg N/(m^3·d).
4. Monosodium glutamate is a flavour popularly known in most Asian countries. It is produced through fermentation of rice, starch and molasses. Wastewater generated from these processes usually contains high suspended solids (SS) (200–10,000 mg/L), COD (1500–60,000 mg/L), NH_4^+-N (200–15,000 mg/L) and sulphate (3000–70,000 mg/L). Traditional treatment usually involves physico-chemical and biological methods in succession. After the physico-chemical step, SS, COD and NH_4^+-N are reduced to 200–270, 1000–1400 and 250–350 mg/L, respectively. The ANAMMOX process was attempted on this water with preliminary nitritation, and a total nitrogen removal rate of 0.46 kg N/(m^3·d) was obtained (Chen et al. 2007). The overall performance of ANAMMOX was far better than the traditional nitrification-denitrification.
5. Domestic sewage treatment using a one-stage ANAMMOX process: Recently anaerobic ammonium oxidation (ANAMMOX) had been effectively used to treat domestic sewage on campus; this was achieved by using a one-stage reactor; partial nitrifying sludge was mixed with ANAMMOX granular sludge at an average temperature of 30 °C. The process started off at 40 days of inoculation; after 70 days of nitrogen load acclimation, it was observed that *Acinetobacter*, including *Candidatus kuenenia*, became the dominant strain of the system within the reactor, which displayed high efficiency and a steady nitrogen removal performance. At an influent chemical oxygen demand (COD), NH4$^+$-N content, total nitrogen (TN) content, hydraulic retention time (HRT), temperature and reactor dissolved oxygen (DO) content of 100, 60 and 70 mg/L, 6 h, 30 ± 1 °C and below 0.6 mg/L, respectively. The removal rates of COD, NH_4^+-N and TN were approximately 89%, 96.7% and 70%, respectively (Yuan et al. 2020).

15.4.1 The Test Device and Method

In the experiment, an up-flow ANAMMOX reactor was utilized to treat wastewater. A schematic diagram of the device is presented in Fig. 15.2, which mainly includes the main reactor (effective volume, $V_1 = 5.5$ L), regulating tank (effective volume, $V_2 = 2.0$ L) and raw water tank (effective volume, $V_3 = 300$ L). During the operation of the reactor, wastewater from the crude water tank enters the regulating tank through the water delta siphon to change the pH and pre-aeration. Wastewater containing a specific measure of oxygen enters the main reactor to undergo incomplete nitrification and ANAMMOX reaction, which degrades pollutants in the wastewater. After partition by the three-stage separator at the top of the reactor, one part of the water floods from the water outlet, and the other part flows back to the regulating tank through the reflux siphon. Setting the return water quickens the rising pace of the wastewater in the main reactor so that the microorganisms in the upper layer of the reactor can likewise adequately remove the pollutants.

The experiment was partitioned into three stages. Artificial wastewater was used in the first two stages, and domestic wastewater from Guilin University of Technology was used in the third stage (Li et al. 2019). The composition of the wastewater used in the three experimental stages is shown in Table 15.6. During the experiment, the pH was adjusted using 0.4 mol/L H_2SO_4 and 0.5 mol/L $NaHCO_3/Na_2CO_3$. The $NH4^+$-N in the artificial wastewater was provided by NH_4HCO_3, and specific amounts of KH_2PO_4, $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ were added to give microelements for the microorganisms. During the experiment, the reactor temperature was

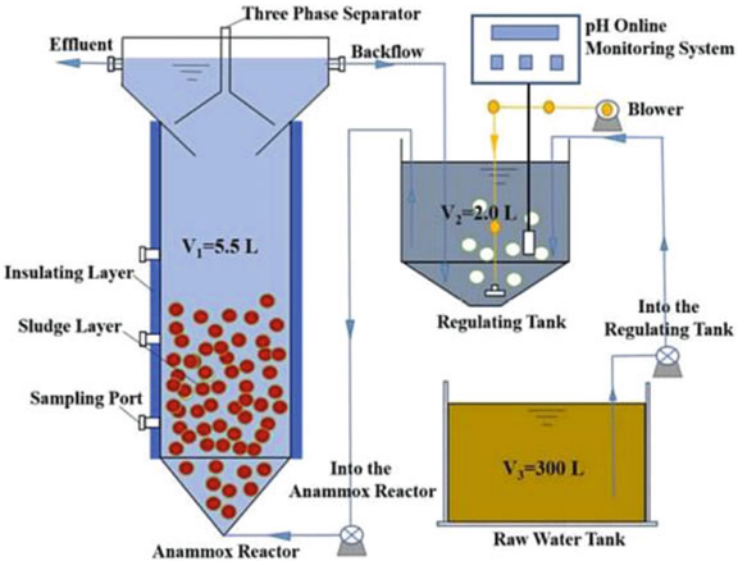

Fig. 15.2 Schematic diagram of the reactor (Yuan et al. 2020)

Table 15.6 Composition of wastewater

Parameter	Unit	Start-up stage	High nitrogen load acclimation stage	Domestic sewage
Chemical oxygen demand (COD)	mg/L	–	–	140–160
NH4-N	mg/L	200–250	200 ± 5	50–70
Total nitrogen (TN)	mg/L	200–250	200 ± 5	70–80
Total phosphorus (TP)	mg/L	–	–	3–4
KH_2PO_4	mg/L	–	25	–
$NaHCO_3$	mg/L	1000	1000	–
$CaCl_2-2H_2O$	mg/L	113	113	–
$MgSO_4-7H_2O$	mg/L	100	100	–
pH		7.5–7.6	7.5–7.6	6.7–8.2
Temperature	°C	30 ± 1	30 ± 1	23.2–26.7

Yuan et al. (2020)

set at 30 ± 1.0 °C, which is reached during the time in parts of southern China (Yuan et al. 2020).

This methodology was utilized to decide the treatment effect and explicit working states of a one-phase ANAMMOX reactor to treat real local sewage and gives a specialized premise to the use of one-phase ANAMMOX reactors in local sewage treatment. To forestall DO from hindering ANAMMOX at the reactor start-up period, the DO substance of the directing tank was controlled to be underneath 0.6 mg/L (Strous et al. 1998; Yin et al. 2016; Yuan et al. 2020). Be that as it may, during the upward progression of wastewater, due to the nearness of incomplete nitrification and other facultative anaerobic microorganisms, the DO in the primary power source diminished in the vertical heading. The DO in the upper layer of the primary power source was beneath 0.1 mg/L.

15.4.2 Application of ANAMMOX and Partial Denitrification Coupling Process

The application of ANAMMOX and partial denitrification coupling in nitrogen removal from wastewaters containing nitrate (such as municipal wastewater, industrial wastewater and aquaculture wastewater) is divided into separated and combined processes.

15.4.2.1 Separated Process

The separated process of ANAMMOX and partial denitrification coupling is inoculated with denitrifying bacteria and ANAMMOX with absolute advantages in two commonly autonomous reactors and at the same time produces NO_2^--N and nitrogen removal processes, which can effectively avoid space rivalry. Organic matter is completely used by denitrifying bacteria in the denitrification reactor, lessening the concentration of organic matter flowing to the ANAMMOX reactor. Wastewater containing nitrate (such as aquaculture wastewater, industrial wastewater and municipal wastewater) first flowed into the partial denitrification reactor to reduce nitrate to NO_2^--N while consuming organic matter as the electron donor. Then, the wastewater NO_2^- was adjusted before flowing into the ANAMMOX reactor (NO_2^--N/NH_4^+-N = 1.32) and was utilized to expel nitrogen, and part of the effluent came back to the partial denitrification reactor for profound nitrogen removal (Du et al. 2019a, b, c, d).

Cao et al. (2016) utilized a separated reactor to treat high concentration nitrogen-containing wastewater (NO_3^--N = 820 mg L^{-1}) in which the total nitrogen (TN) in the effluent was under 20 mg L^{-1} and the TN expulsion rate was as high as 97.8%. It was demonstrated that the removal rate of NH_4^+-N peaks at 96.7% while treating domestic sewage, and this procedure can adequately solve the problem of nitrogen deposit in sewage.

Du et al. (2019a, b, c, d) streamlined the mixed volume proportion of influent NO_3^--N and sewage and the amount of organic matter to treat high-nitrogen wastewater (NO_3^--N = 1000 mg L^{-1}); the TN in the effluent was less than 11 mg L^{-1}, and the removal rate of TN was as high as 95.8%. Meanwhile, the contribution rate of ANAMMOX to TN nitrogen removal was 78.9%. In addition, Wang et al. (2019a, b) proposed a new process, EPDPR (endogenous partial denitrification and denitrifying phosphorus removal), in which the rate of phosphorus removal is as high as 92.3% and the output of 79.2% of NO_2^--N is the best for the post-ANAMMOX process. The suitable substrate ratio (NO_2^--N/NH_4^+-N = 1.32) and high purification capacity (TN < 6 mg L^{-1}, TP < 0. mg L^{-1}) provide new ideas for removing nitrogen, phosphorus and other nutrients from sewage. A mainstream literature search revealed that the reason why the separated process is stable and the TN removal rate is high is because the ANAMMOX makes a more prominent commitment to the TN removal rate (Cao et al. 2019; Du et al. 2016; Du et al. 2019a, b, c, d; Ji et al. 2018).

15.4.2.2 Combined Process

The combined process of ANAMMOX and partial denitrification coupling begun by inoculating the ideal partial denitrification floc sludge and ANAMMOX granular sludge (Zhang et al. 2019a, b, c), and it has also been revealed that the inoculated partial denitrification sludge is adjusted from PN sludge (Wang et al. 2019a, b).

Zhang et al. (2019a, b, c) reported a new procedure called completely anaerobic ammonium removal over nitrite (CAARON); they looked at the synchronous expansion and addition of acetate at low C/N with that of asynchronous addition and affirmed that the asynchronous expansion of acetate derivation improved and optimized the reactor. The distribution of the bacterial nitrate-to-nitrite transformation ratio (NTR) was 97.4%, and the ANAMMOX nitrogen removal contribution rate was 85.8%. Additionally, Du et al. (2019a, b, c, d) carried out the DEAMOX reaction in an SBR, with the provision and supplying acetate at various periods to the reactor also accomplished a nitrogen removal rate of up to 95.8%. Zhang et al. (2019a, b, c) studied the economic feasibility, potential challenges and engineering feasibility of the PN/AMX and PDN/AMX processes; the researchers noticed that bacteria demonstrated good capture of soluble COD, which is another approach control the impacts of organic matter on ANAMMOX bacteria. Qin et al. (2017) concentrated on the effects of glucose on DEAMOX nitrogen removal performance in an upstream continuous consideration reactor and confirmed that dissolved COD significantly affected the biological communities. When the concentration of glucose was 56.4 mg L^{-1}, the system TN removal rate was 98.6%.

15.4.3 Reactor Management

The methods of managing the reactor in the separated and combined processes include:

1. Selection of reactor type and seed sludge: Given that the processing has low carbon request and negligible sludge creation, researchers as a rule make use of SBR or UASB as the ANAMMOX and partial denitrification coupling starting reactor (Du et al. 2017; Zhang et al. 2019a, b, c; Wang et al. 2019a, b; Du et al. 2020). ANAMMOX granular sludge and partial denitrification floc sludge are inoculated as seed sludge, or ANAMMOX sludge is cultured to acquire the target strain.
2. Optimization of hydraulic retention time (HRT) and proper ventilation: Increasing the wastewater HRT adequately and appropriately in the reactor can afford higher TN and COD removal rates, and even the difficult-to-degrade COD will be assimilated and expended in a higher HRT. This strategy effectively enhances an adequate measure of partial denitrification and ANAMMOX useful microorganisms (Du et al. 2019a, b, c, d; Zhang et al. 2019a, b, c; Du et al. 2020). Advancing air circulation can evade unreasonable creation of extracellular polymers (EPS), which is beneficial to the stability of the microbial community and nitrogen removal (Du et al. 2020).
3. Selection of organic matter feeding methods and addition of microbial carriers: Organic matter feeding strategies are distributed and conveyed constantly and irregularly. The richness and metabolism of continuous supply of organic matter ANAMMOX bacteria will be constrained, decreasing the nitrogen expulsion

capacity of the reactor. Intermittent feeding is generally utilized in the consolidated procedure and performs well, with a high TN load nitrogen removal effect (Zhang et al. 2019a, b, c; Du et al. 2019a, b, c, d). Partial denitrification and ANAMMOX bacteria can be effectively held on the microbial bearer, expand the particular surface area of the microorganism, increase the mass exchange rate and upgrade and enhance the nitrogen removal effect (Li et al. 2019; Niu et al. 2016).

15.4.4 Engineering Application

Research on ANAMMOX and partial denitrification coupling process is still in its infancy. The procedure has not been advocated or applied to nitrogen evacuation for municipal wastewater treatment; as a result, it has been rarely reported to date. Despite the fact that the engineering application of ANAMMOX has been expanding step by step in the past 10 years, the development of ANAMMOX and some denitrification coupling processes have slacked, and laboratory-scale research has a positive effect on engineering applications (Cao et al. 2019; Du et al. 2019a, b, c, d; Du et al. 2020; Wei et al. 2019).

Wang et al. (2010) revealed simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) treatment of landfill leachate in a full-scale (192 m^3) sewage treatment plant. The TN removal was around 83.9%, which affirms that mass balance of the system is the best approach for the assessment of ANAMMOX and nitration processes and that molecular tools and tracer studies with named nitrogen are valuable methodologies for the evaluation of ANAMMOX.

In view of an AAO procedure, Li et al. (2019) added carriers to the reactor to enrich ANAMMOX bacteria in the anoxic zone, forming the An-AAO process. ANAMMOX species advancement in situ builds the nitrogen removal rate, and it has been demonstrated that ANAMMOX has a high nitrogen expulsion commitment rate and is the way into the security and designing utilization of the denitrification coupling process, which was effectively utilized in urban sewage treatment plants.

15.4.5 Other Applications

Aquatec Maxcon is presently into wastewater treatment by Anammox process, although this innovative process is patented and supported by Paques BV of the Netherlands. Their potential areas of applications include sludge digester concentrate, slaughterhouses/rendering plants, distilleries, potato processing, semiconductor industry, tannery, fermentation, glutamate, enzymes, ethanol, yeast and fertilizers.

15.4.5.1 Advantages

1. ANAMMOX has high nitrogen removal efficiency.
2. It requires no additional organic carbon source.
3. It has a high load, low excess sludge output and low operating costs.
4. Power consumption is reduced by up to 60% as compared to conventional nitrification/denitrification methodology.
5. No additional carbon source needed.
6. Overall operational cost is reduced by up to 90% compared to conventional nitrification/denitrification methods.
7. It reduces the emission of CO_2 by approximately 88%.
8. ANAMMOX bacteria easily form stable self-aggregated biofilm (granules) allowing reliable operation of compact systems characterized by high biomass concentration and conversion rate up to 5–10 kg N m^{-3} (van Loosdrecht 2008).

15.4.5.2 Disadvantages

1. ANAMMOX processes has a slow doubling time which is between 10 and 14 days, due to this, it becomes slow to grow in the sludge for a wastewater treatment reactor.
2. It has a long recovery time: that is to say that the recovery time after the loss of sludge by accident is longer than in conventional nitrogen removal systems, although this slow growing rate can also be an advantage due to the reduction of surplus sludge that needs to be removed and treated.

15.5 Conclusion

ANAMMOX process is an economical and energy-saving biotechnology that encompasses a great potential in the treatment of ammonium-rich wastewaters, especially after its successful case in the treatment of sludge digest liquids. Long start-up time of this process severely limits its application, but this process will be improved through inoculation with pre-cultivated ANAMMOX sludge, selection of reactors with efficient biomass retention, adjustment of nutrient balance and environmental conditions. At present, the ANAMMOX process continues to be confined to some forms of wastewaters (sludge digestate and animal wastewaters). Even though domestic treatment has been recently applied, still more wide application is feasible if the biomass is largely enriched and more adapted to the organic matters. Some species from seawaters, which is psychrophilic, may be investigated for the application to cold regions. Multiple process parameters (such as dissolved oxygen, temperature, redox potential, pH, carbon-to-nitrogen ratio and sludge) can be adjusted to improve the rector condition and the ANAMMOX process which in turn will modify the nitrogen removal performance.

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Chapter 16
Microbial Bioremediation: A Cutting-Edge Technology for Xenobiotic Removal

Jaskiran Kaur and Naga Raju Maddela

Contents

Abstract Industrialization, urbanization, and the use of modern technology in agriculture have its pros and cons. On one hand, they improve the standard of living but impact the structure and function of different ecosystems drastically. In a broad sense, a decline in crop productivity, impairment in activity of soil microbes, death of aquatic fauna, as well as carcinogenicity and mutagenicity in humans and animals are some of the ill effects due to xenobiotic presence in the environment. It is thus imperative to develop certain strategies that can notably ensure the perspective of development without compromising the health of the ecosystem. Among the various physical and chemical methods for xenobiotic degradation, bioremediation using microorganisms is unequivocally an economical and ecologically sound approach. This chapter emphasizes the applicability of the bioremediation process for the effective degradation of different classes of xenobiotic compounds like pesticides, dyes, phenols, pharmaceuticals, etc. The up-to-date information about the

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involvement of two major microbes, namely, bacteria and fungi as well as enzymes from different sources, are described in the context of xenobiotic degradation and detoxification. Last but not the least, the various factors that come into play for significant removal of a xenobiotic are also explained in a well-defined manner.

Keywords Xenobiotics · Pesticides · Bioremediation process · Dye degradation · Enzymes

16.1 Introduction

Due to the industrial revolution together with urbanization and the introduction of contemporary agricultural practices, a remarkable advancement of economic growth has been apparent during the past few years. As a consequence, a plethora of environment-unfriendly xenobiotic compounds are generated daily. The term xenobiotics signifies those compounds which are considered as foreign to the biological system (Loredana et al. 2017). More precisely, these include a wide range of man-made chemical substances which are manufactured in the laboratory, for instance, pharmaceuticals, pesticides, hydrocarbons, artificial sweeteners, plastics, lignin, aromatics, and certain solvents like phenol and carcinogens as well (Rieger et al. 2002). The prevalence of certain elements has been experienced in the structure of xenobiotics which are not generally exist in natural environments. Examples of such elements include aromatic sulfonic acids (present in dyes), diazo bond, and polychlorination of either an alkane or aromatic compound (Knapp and Bromley-Challoner 2003).

Although xenobiotics are present in very low concentrations (ng/L to μg/L) in the environment but the existence of such toxic compounds are regarded as major havoc to environmental integrity. Being recalcitrant, they accumulate in the hydrosphere, lithosphere, and atmosphere where they are known to negatively impact the flora, fauna, and humans (Gangola et al. 2018). When xenobiotic-contaminated effluents from industries like oil refineries, food industries, paint, pesticides, and textile are discharged into the surrounding water bodies, the dissolved oxygen gets significantly depleted (Garg and Tripathi 2017; Lellis et al. 2019). On top of that, the colored effluent from the textile industries blocks the sunlight's entry into the waterways, thus impairing the photosynthesis in aquatic plants (Imran et al. 2015; Hassan and Carr 2018). On the other hand, the spray of pesticides and herbicides causes the introduction of these toxic compounds into the agricultural lands. Interminable pesticide consumption is responsible for the impairment in soil properties and reduction in the population of soil microbes (El-Ghany and Masmali 2016). Moreover, they also flow into lakes and rivers through surface runoff, thus making the water infected (Casara et al. 2012; Pande et al. 2020). These xenobiotics persist in the environment for longer durations which ultimately enter into the food chain,

hence causing biomagnification. It has been observed that long-term exposure towards xenobiotics can induce various tumorigenic, neurotoxic, genotoxic, immunotoxic, and mutagenic effects in humans and animals (Terry 2012). The possible effects of xenobiotic presence in the environment on various communities are delineated in Fig. 16.1.

Environmental restoration is the prime necessity in the present scenario. To achieve this motive, it is essential to tranquilize the deleterious effects of xenobiotics on air, water, and soil. For reducing the toxicity content of xenobiotics, many physical and chemical treatment processes such as photolysis, advanced oxidation, ozonation, hydrolysis, membrane filtration, electrocoagulation, adsorption, and flocculation are put into practice (Kaneco et al. 2006; Foo and Hameed 2010; Behera et al. 2011; Plakas and Karabelas 2012; Adak et al. 2019). But considering the problems of sludge generation, high operational cost, and generation of toxic degradation products associated with the physicochemical methods, the bioremediation method is of particular interest for eco-friendly and cost-effective treatment of xenobiotics (Lopez et al. 2004; Linley et al. 2012; Youssef et al. 2016). Furthermore, the problem of toxic degradation product generation is not associated with the bioremediation method. Many microorganisms, namely, bacteria and fungi as well as microbial enzymes, are the key players in the bioremediation process.

However, the conditions under which the microbes and enzymes can reveal maximum degradation capability are an important point in question. In this chapter, comprehensive knowledge concerning the sources of xenobiotics is provided. An attempt is made to evaluate the various microbes responsible for carrying out the degradation of various xenobiotic compounds, for example, pesticides, pharmaceuticals, dyes, phenols, etc. Finally, the different nutrient and physicochemical conditions underpinning the bioabsorption, transformation, and mineralization of xenobiotics by microbes are extensively addressed.

16.2 Classification and Sources of Xenobiotics

Every day huge quantities of several hazardous substances (the xenobiotics) are released into the environment (Fig. 16.2). The potential sources of different types of xenobiotics are documented in Table 16.1. Many industries, namely, pharmaceuticals, paper and pulp bleaching, petrochemical, coal refineries, and textile industries, play an important role in the introduction of these toxic recalcitrant substances. One of such toxic compound is dyes that are increasingly being used by industries during several industrial processes like for coloration of fabrics in textile industries, for hair, nails, lip coloring, eye and facial makeup in cosmetic industries, as well as for photosensitization in the photographic industry. Dyes can persist in the environment for longer durations (Waller et al. 2000; Guerra et al. 2018; Lellis et al. 2019). Some of the most commonly used dyes in these industries comprise of azo-type dyes during the industrial processes. Around 80% of azo dyes used in textile industries are incapable of binding to the fabric and are released as such in the effluent (Rehman

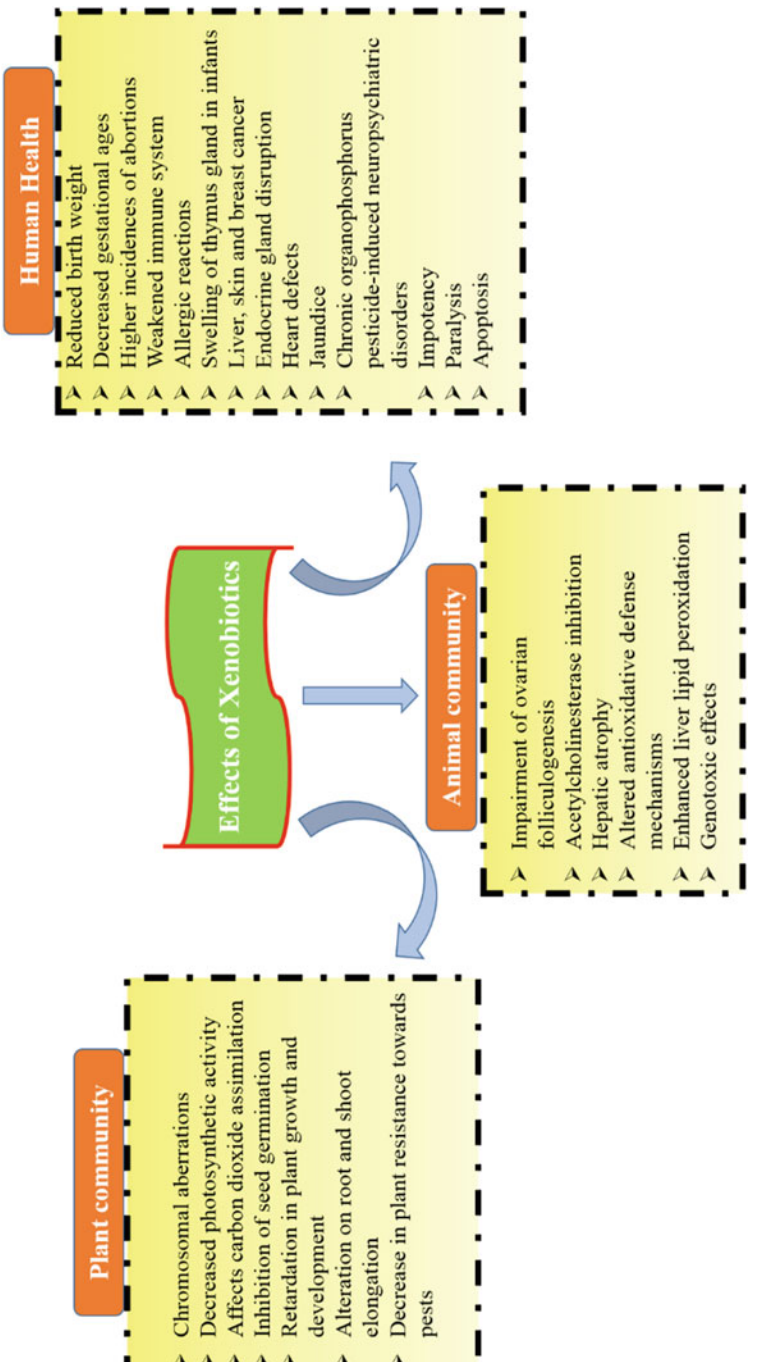

Fig. 16.1 Effects of xenobiotics on the biotic communities

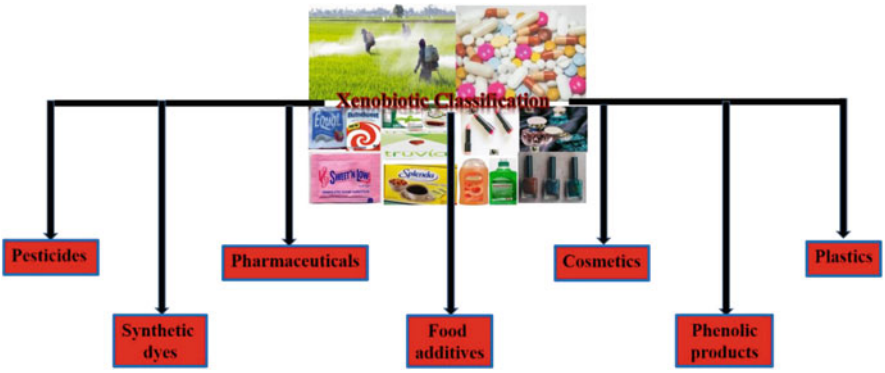

Fig. 16.2 Classification of xenobiotic

et al. 2018). As per reports, around 280,000 tons of textile dyes are lost in the wastewater globally every year (Choudhary et al. 2020).

Likewise, some other industries like petrochemical and pesticide industries are also responsible for the release of an extensive list of xenobiotics which due to their recalcitrant property tends to concentrate in the environment. For example, the petrochemical industry which is considered as one of the fastest-growing industrial sectors generates hazardous wastes such as polycyclic aromatic hydrocarbons (anthracene and naphthalene). The effluents from petrochemical industries such as oil refineries as well as plastic industries and olive processing plants are also rich in polyphenolics (Aggelis et al. 2002). Another class of xenobiotics, that is, pharmaceutical drugs, including human and veterinary antibiotics, hormones, analgesics, anticonvulsants, antihistamine, antidepressants, and β-blockers, are persistently discharged into the terrestrial and aquatic ecosystems by households, landfills and from effluents released by hospitals, sewage treatment plants, and municipal and industrial facilities (Barnes et al. 2004; Watkinson et al. 2009; Fatta-Kassinos et al. 2011; Chakraborty et al. 2020). Overall, it has been estimated that these industrial plants are responsible for the deposition of approximately 300–400 million tons of toxic sludge, solvents, and heavy metals into the environmental surroundings (Xiao et al. 2015).

Besides the industrial sectors, the contribution of agriculture in deteriorating the environment through the release of xenobiotics is not a matter to be ignored. It has been estimated that by 2025, the world population will reach 8 billion. The continuous ever-increasing population exerts tremendous pressure on the agriculture sector to employ different methods to produce food on a large scale. As a result, the farmers are using pesticides—the chemicals to kill pests of crops which can be weeds, insects, rodents, fungi, etc., to increase the agricultural productivity. A wide variety of pesticides like organophosphates, organochlorines, carbamate, and morpholine successfully control the pest establishment, but due to their broad-spectrum activity, certain nontarget organisms get severely affected.

Table 16.1 Sources involved in the generation of various xenobiotics

Source	Type of xenobiotic released into the environment	Examples of xenobiotics	References
Paper and pulp bleaching	Chlorinated organic compounds	di-, tri-, tetra-, and pentachlorophenols, tetrachloroguaiacols, and tetrachlorocatechols	Tana (1988), Singh (2017)
Sewage treatment plant	Pharmaceuticals	Ofloxacin, ciprofloxacin, erythromycin, diclofenac, ibuprofen, carbamazepine, bezafibrate, atenolol, acetaminophen, oxytetracycline, tylosin, sulfamethoxazole, amoxicillin, diazepam, trimethoprim, clindamycin, lincomycin	Rosal et al. (2010), Zuccato et al. (2010), Subedi et al. (2017)
Hospital wastewater treatment plant	Pharmaceuticals	Acetaminophen, ciprofloxacin, norfloxacin, tetracycline, atenolol, ketoprofen, ibuprofen, estrone, estriol	Kanama et al. (2018)
Municipal wastewater treatment plant	Pharmaceuticals	Fenbendazole	Sim et al. (2013)
Intensive agriculture	Herbicides	Pendimethalin, butachlor, bensulfuron-methyl, pretilachlor	Das et al. (2011), Pinto et al. (2012), Singh (2017), Mohanty and Jena (2019)
	Pesticides and insecticides	Benzimidazoles, methyl parathion, morpholine, chlorpyrifos, aldrin	Singh (2017), Rayu et al. (2017), Doolotkeldieva et al. (2018)
Paper mill	Phenol	–	Sachan et al. (2019)
Chemical and pharmaceutical industry	Synthetic polymers, phenols	–	Singh (2017), Varsha et al. (2011)
Petrochemical industry	Phenol	–	Liu et al. (2016)
Plastic industry	Phenol	–	Liu et al. (2016)
Paint industry	Organic solvents	Toluene, xylene, styrene, ethylbenzene	Moro et al. (2010)
Textile industry	Azo dyes	Black B, Turq Blue GN, Tectilon Yellow 2G, Yellow HEM, Red HEFB, and Navy HER	Tufekci et al. (2007), Acuner and Dilek (2004)
Textile industry	Heavy metals	Ni, Cu, Cr, Pb, Cd, Zn	Khan and Malik (2018)

16.3 Xenobiotic Bioremediation Utilizing Microbes

Bioremediation technology nowadays has achieved stupendous attention for the removal of recalcitrant compounds from soil and water. In this technology, a myriad of microorganisms which are either inhabitant of xenobiotic contaminated sites or genetically modified microbes with amplified biodegradability potential are exploited. Usually, the bioremediation processes involve various reactions including oxidation-reduction, hydrolysis, hydroxylation, conjugation, sulfation, and methylation for the degradation or biotransformation of xenobiotics. Varied microorganisms are likely to degrade diverse xenobiotics present in different sources across the world which are given in detail in the following sections.

16.3.1 Role of Bacteria for Xenobiotic Removal

The role of microbes in bioremediation and their resistance to xenobiotic toxicity has been well documented by various researchers. Table 16.2 summarizes the various studies on the use of bacteria for the removal of a wide range of xenobiotics. Heterogeneous bacteria are known to make use of phenol as a carbon and energy source. Most widely recognized among the bacterial population are *Acinetobacter calcoaceticus*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* (Liu et al. 2016; Mohanty and Jena 2017; Maniyam et al. 2020). From several different environments which most often include agricultural wastewater, natural river biofilms, agricultural soil and sugarcane farm soil, etc. (El-Helow et al. 2013; Tien et al. 2017; Fareed et al. 2017), pesticide degraders have been frequently isolated.

The hydrolysis of labile methylcarbamine linkage with the production of metabolites such as carbofuran-7-phenol and methylamine occurred during the degradation of pesticide carbofuran by bacteria (Yan et al. 2007). The bacteria *Sphingomonas* sp. can degrade carbofuran into various metabolites, for example, 2-hydroxy-3-(3-methylpropan-2-ol) phenol and red intermediates (Park et al. 2006).

The *Pseudomonas aeruginosa* that is isolated from desert soil can simultaneously degrade cadmium (Cd) and Reactive Black 5 (RB5) which are the common xenobiotics found in the industrial effluent (Louati et al. 2020). It has been seen that the Cd is removed by bacteria via biosorption mechanism wherein the metal binds on the microbial surface through processes like electrostatic interaction, complex formation, ion exchange, and precipitation (Hansda et al. 2016; Ayangbenro and Babalola 2017). Apart from that, the extracellular polymers synthesized by *Pseudomonas* sp. are involved in metal chelation (Gupta and Diwan 2017). As per the study by Giovanella et al. (2017), the reduction, biosorption, production of siderophore, and biofilm development are the main mechanisms responsible for metal removal by *Pseudomonas* sp.

The xenobiotic removal by free cells nonetheless is a prime concern due to the complications of activity loss, cell separation, and problem in isolating strain having

Table 16.2 List of various microbes with xenobiotic degrading potential

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
Bacteria	*Gordonia* sp. JAAS1	Chlorpyrifos	Paddy soil	• Temperature—28 °C • Incubation time—24 h • Pesticide concentration—110 mg/L	100%	Abraham et al. (2013)
	Sphingobacterium sp. JAS3	Chlorpyrifos	Paddy soil	• Temperature—30 ± 2 °C • Incubation time—24 h • Pesticide concentration—300 mg/L	100%	Abraham and Silambarasan (2013)
	Bacillus subtilis Y242	Chlorpyrifos	Agricultural wastewater	• Temperature—30 °C • Incubation time—48 h • Pesticide concentration—150 mg/L	95.1%	El-Helow et al. (2013)
	Mesorhizobium sp. HN3	Chlorpyrifos	Soil	• Temperature—37 °C • pH—7 • Incubation time—5–7 days • Pesticide concentration—100 mg/L	100%	Jabeen et al. (2015)

	Acinetobacter calcoaceticus PA	Phenol	Oil refinery wastewater	• Temperature—30 °C • Incubation time—48 h • Agitation speed—150 rpm • Phenol concentration—800 mg/L	91.6%	Liu et al. (2016)
	Pseudomonas japonica I-15	Reactive Black 5 dye	Activated sludge	• Temperature—28 °C • Daily light integral—240 μmol/m^2/s^1 • Relative humidity—61% • Incubation time—48 h • Dye concentration—150 mg/L	81%	Shafqat et al. (2017)
	Bacillus sp. SR-2-1/1	Azo dyes	Rhizosphere samples of sorghum plants grown at textile wastewater-contaminated soil	• Temperature—30 °C • pH—7 • Incubation time—48 h • Dye concentration—150 mg/L	80–90%	Mahmood et al. (2017)
	Sulfitobacter dubius	Lindane	Demosponge *Hymeniacidon perlevis* associated	• Temperature—22 °C • Incubation time—12 days • Pesticide concentration—0.05 mg/L	97%	Loredana et al. (2017)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
	Alteromonas australica	Lindane	Demosponge *Hymeniacidon perlevis* associated	• Temperature—22 °C • Incubation time—12 days • Pesticide concentration—0.05 mg/L	97%	Loredana et al. (2017)
	Pseudovibrio ascidiaceicola	Lindane	Demosponge *Hymeniacidon perlevis* associated	• Temperature—22 °C • Incubation time—12 days • Pesticide concentration—0.05 mg/L	95%	Loredana et al. (2017)
	Xanthomonas sp. 4R3-M1	Chlorpyrifos	Sugarcane farm soils	• Temperature—28 °C • Incubation time—6 days • Pesticide concentration—10 mg/L	80%	Rayu et al. (2017)
	Pseudomonas sp. 4H1-M3	Chlorpyrifos	Sugarcane farm soils	• Temperature—28 °C • Incubation time—6 days • Pesticide concentration—10 mg/L	90%	Rayu et al. (2017)

	Rhizobium sp. 4H1-M1	Chlorpyrifos	Sugarcane farm soils	• Temperature—28 °C • Incubation time—6 days • Insecticide concentration—10 mg/L	75%	Rayu et al. (2017)
	Pseudomonas sp.	Phenol	Sewage and wastewater discharged site	• Temperature—30 to 32 °C • pH—6.8 to 7.2 • Incubation time—168 h • Agitation speed—150 rpm • Phenol concentration—1000 mg/L	>98%	Mohanty and Jena (2017)
	Ochrobactrum sp.	Erythromycin	Soil sample	• Temperature—32 °C • pH—6.5 • Incubation time—72 h • Erythromycin concentration—100 mg/L	97%	Zhang et al. (2017)
	Sphingobacterium multivorum	Carbofuran	Natural river biofilms	• Temperature—25 °C • pH—7 • Incubation time—7 days • Pesticide concentration—50 mg/L	73.1%	Tien et al. (2017)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
	Thermus thermophilus	Ciprofloxacin	Pharmaceutical sludge	• Temperature—65 to 80 °C • pH—6.5 • Incubation time—72 h • Antibiotic concentration—5 mg/L	>55%	Pan et al. (2018)
	Bradyrhizobium sp. GLC_01	Ciprofloxacin	Activated sludge	• Temperature—25 °C • pH—6.5 • Incubation time—8 days • Agitation speed—150 rpm • Antibiotic concentration—0.05 mg/L	70.4%	Nguyen et al. (2018)
	Arthrobacter soli BS5	Reactive Black 5	Native of soil irrigated with textile industry wastewater	• Temperature—37 °C • pH—5 to 9 • Incubation time—120 h • Dye concentration—50 µg/mL	98%	Khan and Malik (2018)
	Serratia liquefaciens	Azure B	Soil sample	• Temperature—30 °C • Incubation time—48 h • Dye concentration—100 mg/L	>90%	Haq and Raj (2018)

	Neisseria sp. (EK-5)	Novacron Orange FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	19%	Karim et al. (2018)
	Neisseria sp. (EK-5)	Novacron Brilliant Blue FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	43% decolorization	Karim et al. (2018)
	Neisseria sp. (EK-5)	Novacron Super Black G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	65% decolorization	Karim et al. (2018)
	Neisseria sp. (EK-5)	Bezema Yellow S8-G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	30% decolorization	Karim et al. (2018)
	Vibrio sp. (EK-6)	Novacron Orange FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days	40% decolorization	Karim et al. (2018)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
				• Dye concentration—100 mg/L		
	Vibrio sp. (EK-6)	Novacron Brilliant Blue FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	67% decolorization	Karim et al. (2018)
	Vibrio sp. (EK-6)	Novacron Super Black G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	65% decolorization	Karim et al. (2018)
	Vibrio sp. (EK-6)	Bezema Yellow S8-G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	50% decolorization	Karim et al. (2018)
	Vibrio sp. (EK-6)	Bezema Red S2-B	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days	42% decolorization	Karim et al. (2018)

				• Dye concentration—100 mg/L		
	Bacillus sp. (EK-7)	Novacron Orange FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	25% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-7)	Novacron Brilliant Blue FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	83% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-7)	Novacron Super Black G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	35% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-7)	Bezema Yellow S8-G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	55% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-7)	Bezema Red S2-B	Dyeing effluent	• Temperature—37 °C • pH—7	41% decolorization	Karim et al. (2018)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
				• Incubation time—6 days • Dye concentration—100 mg/L		
	Bacillus sp. (EK-9)	Novacron Orange FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	39% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-9)	Novacron Super Black G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L.	40% decolorization	Karim et al. (2018)
	Bacillus sp. (EK-9)	Bezema Yellow S8-G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	47% decolorization	Karim et al. (2018)
	Aeromonas sp. (EK-13)	Novacron Orange FN-R	Dyeing effluent	• Temperature—37 °C • pH—7	27% decolorization	Karim et al. (2018)

				• Incubation time—6 days • Dye concentration—100 mg/L		
	Aeromonas sp. (EK-13)	Novacron Brilliant Blue FN-R	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	90% decolorization	Karim et al. (2018)
	Aeromonas sp. (EK-13)	Novacron Super Black G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	23% decolorization	Karim et al. (2018)
	Aeromonas sp. (EK-13)	Bezema Yellow S8-G	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	38% decolorization	Karim et al. (2018)
	Aeromonas sp. (EK-13)	Bezema Red S2-B	Dyeing effluent	• Temperature—37 °C • pH—7 • Incubation time—6 days • Dye concentration—100 mg/L	41% decolorization	Karim et al. (2018)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
	Bacillus polymyxa	Aldrin	Soil	• Temperature—25 °C • pH—7.2 • Incubation time—12 days • Insecticide concentration—0.2 mg/mL	48.2%	Doolotkeldieva et al. (2018)
	Pseudomonas fluorescens	Aldrin	Soil	• Temperature—25 °C • pH—7.2 • Incubation time—12 days • Insecticide concentration—0.2 mg/mL	43.2%	Doolotkeldieva et al. (2018)
	Serratia ureilytica strain AS1	Butachlor	Herbicide-contaminated soil	• Temperature—32.5 °C • pH—7.5 • Incubation time—10 days • Herbicide concentration—500 mg/L	100%	Mohanty and Jena (2019)
	Rhodococcus UKMP-5M	Phenol	Petroleum-contaminated soil	• Temperature—30 °C • pH—7.4 • Incubation time—24 h	89%	Suhaila et al. (2019)

				• Phenol concentration—0.5 g/L		
	Rhodococcus strain UCC 0016	Methyl red dye	Palm oil mill effluent	• Temperature—30 °C • pH—7 • Incubation time—24 h • Incubation at static condition • Dye concentration—0.5 g/L	100%	Maniyam et al. (2020)
	Enterobacter sp.	Carbofuran	Agricultural soil	• Temperature—37 °C • Incubation time—68 h • Insecticide concentration—100 mg/L	100%	Mustapha et al. (2020a)
Fungi	*Trametes versicolor*	Norfloxacin	–	• Temperature—30 °C • Incubation time—7 days • Agitation speed—150 rpm • Antibiotic concentration—2 mg/L	>90%	Prieto et al. (2011)
	Trametes versicolor	Ciprofloxacin	–	• Temperature—30 °C • Incubation time—7 days • Agitation speed—150 rpm	>90%	Prieto et al. (2011)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
				• Antibiotic concentration—2 mg/L		
	Lentinula edodes	Pendimethalin	Loamy sand soil and a biomixture	• Temperature—25 °C • Incubation time—10 days • Agitation speed—150 rpm • Pesticide concentration—25 mg/L	~ 96%	Pinto et al. (2012)
	Penicillium brevicompactum	Terbuthylazine	Loamy sand soil and a biomixture	• Temperature—25 °C • Incubation time—10 days • Agitation speed—150 rpm • Pesticide concentration—25 mg/L	99.5%	Pinto et al. (2012)
	Trametes versicolor	Flumequine	–	• Temperature—28 °C • Incubation time—4 days • Antibiotic concentration—12 μg/mL	100%	Cvancarova et al. (2013)
	Irpex lacteus	Flumequine	–	• Temperature—28 °C • Incubation time—4 days	90%	Cvancarova et al. (2013)

				• Antibiotic concentration—12 μg/mL		
	Dichomitus squalens	Flumequine	–	• Temperature—28 °C • Incubation time—4 days • Antibiotic concentration—12 μg/mL	90%	Cvancarova et al. (2013)
	Pestalotiopsis sp. NG007	Reactive Green 19	Textile industry wastewater	• Temperature—30 °C • pH—3 to 12 • Salinity—0 to 10% w/v • Incubation time—24 h	94% decolorization	Yanto et al. (2014)
	Pestalotiopsis sp. NG007	Reactive Orange 64	Textile industry wastewater	• Temperature—30 °C • pH—3 to 12 • Salinity—0 to 10% w/v • Incubation time—24 h	54% decolorization	Yanto et al. (2014)
	Pestalotiopsis sp. NG007	Reactive Red 4	Textile industry wastewater	• Temperature—30 °C • pH—3 to 12 • Salinity—0 to 10% w/v • Incubation time—24 h	47% decolorization	Yanto et al. (2014)
	Phanerochaete chrysosporium	Amido Black 10B	–	• Temperature—37 °C • pH—3 to 7 • Incubation time—	98% decolorization	Senthilkumar et al. (2014)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
				3 days • Agitation speed—150 rpm		
	Penicillium citrinum	Chlorfenvinphos	Untreated surface water	• Temperature—27 °C • Incubation time—82 days • Agitation speed—100 rpm • Pesticide concentration—250 µg/L	100%	Oliveira et al. (2015)
	Penicillium ochrochloron AMDB-12	Reactive Blue 13	Acidic mine drainage	• Temperature—40 °C • pH—2 • Contact time—120 min • Agitation speed—150 rpm • Dye concentration—50 ppm	55%	Aytar et al. (2016)
	Penicillium ochrochloron AMDB-12	Reactive Blue 72	Acidic mine drainage	• Temperature—40 °C • pH—2 • Contact time—120 min • Agitation speed—150 rpm • Dye concentration—50 ppm	61%	Aytar et al. (2016)

	Aspergillus fumigatus A23	Simulated textile effluent	–	• Temperature—40 °C • pH—4 • Incubation time—7 days • Agitation speed—100 rpm	86%	Dharajiya et al. (2016)
	Phanerochaete chrysosporium	Simulated textile effluent	–	• Temperature—30 °C • pH—5 • Incubation time—7 days • Agitation speed—100 rpm	62% decolorization	Dharajiya et al. (2016)
	Trichoderma harzianum	Malathion	Soil samples	• Temperature—20 to 40 °C • Incubation time—20 days • Insecticide concentration—10 to 40 mg/L	>90%	El-Ghany and Masmali (2016)
	Metarhizium anisopliae	Profenofos	Soil samples	• Temperature—20 to 40 °C • Incubation time—20 days • Insecticide concentration—40 mg/L	63.6%	El-Ghany and Masmali (2016)
		Diazinon	Soil samples	• Temperature—20 to 40 °C • Incubation time—20 days • Insecticide	85.6%	El-Ghany and Masmali (2016)

(continued)

Table 16.2 (continued)

Type of microbes	Microorganisms degrading xenobiotic	Target xenobiotic compound	Site of isolation	Culture conditions	Percent removal	References
				concentration—10 mg/L		
		Malathion	Soil samples	• Temperature—20 to 40 °C • Incubation time—20 days • Insecticide concentration—20 mg/L	>90%	El-Ghany and Masmali (2016)
	Thamnidium elegans	Reactive Yellow 2	–	• pH—2 • Contact time—39.4 min • Dye concentration—100 mg/L	95%	Akar et al. (2017)
	Pleurotus ostreatus	Ciprofloxacin	–	• Temperature—25 °C • Incubation time—14 days • Antibiotic concentration—500 ppm	95%	Singh et al. (2017)
	Aspergillus sp. TS-A CGMCC 12964	Mordant Yellow 1	Activated sludge of a textile factory	• Temperature—30 °C • pH—6 • Incubation time—1 h • Agitation speed—160 rpm • Dye concentration—50 mg/L	98.6%	Kang et al. (2018)

the ability to withstand high toxic concentrations of xenobiotics (Kathiravan et al. 2010; Fareed et al. 2017). To overcome this limitation, many researchers immobilized the free bacterial cells into the numerous organic materials as well as inorganic materials. Mustapha et al. (2020a) reported efficient degradation of carbofuran even at 250 mg/L concentration, whereas the free cells are unable to tolerate such a high concentration of carbofuran. Similarly, several other pesticides, for example, carbofuran, carbamates, pendimethalin, profenofos, atrazine, and cypermethrin, are found to be competently degraded by immobilized cells of bacteria contrary to free bacterial cells (Kadakol et al. 2011; Tallur et al. 2015; More et al. 2015; Talwar and Ninnekar 2015; Kumar et al. 2017; Fareed et al. 2017). Besides, Wang et al. (2017) confirmed the degradation of di-n-butyl phthalate using *Acinetobacter* species strain LMB-5 coated with magnetic nanoparticles.

16.3.2 Role of Fungi in Xenobiotic Removal

The fungi as a probable candidate for xenobiotic removal are attracting profound attention in contemporary times. The specific activity and growth morphology make fungal species a proficient degrader of xenobiotics (Mollea et al. 2005). *Aspergillus*, *Galactomyces geotrichum*, *Podoscypha elegans*, and *Scheffersomyces spartinae* are routinely used for detoxification of recalcitrant compounds (Ali et al. 2008; Waghmode et al. 2011; Tan et al. 2016; Dharajiya et al. 2016; Chaudhry et al. 2014; Pramanik and Chaudhuri 2018). Fungi such as *Aspergillus niger* cleave the carbon-phosphorus bond of organophosphonates, thereby releasing phosphate ions (Adelowo et al. 2015). Carbon-phosphorus bond cleavage is indicated to be the first step during the degradation of organophosphonate pesticides.

Among the fungal population, the white-rot fungi outclass as a major xenobiotic degrader. Many xenobiotic compounds, namely, pharmaceuticals, dyes, pesticides, phenols, lignin, etc., are converted into nontoxic metabolites using different white-rot fungi such as *Trametes versicolor*, *Trametes polyzona*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Bjerkandera adusta*, and *Cerrena unicolor* (Singh et al. 2010; Prieto et al. 2011; Asgher et al. 2016; Singh et al. 2017; Zhang et al. 2018; Bouacem et al. 2018; Lueangjaroenkit et al. 2019). The xenobiotic degradative ability of varied fungal species is evident from Table 16.2.

16.4 Bioremediation with Microbial Enzymes

Microbial enzymes have drawn profound attention when it comes to xenobiotic degradation. Reductase, laccase, peroxidase, oxidase, and hydrolase are some of the enzymes isolated from either bacteria or fungi which are relevantly functional in the degradation of a wide variety of substrates, for example, dyes, benzene, pharmaceuticals, pesticides, phenolic compounds, and polycyclic aromatic hydrocarbons

Table 16.3 Microbial enzymes employed for xenobiotic degradation

Enzyme used	Source of enzyme	Type of xenobiotic degraded	References
Alkaline phosphatase	*Spirulina platensis*	Chlorpyrifos	Thengodkar and Sivakami (2010)
Laccase	*Trametes versicolor*	Norfloxacin and ciprofloxacin	Prieto et al. (2011)
Laccase	*Trametes versicolor* and *Phanerochaete chrysosporium*	Benzo[a]pyrene	Qian and Chen (2012)
Laccase	*Trametes versicolor*	Acid Violet 7, Acid Red 1, Allura Red AC, Orange G, and Sunset Yellow FCF	Legerska et al. (2018)
Manganese peroxidase	*Phanerochaete chrysosporium*	Sulfamethoxazole	Gao et al. (2018)
Laccase	*Pycnoporus sanguineus*	Sulfamethoxazole, ciprofloxacin, norfloxacin	Gao et al. (2018)
Manganese peroxidase	*Aspergillus* sp. TS-A CGMCC 12964	Mordant Yellow 1	Kang et al. (2018)
Lignin peroxidase	*Bjerkandera adusta* strain CX-9	Acid Blue 158, Cibacet Brilliant Blue BG, Polymeric dye R, Remazol Brilliant Blue Reactif, Remazol Brilliant Violet 5R, Indigo Carmine, and Methyl Green	Bouacem et al. (2018)
Manganese peroxidase	*Bjerkandera adusta* strain CX-9	Acid Blue 158, Cibacet Brilliant Blue BG, Polymeric dye R, Remazol Brilliant Blue Reactif, Remazol Brilliant Violet 5R, Indigo Carmine, and Methyl Green	Bouacem et al. (2018)
Laccase	*Trametes* sp. MA-X01	Direct Blue 53, Direct Blue 14, Acid Orange 10, Acid Red 18, Acid chrome blue K, and Janus green B	Wang et al. (2018)
Laccase	*Kluyveromyces dobzhanskii*, *Pichia manshurica*	Malachite green and methyl red	Wakil et al. (2019)
Laccase	*Pseudomonas mendocina*	Mixed azo dye (reactive red, reactive brown, and reactive black)	Sridharan et al. (2019)
Laccase	*Trametes polyzona* KU-RNW027	Remazol brilliant blue, Remazol navy blue, and Remazol brilliant yellow, Remazol red, tetracycline, doxycycline, amoxicillin, and ciprofloxacin	Lueangjaroenkit et al. (2019)
Manganese peroxidase	*Trametes polyzona* KU-RNW027	Remazol brilliant blue, Remazol navy blue, and Remazol brilliant yellow, tetracycline, doxycycline, amoxicillin, and ciprofloxacin	Lueangjaroenkit et al. (2019)

(Weng et al. 2013; Zafra and Cortes-Espinosa 2015; Lellis et al. 2019). Table 16.3 enlists the studies concerning the use of bacterial and fungal enzymes for xenobiotic degradation or detoxification. The function of manganese peroxidase is to oxidize

Mn^{2+} to Mn^{3+} in the presence of H_2O_2 which thereby causes oxidation of various toxic compounds (Chen et al. 2015b; Bilal et al. 2016). The enzyme lignin peroxidase brought about the oxidation of aromatic compounds through the generation of free radicals in the presence of H_2O_2 (Bilal et al. 2017). Another vital enzyme laccase is a member of blue copper oxidases containing four copper atoms and is mainly found in fungi and higher plants. At the enzyme active site, copper atoms of four different types, that is, type I, type II, and two type III, are present which accounts for the catalytic activity of laccase (Wang et al. 2018). The type I copper atom of laccase oxidized the substrate which in turn loses electron that is accepted by type I.

From type I, the electrons are transported to types II and III where molecular oxygen is reduced to two molecules of water and substrate produces free radicals (Zucca et al. 2016; Wang et al. 2018; Lellis et al. 2019). Laccase is a efficient xenobiotic degrader unlike other enzymes as the requisite of cofactor, namely, hydrogen peroxide for substrate oxidation is not there (Liebminger et al. 2009; Bayramoğlu et al. 2010). Along with it, they have specificity for many substrates and hence can be increasingly useful for oxidizing diverse toxic pollutants (Saito et al. 2003; Aslam et al. 2012).

Immobilization of enzymes is a predominant research focus during the last decades. Enzymes when immobilized have better stability and activity as well as recovered from the suspension without difficulty (Shah and D'Mello 2007; Hebert and Rochefort 2008; Roman-Gusetu et al. 2009). A variety of carriers are available for immobilization of enzymes including alginate beads, glass beads, mesoporous silica, porous poly(GMA/EGDMA) beads, chitosan beads, poly(ethyleneimine) microcapsule, and nanoparticles (Dominguez et al. 2007; Mureseanu et al. 2009; Arica et al. 2009; Roman-Gusetu et al. 2009; Bilal et al. 2016; Sridharan et al. 2019). The nano-sized materials as a support material for immobilization are usually preferred nowadays as they provide large surface area for attachment and also ensure easy separation of enzymes in a magnetic field (Sridharan et al. 2019). Bayramoğlu et al. (2010) immobilized *Trametes versicolor* laccase onto the poly(4-vinylpyridine) grafted and Cu(II) chelated magnetic beads. The immobilized enzyme showed activity at a broader pH and temperature. Besides, it is possible to reuse immobilized enzymes for five cycles for dye degradation which is not possible in the case of free enzymes.

16.5 Factors Influencing the Biodegradation Ability of Microbes

The microbes need specific conditions for optimum growth and efficacious xenobiotic removal. The behavior of microbes varies depending upon different environmental factors such as pH, temperature, nutrients availability, and physicochemical conditions for xenobiotic degradation (Nagase et al. 2006; Tien et al. 2017). For

example, the carbofuran pesticide degrading ability of *Sphingobacterium multivorum* was found to be maximum at pH 7 and a temperature of 25 °C (Tien et al. 2017). The bacterial genus *Enterobacter* efficiently removes 92.5 mg/L carbofuran at pH 6 and temperature 27.5 °C and nitrogen source of 0.45 g/L (Mustapha et al. 2020b). Likewise, the laccase enzyme secreted by bacteria *Pseudomonas mendocina* revealed better degradation of azo dyes at pH 5.8 and temperature 20 °C (Sridharan et al. 2019). The laccase enzyme from macro fungus *Podoscypha elegans* can carry out an effective degradation of azo dyes (Congo Red, Orange G, Direct Blue 15, Direct Yellow 27, and Rose Bengal) at a broad pH range of 5.5–7 (Pramanik and Chaudhuri 2018). Other enzymes, that is, manganese peroxidase, isolated from white-rot fungi, *Trametes polyzona* KU-RNW027, have exhibited optimal activity at pH 4.5 (Lueangjaroenkit et al. 2019).

The availability of nutrients, namely, glucose, iron, manganese, magnesium, phosphorus, and other trace elements, ameliorates the xenobiotic degradation activity of microbes. Bacterial species such as *Sphingobacterium* sp. strain D6 and *Sphingomonas* exhibited higher degradation of DDT and methomyl in a media enriched by glucose than that of glucose devoid media (Fang et al. 2010; Chen et al. 2015a). Conversely, no significant difference was observed during the degradation of carbofuran by *Sphingobacterium multivorum* cultured in a medium with or without sugar (Tien et al. 2017). Furthermore, the presence of phosphorus is considered to be an essential requirement for better biodegradation of antibiotics and oil/hydrocarbons (Abu and Atu 2008; Nnenna et al. 2011). In fungi, *Phanerochaete chrysosporium*, the availability of nitrogen is considered to be favorable for the effective degradation of polycyclic aromatic hydrocarbons (Mollea et al. 2005). The activity of fungal enzymes such as manganese peroxidases and lignin peroxidase increases in the presence of manganese and hydrogen peroxide at a concentration of less than 1 mM during dye degradation (Kang et al. 2018). Also, surplus substrates enhance the activity of manganese peroxidases to a greater extent. The exhaustive list of these substrates includes 2,6-dimethoxyphenol, *o*-dianisidine, veratryl alcohol, 2,4-dichlorophenol, commercial humic acid, levodopa, signayl alcohol, 2,6-dichlorophenol, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), coniferyl alcohol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, guaiacol, and pyrogallol (Bouacem et al. 2018). Other important nutrients like magnesium, copper, manganese, and zinc stimulate the activity of laccase enzyme isolated from the genus *Trametes*. On the other hand, laccase works best in the absence of certain elements like lead, potassium, sodium, and calcium as well as protein reductants including β-mercaptoethanol, L-cysteine, dithiothreitol, and sodium azide (Aslam et al. 2012; Wang et al. 2018).

Also, the presence of heavy metals might either have a positive or negative effect on the microbial degradation of xenobiotics. In a study by Mustapha et al. (2020a), most severe effects are noticed when the media is supplemented with mercury and copper at a concentration of 1 mg/L during degradation of carbofuran by *Enterobacter* sp. However, another heavy metal, that is, cadmium, exhibited a negligible toxic effect at this concentration. It has been reported that the low

concentration of around 1mM of silver have increased the activity of laccase enzyme (Lueangjaroenkit et al. 2019).

In some cases, the preservation of microbes has proved to be worthwhile during pesticide degradation. Tien et al. (2017) reported increased carbofuran degradation activity of microbial consortia (*Comamonas jiangduensis*, *Pseudomonas fulva*, *Stenotrophomonas* sp., and *Thermolithobacter* sp.) after preservation at 25 °C for 1 month. Despite that, some bacteria like *Sphingobacterium* sp., *Sphingomonas* sp., and *Pseudomonas* sp. showed a decrease in methomyl pesticide degradation after preservation (Chen et al. 2015a).

16.6 Conclusion

Development particularly in terms of industrialization compromises environmental integrity on a large scale. The entry of xenobiotics into the environment is one of the deleterious consequences of industrialization. Hence, it is obligatory to devise certain techniques through which the toxicity of xenobiotics can be controlled effectively. Bioremediation by microorganisms and microbial enzymes is considered to be one such cost-effective technique that removes a vast number of environmental pollutants. Being environment friendly, this field is flourishing day by day. With the advent of genetic engineering technology, it has also become possible to improve the xenobiotic degradation efficiency of microorganisms. The success of bioremediation, however, depends upon the various environmental conditions and nutrients requirement which must be taken care of.

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Chapter 17
Conventional Wastewater Treatment Processes

Pabbati Ranjit, Vulise Jhansi, and Kondakindi Venkateswar Reddy

Contents

Abstract The concept of wastewater treatment is generally to allow anthropoid and industrial effluents to be inclined of without hazard to human health or impermissible harm to the natural environment. Conventional wastewater treatment is a combination of physical, chemical, and biological processes to remove suspended solids, dissolved solids, biological decomposition of organic matter, and nutrients from wastewater. Broadly, there are various degrees of treatment in sequence to increase the treatment level, which are preliminary, primary, secondary, and tertiary and/or advanced wastewater treatment.

Keywords Wastewater · Preliminary · Primary · Secondary and tertiary treatment

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17.1 Introduction

Water is an inorganic, colorless chemical substance. It is available in three states, that is, liquid, solid, and gaseous, and is also a major constituent of our Earth's hydrosphere. It is also an essential fluid of most of the living forms. It covers almost 70% of Earth's surface (Gleick 1993). It is estimated that about 97% of the total water is in the oceans and inland seas with giant salt content. This water is insufficient to anthropoid consumption. Around 2% of water is found in glaciers and ice caps. Only 1% of water on this Earth is available for human consumption as ground water, lakes, rivers, and soil moisture (Satyanarayana 2008). Day by day, human need for water is increasing due to which availability of water is becoming scarce.

Water is an essential nutrient on earth. It is absolutely essential for the existence of life (animal and plant). Life does not exist without water. It is needed for multiple purposes such as cooking, bathing, washing, and other human activities. It should be noted, water which we consume and use for additional purposes should be clean, that is, it must be free from germs and other harmful chemicals. Water that is safe for drink or to use for food preparation is called as potable water. Water that is not safe for drinking is called as non-potable water whose consumption leads to many infectious diseases (Griffiths et al. 1991).

Water that has been polluted by human use can be considered wastewater. Water is used for many purposes like domestic, industrial, commercial, and agricultural activities of mankind. Wastewater carries any kind of physical, chemical, and biological pollutants (Tilley et al. 2014).

17.2 Types of Wastewater

The characteristics of wastewater differ depending upon the source and release. The major wastewater contaminants and their significance are shown in Table 17.1.

Types of wastewater include the following:

- Domestic wastewater (released from households).
- Municipal wastewater (released from communities).
- Industrial wastewater (released from industries) (Tilley et al. 2014).

The main aim of wastewater treatment is to eliminate suspended solids as much as possible and the remaining water, called effluent, is discharged back to the environment. This process can be termed as water reclamation as treated wastewater can be used for other purposes (Metcalf and Eddy 2003). The purpose of wastewater treatment is to remove suspended solids (less than 50 mg/L), biodegradable organic matter (BOD_5 at 20 °C less than 30 mg/L), and to kill pathogens.

Table 17.1 Major wastewater contaminants and their significance. Reproduced from (Shon et al. 2011)

Contaminant	Source	Environmental significance
Suspended solids	Domestic use, industrial waste, erosion by infiltration/inflow	Cause sludge deposit and anaerobic condition in aquatic environment
Biodegradable organics (starch, fats, proteins, alcohols, aldehydes, and esters)	Domestic and industrial waste	Cause biological degradation, which may use up oxygen in receiving water and results in undesirable conditions
Pathogens (bacteria, viruses, fungi, etc.)	Domestic waste	Transmit communicable diseases
Nutrients (nitrogen and phosphorous)	Domestic and industrial waste	May cause eutrophication
Refractory organics (BOD: COD)	Industrial waste	May cause taste and odor problems, may be toxic or carcinogenic
Heavy metals (Hg, Cd, Pb)	Industrial waste, mining, etc.	Are toxic, may interfere with effluent reuse
Dissolved inorganic solids(Ca, Mg, K, sodium bicarbonates, chlorides, and sulfates)	Increases above level in water supply by domestic and/or industrial use	May interfere with effluent reuse

17.3 Process of Wastewater Treatment

There are four major steps involved in the wastewater treatment processes, which include preliminary treatment, primary treatment, secondary treatment, and tertiary or advanced treatment.

17.3.1 Preliminary Treatment

Preliminary treatment involves the removal of floating materials such as leaves, papers, wood pieces, sticks, tree branches, diapers, napkins, glass bottles, plastics, dead animals, and other heavy settleable inorganic solids such as sand, grit, fats, oils, grease substances, etc. There are some specified devices designed particularly for performing preliminary treatment including, screeners, grit chambers, and skimming tanks.

Screeners Screening is an essential step in wastewater treatment in order to remove floating objects such as sticks, dead animals, wood pieces, tree branches, papers, clothes, diapers, napkins, glass bottles, plastic bottles, coconut shells, and other large-sized floating materials. It is the first step in treatment processes; screener is a device with a uniform size opening for removal of floating matter in the wastewater. Floating objects retained by screens are called screenings. Objects which are not

Table 17.2 Design aspects of screens. Reproduced from (Amina 2017)

Screen type	Size of opening (mm)	Solids captured per million liter of sewage	Screening type	Solids disposal
Coarse screens/racks (steel bars)—bar screens	> 50 mm	6 liters	Rags, wood, paper, etc.	Incineration burial, dumping
Medium screens (steel bars)—bar screens	6–40 mm	30–90 liters	Contains some organic material	Incineration or burial but no dumping
Fine screens (brass or bronze plates or wire mesh)—disc or drum type	< 6 mm	20% SS from sewage removed	Frequent cleaning. Used for industrial wastewater	Properly disposed

removed clog and damage the pumps, valves, pipe lines, and other appurtenances. The screening equipment has parallel bars, rods gratings, wire meshes, or perforated plates, and the openings may be of any shape mostly of circular or rectangular depending upon the size (Srinivas 2008). Depending upon the size of opening, screeners are classified into course screens, medium screens, and fine screens. Design aspects of these screens are shown in Table 17.2.

Grit Chambers Grit chambers are long, narrow tanks designed to slow down the flow so that solids such as sand, eggshells, coffee grounds, etc. will settle out of the water (Ambulkar and Nathanson 2019). The heavy inorganic materials like sand, ash, etc., are removed by grit chambers. This technique is based on the principle of sedimentation due to gravitational forces (Satyanarayana 2008). Grit causes excessive wear and tear on pumps and equipment. Its removal is particularly important in cities with combined sewer systems, which carry a good deal of silt, sand, and gravel that wash off streets or land during a storm (Ambulkar and Nathanson 2019). Grit chambers are usually located ahead of pumps or comminuting devices and, if mechanically cleaned, should be preceded by coarse bar rack screens.

Skimming Tanks Skimming tank is arranged to remove several greasy and oily materials (fats, oils, waxes, soaps, etc.) from the sewage. These oily and greasy materials from domestic and industrial outlets are released into the sewage. A skimming tank is fitted with baffle walls that divide the tank. It is divided into three compartments that are interconnected. The compressed air is pushed from the floor of the tank, leading to the coagulation of raising air bubbles and solidification of the oily and greasy materials present in the sewage. This removed material is pushed to the side compartment, referred to as stilling compartment, and from there it is removed either manually or mechanically.

17.3.2 Primary Treatment

It is carried out majorly in order to remove the small-sized inorganic matter and large-sized settleable organic matter, 60–65% of suspended solids, and 30–35% of BODs. It is a physical process that basically includes sedimentation and coagulation.

Sedimentation It is a physical treatment process using gravity to eliminate suspended solids from water. The primary purpose is to produce a clarified effluent, and it is the mostly used unit operation in wastewater treatment process. Sedimentation process is carried out twice: once before secondary treatment, called as primary sedimentation, and then after secondary treatment, called as secondary sedimentation. In this process, solid particles present in the sewage get settled down due to the gravitational force. Majority of the solid particles are organic compounds and maintain the suspended state in a flowing sewage. This flow of sewage is stopped and is stored in a tank known as sedimentation tank, facilitating the solid particles to settle down at the bottom. According to the tendency of particles, the process of settling is of four types: discrete settling, flocculant settling, zone or hindered settling, and compression.

1. Discrete settling: The constant particles that do not change their size, shape, and weight are called as discrete or granular particles.
   Example: Use of grit in sewage.
2. Flocculant settling: The particles that change their size, shape, and weight losing their original identity are called as flocculant particles.
   Example: Settling of bioflocs in secondary sedimentation tanks.
3. Zone or hindered settling: The mass of these particles can settle as a unit or zone even after flocculation. The particles remain in a fixed position with respect to each other. In this process of settling, the congregation of particles increases from top to bottom resulting in thickening of sludge.
   Example: Secondary sedimentation tank (activated sludge process).
4. Compression: Settlement of particles in the lower layers occur by compressing the weight of the particles on the upper layers, which facilitates the thickening of the sludge at the bottom.
   Example: Sludge thickening (wastewater treatment plant).

Several ways of classifying sedimentation tanks include the following:

- Based on shape: Rectangular, circular, and square.
- Based on flow of sewage: Longitudinal, vertical, radial, and spiral.
- Based on purpose and position: Primary, secondary, coagulation-cum-sedimentation tanks, grit chambers, septic and Imhoff tanks.
- Based on operation: Batch and continuous flow type.

Coagulation It is a chemical process in which charged particles are destabilized by the addition of chemical agents. Every time it is not possible to remove the colloidal wastes in sewage by plain sedimentation. Sometimes it is mandatory to use chemical

coagulants to help sedimentation. This procedure is termed as chemical precipitation or coagulation-aided sedimentation or coagulation. By using this technique, about 60–80% of the suspended particles can be easily removed. Types of chemical agents used in coagulation: coagulants and coagulant-aids.

1. Coagulants: Coagulants (positively charged) are chemicals that form insoluble and gelatinous precipitates with the colloidal particles (negatively charged). Example: alum (aluminum sulfate), iron salts, lime and soda ash, sodium silicate, and sodium aluminate.
2. Coagulant-aids: These chemicals facilitate the coagulation process. They enhance the action of coagulants and thus reducing the amount of sludge formed. Example: activated silica, weighting agents such as silica, powdered limestone, and polyelectrolytes (Ambulkar and Nathanson 2019).

17.3.3 Secondary Treatment

Secondary treatment is also known as biological treatment of wastewater. If the BOD/COD $\geq$0.6, then biological treatment can be applied for industrial wastewater. Secondary treatment is useful for the removal of 95% of suspended solids, 5 day BOD and decomposition of organic matter. Various types of microorganisms such as bacteria, algae, fungi, and protozoa are used for the decomposition of organic matter. For balance growth of microbes in a biological treatment, the ratios of BOD:N:P for aerobic systems and anaerobic systems are 100:5:1 and 100:2.5:0.5. Microbes can feed on the unstable organic matter and decomposed to solid inorganic forms. Secondary treatment or biological treatment processes of wastewater are broadly classified as *aerobic, anaerobic* and *pond* processes shown in Fig. 17.1. Depending upon the nature and use of microorganisms, the biological processes are further classified as "suspended growth systems" and "attached growth systems" (Satyanarayana 2008).

17.3.3.1 Aerobic Process

Aerobic treatment of wastewater requires oxygen to break down organic contaminants and other pollutants such as nitrogen and phosphorous. It is further classified into two sub categories.

(a) Aerobic suspended growth treatment: There are many methods involved under this treatment process. The most important suspended growth biological systems used for this treatment are below:

1. Activated sludge process
2. Aerated lagoons
3. Sequencing batch reactor

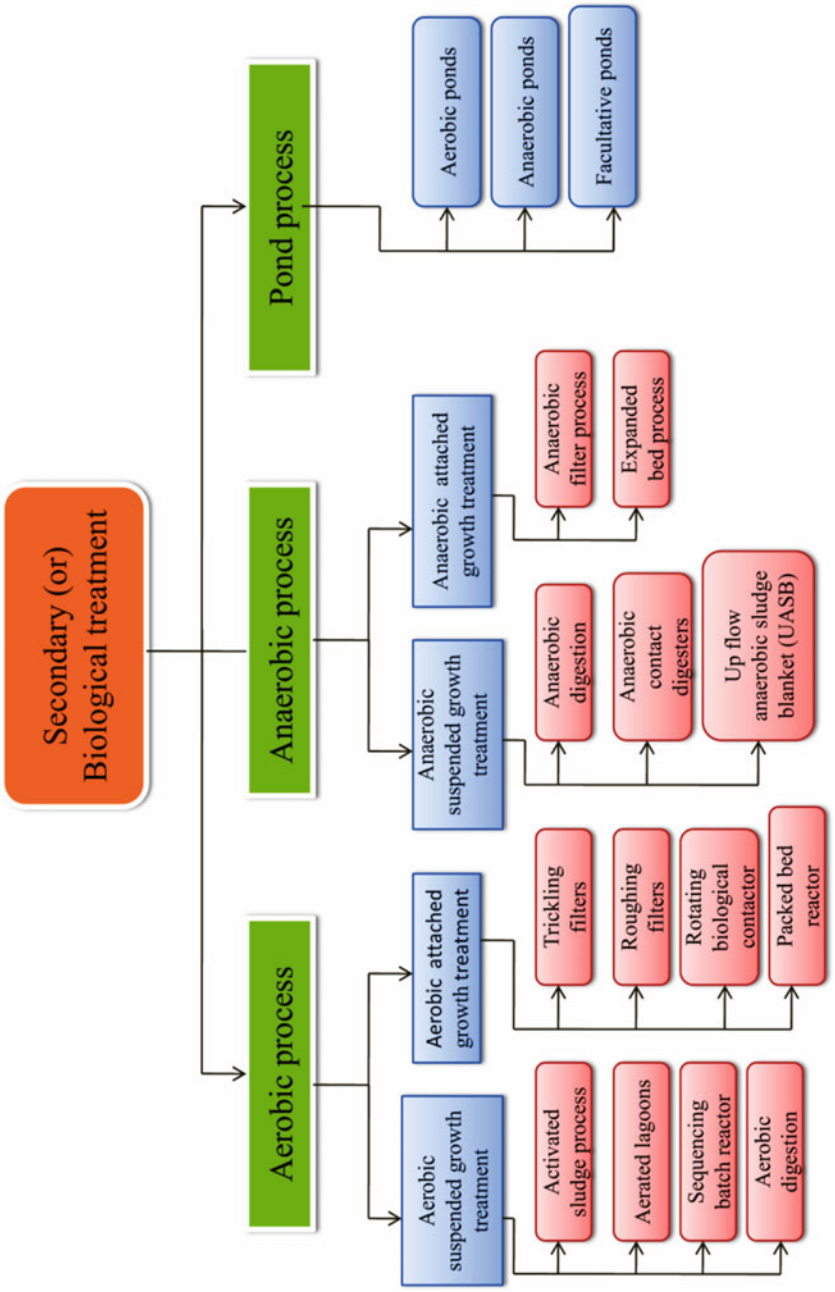

Fig. 17.1 A flow chart of various secondary (or) biological treatment processes

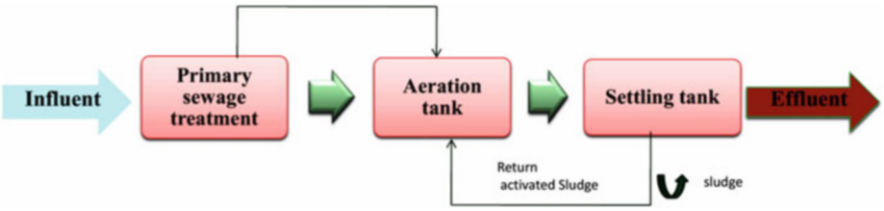

Fig. 17.2 Flow chart showing activated sludge process

4. Aerobic digestion

(1) Activated sludge process: This process is considered to be the most used modern biological treatment process. Aeration of the sewage containing organic matter with microbes is carried out in an aeration tank with the help of a mechanical aerator. The contents of the reactor are called as "mixed liquor" (Satyanarayana 2008). The microorganisms during the onset of favorable aerobic conditions metabolize the suspended particulate matter. Microbes utilize a portion of the organic matter and synthesize new bacterial cells, and the remaining of the organic matter gets converted into CO_2 (carbon dioxide) and H_2O (water). These newly formed bacterial cells aggregate together to form flocs, which is technically called as sludge. Now this separated sludge loses connection with the organic matter and becomes activated. This activated sludge is segregated from the settling tank to aeration tank and recycled. This recycled activated sludge serves as an inoculum, whereas the excess sludge gets eliminated (Fig. 17.2). In order to carry out this activated sludge process in an efficient way, a continuous oxygen supply needs to be maintained. This is achieved either by using rotating paddles or through mechanical aeration. The growth of protozoa is an indicator of activated sludge performance. The waste sludge that is eliminated can be used as a fertilizer in various agricultural lands. The activated sludge process can be categorized as conventional activated sludge process and modified activated sludge process.

Conventional activated sludge process

In this process, a separation tank (settling or sedimentation tank) and sludge removal line are required. Following the primary treatment, the sewage is released into head of the tank, and oxygen is supplied throughout the tank uniformly.

Modified activated sludge process

In this process, several modifications have been done to improve aeration efficiency. Aeration can be provided by step aeration, tapered aeration, high rate aeration by complete mixing, and extended aeration.

Factors affecting the activated sludge process:

- Type of reactor
- Inadequate air supply in aeration tank
- Food/microorganism ratio
- Nutrients
- Sludge recirculation rate

- Low pH value
- Temperature
- Prolonged detention of sludge in secondary settling tank
- Industrial sewage which favors the growth of filamentous fungi

Advantages:

- It is a low cost, very compact, and an efficient biological sewage treatment process.
- Under ideal conditions, 95% of BOD, 98% of bacteria, and 95% of suspended organic matter can be removed through this process.
- Even the waste sludge can be used as an effective fertilizer in many agricultural lands.

Disadvantages:

- Large amount of sludge production becomes difficult to handle sometimes.
- Require high amount of power supply.

(2) Aerated lagoons: These are also called as aerated ponds. It is simply a pond with artificial aeration. In these aerated lagoons, surface aerators are inaugurate. These aerators can control the bad odors released due to the organic materials. Continuous nitrification can be possibly carried out in these aerated lagoons depending upon the temperature. The microbial treatment of this process is compared to activated sludge process. It has the large surface area when compared to the activated sludge process and is more susceptible to temperature effects (Satyanarayana 2008). The aerated lagoons can be divided as facultative aerated lagoons and aerobic aerated lagoons.

Facultative aerated lagoons

In such type of lagoons, the lower part is feasibly anaerobic, whereas the top layers are aerobic. Hence, these are termed as facultative aerated lagoons. In these, few solids depart from the effluent stream, whereas some solids settle down in the lagoon due to insufficient aeration power input. These are also known as partially mixed type aerated lagoons as they can be operated at low rate of aeration.

Aerobic aerated lagoons

These are entirely oxygenic from top to bottom. In these lagoons, the aeration power input is sufficient to keep all the solids of the suspension. There is no settling of solids in these lagoons, and parallelly new solids are formed in this system are equivalent to the number of solids leaving. Further treatment is provided after these lagoons in order to lower the concentration of solids in the effluent (Oliveira and von Sperling 2011).

Advantages of aerated lagoons

- These are simple in operating as the aerator is the only moving piece of the equipment.
- These are majorly used for treating industrial wastes.
- They require only 5–10% as much land as stabilization ponds.

Disadvantages:

- Slighter competent in cold climates.
- If not properly maintained, they supply a birth site area for mosquitoes and other insects.

(3) Sequencing batch reactors: In this process, aeration and sedimentation processes are implemented one by one respectively in the same tank. Simply, it is a modified activated sludge system as activated sludge processes both aeration and sedimentation are carried out in separate tanks. Hence, it is a fill and draw activated sludge process (Satyanarayana 2008). These reactors treat the sewage in batches. Oxygen is sent in the form of bubbles through the mixture of wastewater and activated sludge in sequence to eliminate the organic matter. Effluent can be discharged into surface waters after the treatment. This treatment can be carried out in various stages as follows:

- Filling—This is the first stage; during this stage the inlet valve is opened and the tank gets filled in followed by mechanical mixing. As this stage does not involve air, it is said to be anoxic.
- Aerating or reacting—This is the second stage; at this stage the above mixed liquor is aerated through mechanical pumps or fine bubbles.
- Settling or sedimentation—This is the third stage; the above aerated liquor is allowed to be maintained in an undisturbed state so that the suspended solids starts to settle.
- Decanting—This is the fourth stage; now the outlet valve is opened, through which effluent is discharged out.
- Idle—This is the final stage; the excess sludge can be removed (Satinder Ahuja et al. 2014).

Advantages:

- The effluent produced during the process contains low organic compounds.
- It can effectively remove nutrients such as nitrogen and phosphorous (Satinder Ahuja et al. 2014).

Disadvantages:

- It requires a skilled person for carry out this process.
- This process can eliminate some pathogens (Ghodeif 2013).

(4) Aerobic digestion: In this process of sewage treatment, the volume of sludge is reduced so that it can be further used (Water Environment Federation 2008). The organic sludge produced in the previously discussed systems are undergone aerobic digestion in a specific reactor called aerobic digester (Satyanarayana 2008). It is usually a batch process. Air is sent through the tank followed by constant stirring as to keep the contents assorted. Gases such as carbon dioxide and hydrogen sulfide are given off as they are required to be reduced in odors. Continuous digestion is carried out until the degradable solids percentage fall between 20% and 10% according to

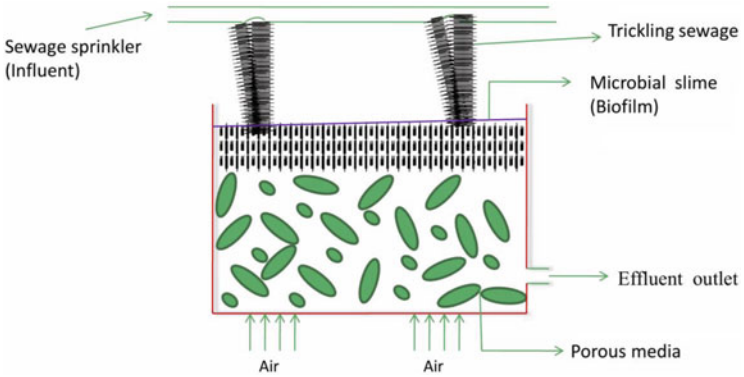

Fig. 17.3 Typical sketch of a trickling filter

local conditions. The non-sewage waste can be further processed by removing wastes like food, cardboard, etc. (Water Environment Federation 2008).

Advantages:

- It is a less time-consuming process.
- This process is less complex when compared to anaerobic digestion.

Disadvantages:

- As blowers and pumps are used for the supply of oxygen, their operating costs are beyond anaerobic digestion.
- Lower energy yield when compared to anaerobic digestion (Water Environment Federation 2008).

(b) Aerobic attached growth treatment: These are the processes mostly used for the elimination of organic matter present in the wastewater or sewage. These processes can also favor nitrification. There are many processes involved under this treatment, which include the following:

1. Trickling filters
2. Roughing filters
3. Rotating biological contactors
4. Packed bed reactors

(1) *Trickling filters:* Trickling filters are mostly used for the secondary treatment of wastewater released from the domestic sources such as households and industrial sewage. They are also called as sprinkling filters (Satyanarayana 2008). A typical trickling filter unit has been fixed with a bed of rocks (porous media), coke, gravel, peat moss, etc. Wastewater is allowed to pass through by spraying (Fig. 17.3). The sewage flows downward as spray and due to which a microbial slime grows and covers the bed of the media (Parker et al. 1989). This slime rich in microbes such as *Pseudomonas*, *Alcaligenes*, yeasts, and fungi (Satyanarayana 2008). The whole unit is maintained in a perfect aerobic condition through splashing and diffusion, etc.

(Parker et al. 1989). As the sewage is allowed to pass through the newly formed microbial slime layer, organic matter used by the microbes. Microbes present on the upper layer of the film carry out the process of oxidation. Accordingly, with the time, the biofilm raise its width and settles at the bottom of the tank (Satyanarayana 2008).

Based on organic loading, trickling filters are classified into

- Low-rate trickling filters: These are mostly used for the treatment of domestic sewage. These are simple units that can produce a consistent effluent quality independent of the influent strength. There is no recirculation of the effluent. It can remove up to 80–85% of the applied BOD (Parker et al. 1989).
- High-rate trickling filters: High-rate trickling filters used for treating industrial waste. Hence, the organic loading per unit area in the filter can be increased due to which flow velocity increases causing continuous sloughing of excess growth. It can remove 65–85% of BOD from the sewage (Parker et al. 1989).
- Super-rate trickling filters: These are also useful for the industrial waste treatment (Satyanarayana 2008).

Factors Affecting the Trickling Filters:

- Type of media
- Organic loading
- Recirculation rate
- Flow distribution
- Filter staging

Advantages:

- These are simple and operated at low costs.
- Effectively works in hot climate.

Disadvantages:

- Excess sludge should be disposed of necessarily.
- Raw sewage cannot be handled by these filters until unless primary sedimentation is done (Satyanarayana 2008).

(2) Roughing filters: These are the filters that are mostly used to reduce the suspended solid organic matter from the wastewater. They not only reduce the pathogens but also reduce the amount of iron and manganese in the wastewater (Nkwonta and Ochieng 2009). They usually operate in downstream processing; if the organic loading is very high, then there will be a continuous shedding of the microbial slime. The planning of the treatment process is increased by recycling the unsettled effluent (Satyanarayana 2008). Based on flow of directions, roughing filters can be classified as follows:

- Downflow roughing filters: Filters have downward flow direction; type of filter medium is sand.
- Upflow roughing filters: Filters have upward flow direction; type of filter medium is gravel.

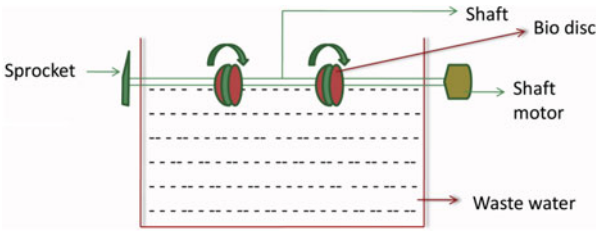

Fig. 17.4 Rotating biological contactor

- Horizontal flow roughing filters: Filters have horizontal flow direction; type of filter medium used in this kind of filter is coconut husk fiber. Among three types, upflow roughing filters are most widely used as they are cheap and easily maintained (Nkwonta and Ochieng 2009).

(3) Rotating biological contactors (RBC): It is the most important system used for the treatment of municipal wastewater or used for the bioprocessing of industrial wastes like vegetable industry. RBC's recent devices work on the principle of aerobic attached growth system performed on the moving or rotating media (Satyanarayana 2008). This process is executed after the completion of primary treatment; flowing wastewater comes in contact with biological film or contactor. During this contact, the pollutants present in the wastewater are eliminated; now water free from pollutants is released into the environment, which can be reused (Fujie et al. 1983).

A typical RBC consists of some parallel discs that are closely spaced and are placed on a rotating shaft (Fig. 17.4). As the wastewater is allowed to flow through this RBC, the organic matter present in that water is degraded by the microbes that grow on these parallel discs (Fujie et al. 1983). The rotating bio discs are generally made up of polystyrene or polyvinylchloride (PVC). The shaft aligned with these discs is allowed to rotate at slow speed. About 40% of the discs are submerged in water. The microbial growth gets attached to the disc, and a biofilm is formed as a result. This bio disc contacts the wastewater with the atmospheric air and carries out oxidation in its rotating motion. Aeration for this system is provided by the rotating motion of the discs due to which the media get exposed to the air after the contact with wastewater, and hence pollutants get eliminated (Fujie et al. 1983).

Factors Affecting RBC (Satyanarayana 2008):

- Rotating speed of the shaft
- Temperature
- Wastewater retention time
- Submergence of disc
- Organic loading

Advantages (Satyanarayana 2008):

- This process is very effective because it can remove about 90% of BOD.
- Low operating costs.

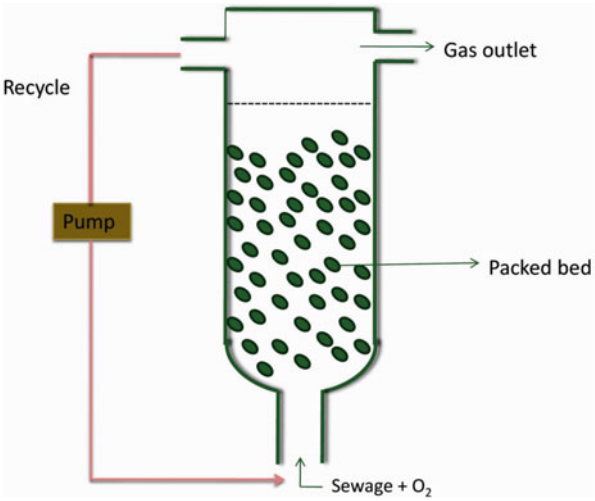

Fig. 17.5 Packed bed reactor

Disadvantages:

- Frequent occurrence of odor.
- Sludge disposal becomes difficult.

(4) Packed bed reactors: These are also called as fluidized bed reactors. They not only serve the purpose of removing BOD but also help in the nitrification. A specific reactor is used in this system, which is filled or packed with a medium (Fig. 17.5). Aeration is induced from the bottom of reactor. When the sewage is allowed to pass through this reactor, the microbes get freely attached on to the medium, thereby forming a biofilm. This formed biofilm removes the suspended organic matter from the sewage by feeding on that organic matter.

17.3.3.2 Anaerobic Process

Anaerobic process does not require oxygen, that is, decomposition of organic matter takes place anaerobically, which leads to formation of end products such as methane and carbon dioxide. It is further classified into two subcategories.

(a) Anaerobic suspended growth treatment: Suspended processes occur in a specialized airtight reactor, called an aerobic digester. Sludge is introduced continuously/intermittently. Mostly used systems under this treatment are

1. Anaerobic digestion
2. Anaerobic contact digesters
3. Upflow anaerobic sludge blankets (UASB)

(1) Anaerobic digestion: Anaerobic digestion is mostly used to treat industrial sewage. Anaerobic digesters are classified into two types, standard-rate digester and the high-rate digester. Digesters are basically designed to treat high-strength organic

wastes. The standard-rate digester reacts without any external heat supply; due to this reaction, time gets prolonged and detention time will be increased. Whereas in high-rate digester, heat is supplied including stirring, which will reduce the detention time. Depending upon the condition, both reactors are arranged in a combined set up to be used. Such process is termed as a two-stage system (Ebrahimi and Najafpour 2016). The process of anaerobic digestion are of three steps.

Hydrolysis: It is an enzyme-catalyzed reaction, in which high-molecular weight compounds (polysaccharides, proteins, lipids, and nucleic acids) are converted to low-molecular weight compounds (monosaccharides, amino acids, fatty acids, purines, and pyrimidines). These compounds serve as substrates for microbial growth and energy supply.

Acidogenesis: The low-molecular weight compounds are further changed into acidic products (lactate, butyrate, and propionate).

Methanogenesis: It is the final stage that involves the production of methane and carbon dioxide from the intermediates formed in the acidogenesis.

Microorganisms to degrade organic matter of sludge or sewage: Consortium of anaerobic microorganisms associated together for degradation of sludge or sewage. It is categorized into two types:

- Acidogenic bacteria: These are also called as acidogens. Various types of acidogenic bacteria such as *Clostridium sp, Corynebacterium sp, Lactobacillus sp, Actinomyces sp, staphylococcus sp, Peptococcus sp, and Escherichia coli* convert low-molecular weight compounds into acidic forms.
- Methanogenic bacteria: These are also called as methanogens and are accountable for converting acid and hydrogen into methane and carbon dioxide, for example, *methanobacterium, methanobacillus*, *methanococcus*, and *methanosarcina* (Ebrahimi and Najafpour 2016).

(2) Anaerobic contact digesters: An anaerobic contact process that is mostly used to treat industrial waste containing high BOD. This process is carried out in a set of reactors that are sequenced in a series. In these reactors, the recycled sludge with the sewage is pumped and then digested under anaerobic conditions. After this, two layers get separated by the use of a clarifier, and the formed supernatant is discharged while sludge is settled and again set for recycling (Satyanarayana 2008).

(3) *Upflow anaerobic sludge blanket*: These digesters are mainly used to overcome the problems caused: filter clogging, low loading rate difficulty, etc. About 200 operating plants were present worldwide. These are mostly used to treat food processing wastes, sugar beet waste, brewery waste, winery waste, etc. (S N Jogdand 2010). It is a strict anaerobic process and forms a blanket of granular sludge. This granular sludge can be processed further by anaerobic microorganisms (Tilley et al. 2014). It is basically a three-phase separator that enables to separate solid (sludge), liquid (water), and gas (methane) under high turbulence conditions (Fig. 17.6). Methane gas formed can be separated through multiple gas hoods of UASB. During treatment, substrate is enabled to pass through an expanded sludge bed that consists of biomass concentration at a very high concentration. Later the remaining substrate is allowed to pass through a less dense biomass called sludge blanket (Tilley et al.

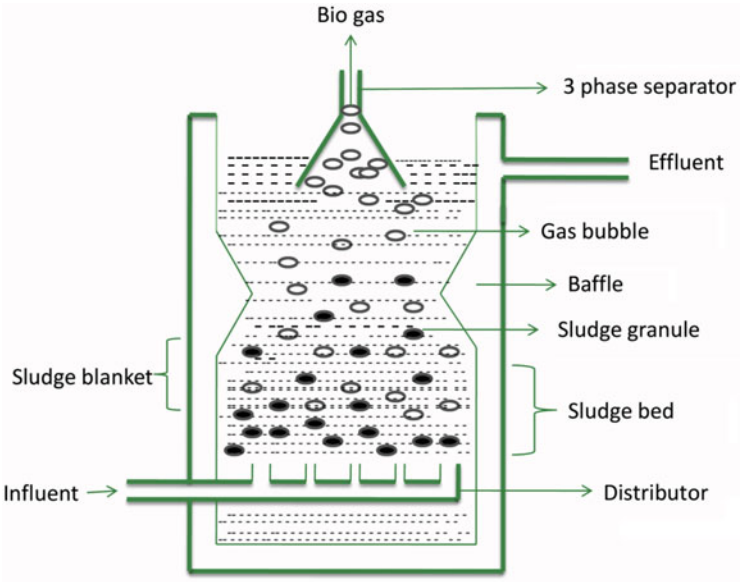

Fig. 17.6 Upflow anaerobic sludge blanket

2014). The influent is introduced from the bottom of the digester. Its level increases upward and meets with the biomass in the sludge bed. The movement of the influent continues upwards, and the remaining substrate acts with biomass present in sludge blanket. To ensure the quality of effluent, the volume of sludge blanket must be maintained at sufficient quantity. After the three-phase separator, the sludge blanket will separate solid particles from the mixture and set a way out for liquid and gas through which they leave the UASB reactor. Now the treated waste was discharged into water bodies, and gas was collected and reused as a biofuel (Tilley et al. 2014).

(b) Anaerobic attached growth process: Attached growth system is a process in which microbes treating the wastes are attached to the media in a reactor. Anaerobic attached growth treatment mainly involves

1. Anaerobic filter process
2. Expanded bed process

(1) Anaerobic filter process: Anaerobic filter process takes place in a special anaerobic digestion tank that consists of filter medium on to which anaerobic microbes attached and establishes themselves. In this process, wastewater flow upwards through the filter media; organic matter present in the wastewater is degraded by active anaerobic microbial population attached to the filter media (Satyanarayana 2008).

(2) Expanded bed process: Reaction occurs in a reactor that consists of a tank filled with a bed of sand, coal, or other aggregates. Bed acts as a medium on to which anaerobic microbes attach and form a biofilm. Wastewater or influent is pumped into

the reactor and passes through the bed. As the influent comes into contact with biofilm, the organic matter from the influent is degraded by the anaerobes on the bed. The effluent is again recycled for maintaining the flow rate (Satyanarayana 2008).

17.3.3.3 Pond Treatment Processes

Pond treatment is a biological process used for treatment of sewage. This process is carried out in special ponds constructed by human in order to reduce the organic waste and remove pathogens from wastewater by using both bacteria and algae (Satyanarayana 2008). These ponds are usually large, shallow basins and are called as stabilization ponds, frequently used for industrial effluents or municipal water treatment. Influent enters one side of the stabilization pond for treatment and exits from other side as effluent after treatment. After treatment, it can be introduced into water bodies or can be used for irrigation purposes. It is a time-consuming process as it is a naturally occurring one. Pond treatment may involve either single pond or a series of ponds in a system each having individual roles in pathogen removal (Ho et al. 2017).

Various types of stabilization ponds:

(a) Aerobic ponds
(b) Anaerobic ponds
(c) Facultative ponds

(a) Aerobic ponds: Aerobic ponds are maintained completely in aerobic conditions. These ponds consist of bacteria and algae in suspension through which organic wastes can be treated. Ponds have a depth of about 0.5–1.5 feet (150–450 mm) and allow the penetration of light throughout the liquid depth. Usually oxygen can be produced by an alga that is grown in the pond. Besides this, it is also maintained in a continuous atmospheric diffusion by pumps. In these ponds, bacteria and algae exhibit symbiotic association. Photosynthesis process is carried out by algae and releases oxygen to keep oxygenic conditions in the pond, while the bacteria degrade organic matter to produce carbon dioxide and nutrients utilized by algae. Growth of protozoa and rotifers are responsible for polishing of effluent (Satyanarayana 2008).

Factors affecting aerobic ponds: species of bacteria and algae, degree of pond mixing, availability of nutrients, sunlight, pH, and temperature.

(b) Anaerobic ponds: Ponds maintained under anaerobic conditions and mainly employed for treatment of the sewage containing high-strength organic solids. Heat conservation is possible because they are very deep (up to 30 feet, i.e., about 9 m). When sewage is introduced into the pond, precipitation and anaerobic conversion of organic waste into carbon dioxide, methane, organic acids, etc. occur. Under favorable conditions, about 75% of BOD removed in anaerobic treatment (Satyanarayana 2008). Factors affecting anaerobic ponds: temperature, HRT, pH, sulfide, toxic compounds, degree of mixing, etc. (Ho et al. 2017).

(c) Facultative ponds: These are the stabilization ponds that are mainly employed in the biological treatment of industrial and domestic wastewater. In these ponds,

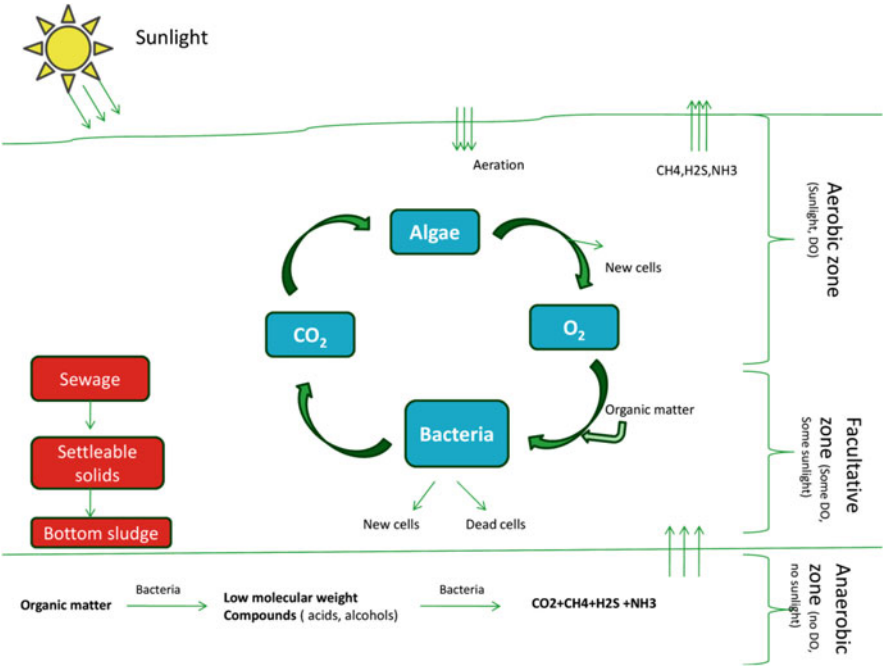

Fig. 17.7 Diagrammatic representation of facultative pond

both aerobic and anaerobic conditions are maintained. Three types of microorganisms are employed in facultative ponds, that is, aerobic, anaerobic, and facultative (both aerobic and anaerobic). A typical facultative pond has three zones, which include the following (Fig. 17.7):

- Surface zone (aerobic zone): Zone having aerobic bacteria and algae existing in a symbiotic relationship.
- Bottom zone (anaerobic zone): Zone having only anaerobic bacteria that decompose the organic matter present in the sewage.
- Intermediate (facultative zone): Zone having partly aerobic, anaerobic, and both types (aerobic and anaerobic) of bacteria.

Process that occurs in facultative ponds: the algae in the aerobic zone execute photosynthesis and release oxygen taken up by aerobes and facultative anaerobes for the oxidation of organic matter. Anaerobes degrade the organic compounds into dissolved organic solids and gases such as carbon dioxide, methane, hydrogen sulfide, etc. The released carbon dioxide is utilized by the algae to carry out photosynthesis, and then hydrogen sulfide combines with the oxygen to form sulfuric acid (Satyanarayana 2008).

17.3.4 Tertiary Treatment

Tertiary treatment removes total suspended solids, total dissolved solids, organic and inorganic matter present in the secondary effluent. It can also remove the specific organic and inorganic constituents, nutrients, and kill the pathogens.

Tertiary treatments are of four types that include the following:

1. Ion exchange
2. Membrane separation techniques
3. Electrochemical techniques
4. Advanced oxidation process

(1) Ion exchange: Ion exchange is one of the water treatment method in which one (or) more undesirable ionic contaminants are removed from water by exchange with another non-objectionable or less objectionable ionic substances. Ion exchange is one of the most appropriate technologies to remove dissolved inorganic ions effectively. In wastewater treatment plant, it is widely used for water softening and removal of nitrogen, heavy metals, and total dissolved solids (Fig. 17.8). Ion exchange resins have the capacity to exchange soluble cations or anions with electrolyte solutions by transferring into the sludge. Resins possess specific metal uptake capacity and hence can easily carry out ion exchange process. In this process, ion exchange resin is suspended in an electrolyte. As water passes through resin bed, these ions get attached to the resin beads due to which the loosely held solution gets released into the water. After some time, the beds get saturated and lose its power so it should be recharged. As a part of this recharge, the resin is flushed with a salt brine solution due to which exchange of ions occurs between salt brine solutions and flushed out water (Keller 2005).

Applications of ion exchange resins:

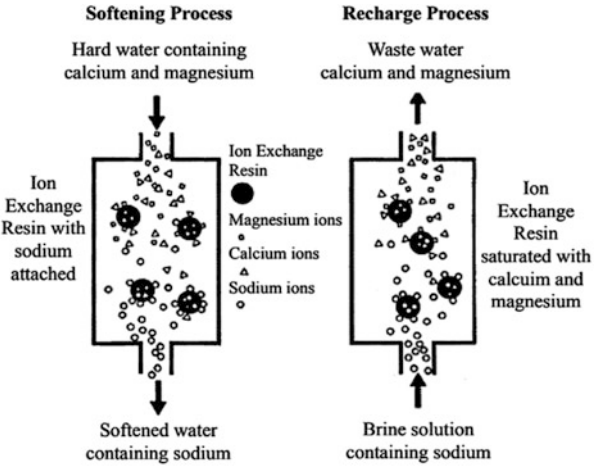

Fig. 17.8 The water softening and recharge process. Modified from Skipton et al. (2008)

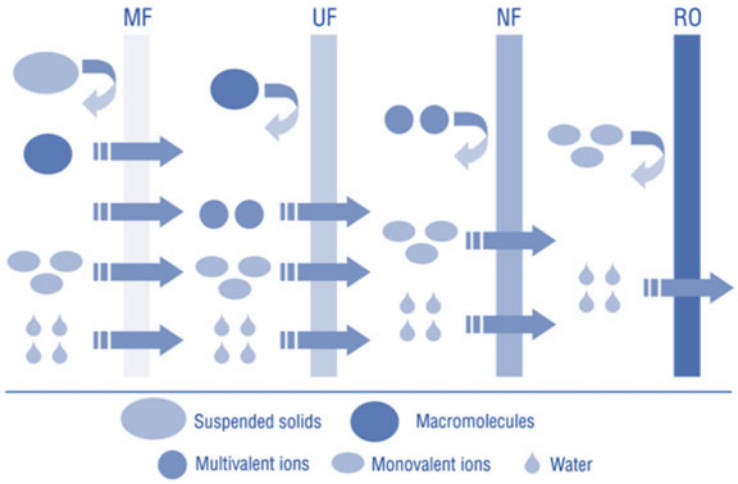

Fig. 17.9 Separation of compounds by membrane filtration processes. Modified from Mo Mukiibi and Feathers (2009)

- For controlling nitrogen: Natural zeolite such as clinoptilolite can be used as ion exchange resin as it has greater affinity for ammonium ions and is inexpensive. It also creates a problem as they form calcium carbonate precipitates within the filter.
- For controlling heavy metals: Natural zeolite such as chabazite is used for removing heavy metals. Chelating resins such as aminophosphonic resins have capacity to remove metals such as copper, nickel, and so on.
- For controlling total dissolved solids: Cationic and anionic exchange resins are used for removing total suspended solids.

(2) Membrane filtration techniques: Membrane filtration work on the principle of filtration. In membrane filtration process, wastewater is allowed to flow through a semipermeable membrane. Membrane filters remove dissolved particles up to 0.0001 to 1 μm. Under high-pressure conditions, membrane acts as a selective barrier that will allow the passage of certain components and will retain other components present in the liquid. It is mostly used in the treatment of seawater, brackish water desalination, etc. (Nqombolo et al. 2018). Membrane filtrations are of various types based on material used for membrane, nature of motive power, detachment mechanism, and minimal size of separation achieved (Fig. 17.9).

(a) Microfiltration (MF)
(b) Ultrafiltration (UF)
(c) Nanofiltration (NF)
(d) Reverse Osmosis (RO)

(a) Microfiltration: It is a physical separation to remove suspended solids and bacteria from water streams through a membrane porosity of between 0.1 and

10 μm. Microfiltration technique is mostly used for treatment of potable water, industrial, and municipal wastewater.

(b) Ultrafiltration: It is used for the removal of particulates and macromolecules from raw water to produce potable water. Filters have membranes containing pore size between 100 and 10 nm. They are usually supplied with pressure between 1 and 10 bar in order to facilitate the separation process.

(c) Nanofiltration: It is used for treating wastewater that consists of low amount of total dissolved solid particles, for example, surface water treatment. This technology is mostly being used in the dairy industries for partial demineralization of products. This process mainly softens the water and removes the organic by-products. Nanofilters have pore size from 1 to 2 nm and require 3–20 bar pressure for the particle separation.

(d) Reverse osmosis: This process utilizes a partially permeable membrane in order to remove unwanted molecules, ions, and salts from the water. It is mostly used to treat fresh water and plays a major role in desalination of seawater. The membranes used in this process have pore size less than 1 nm. As this process uses membranes with less porosity, it is operated usually at high-pressurized conditions (up to 80 bars).

(3) Electrochemical Techniques: This method can be achieved by direct oxidation and reduction reactions by releasing chemicals that can physically remove all the pollutants from the wastewater or through producing chemical species that are reactive. Electrochemical techniques are of two types: electrodialysis and electrocoagulation (Feng et al. 2016).

(a) Electrodialysis: In this technique, transport of salt ions is achieved from the wastewater. This transport occurs over ion-exchange membranes in an applied electric potential difference. This process usually occurred in an electrodialysis cell. This cell usually has a diluent section and a brine part formed by an anion exchange membrane. The cationic membrane is between the two electrodes, and due to electric potential contrast, the negatively charged ions migrate toward anode whose further migration is prevented by the anionic membrane (Fig. 17.10). The positively charged ions migrate toward the cathode whose further migration is prevented by the cationic membrane. This migration of ions occurs equally. Due to this migration, electric current flows between cathode and anode. Electrodialysis results in the increase of ion concentration in the brine compartment with the depletion of ions in dilute compartment (Tedesco et al. 2016).

(b) Electrocoagulation (EC): Electro means to apply an electrical charge to water, and coagulation means the process of changing the particles surface charge, allowing suspended matter to form an agglomeration. It is an advanced, economical, and broad spectrum treatment technology. EC removes the suspended solids, heavy metals, emulsified oils, total petroleum hydrocarbons, grease, latex, bacteria, and other contaminants from water (Tedesco et al. 2016).

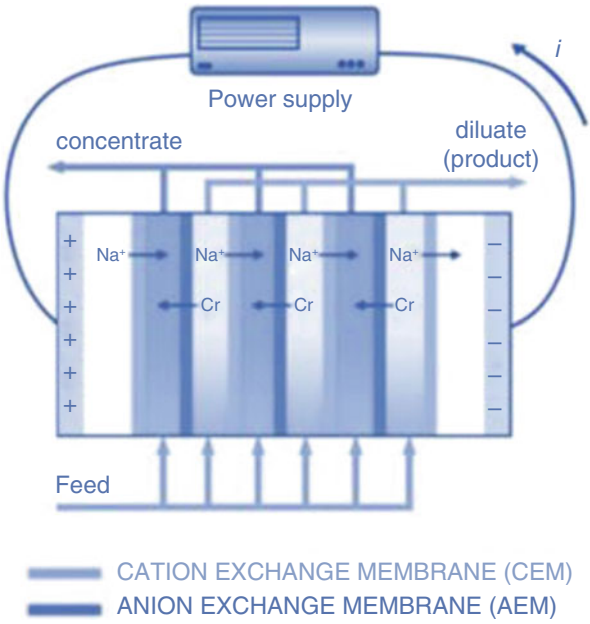

Fig. 17.10 Electrodialysis process (ED). Modified from Michele Tedesco et al. (2016)

(4) Advanced Oxidation Process: Advanced oxidation process is used to oxidize complex organic constituents found in wastewater that are difficult to degrade biologically into simpler end products. It involves generation and use of hydroxide free radical (HO) (Nick Nicholas 2018). Most commonly used advanced oxidation processes are:

(a) Ozone/UV
(b) Ozone/hydrogen peroxide
(c) Hydrogen peroxide/UV

(a) Ozone/UV process: In this process, the photons present in the UV spectrum splits ozone to oxygen and peroxide in the presence of water. The formed peroxide now reacts with the ozone and releases hydroxyl free radical (Tichonovas et al. 2017).

$$O_3 + UV \rightarrow O_2 + O\left(^1D\right)$$

$$O\left(^1D\right) + H_2O \rightarrow HO^{\cdot} + HO^{\cdot}$$

$$O\left(^1D\right) + H_2O \rightarrow HO^{\cdot} + HO^{\cdot} \rightarrow H_2O_2$$

where $O_{3\ =}$ = ozone
UV = Ultraviolet radiation
O_2 = oxygen
O (^{1}D) = excited oxygen atom

$HO^{\cdot}$ = Hydroxyl radical

(b) Ozone/hydrogen peroxide process: In this process, the hydrogen peroxide is used with the ozone in order to enhance the release or formation of hydroxyl radicals (Mansouri et al. 2019). Hydrogen peroxide is a weak acid and dissociates into additional hydrogen peroxide ions, reacts with the ozone, and results in the formation of hydroxyl free radicals (Kuo et al. 1999). It is effective in reducing trichloroethylene (TCE) and perchloroethylene (PCE).

$$H_2O_2 + 2O_3 \rightarrow HO^{\cdot} + HO^{\cdot} + 3O_2$$

(c) Hydrogen peroxide/UV: This process is effective for removal of N-nitrosodimethylamine (NDMA) and other compounds of concern in treated waste water such as PPCPs/ micro constituents (steroidal hormones, veterinary and human antibiotics, human prescription and nonprescription drugs, industrial and house hold wastewater products).

$$H_2O_2 + UV \rightarrow HO^{\cdot} + HO^{\cdot}$$

Applications:

- Used for low COD wastewater because of the cost of ozone and/H_2O_2 required to generate the hydroxyl radicals.
- Used for high-level disinfection and treatment of refractory organic compounds.
- Used for removal of estrogenic, antibiotic, herbicide, pesticides, and viruses.

17.4 Conclusion

The following points were concluded in conventional wastewater treatment processes:

- Preliminary treatment is useful for elimination of coarse solids and other large objects present in wastewater. Hence, this removal increases the operation and maintenance of subsequent treatment units.
- Primary treatment is useful for elimination of settleable inorganic and organic solids, approximately 20–50% of the incoming BOD, 50–70% total suspended solids, and 65% of oil and grease materials.
- Secondary or biological treatment removes the 95% of suspended solids, BOD, and residual organics.
- Tertiary treatment removes the total suspended solids, total dissolved solids, specific organic and inorganic constituents, and nutrients removal (nitrogen and phosphorous).

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Chapter 18
Analytical Techniques/Technologies for Studying Ecological Microbial Samples

Chidinma Lynda Akaihe, Ebubechukwu Nnamdi Dim, Chizoba I. Ezugwu, Emeka Innocent Nweze, and Paul Ekene Chidebelu

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Abstract It is known that most of the microorganisms in the environment are unculturable and using the culture-dependent methods in isolating these organisms may be time consuming among other disadvantages. As a result, methods used in studying microbes have evolved from the traditional culture-dependent methods to culture-independent molecular-based approaches. This chapter discusses the classical culture-independent molecular techniques with an emphasis on the latest information on novel methods in microbial ecological research including the use of "omics" approaches such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics for identifying total microbial community structure and their functions in the environment. It also covers the recent use of a combination of "omics" (multi-omics) approach and the use of improved bioinformatics pipeline for big data analysis and interpretation.

Keywords Quantitative PCR · Metagenomics · Fingerprinting · Multi-omics approach · DNA microarrays

Abbreviations

ARDRA	Amplified ribosomal DNA restriction analysis
cRNAs	coding RNAs
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
HTS	High-throughput sequencing
ISR	Intergenic spacer region
MS	Mass spectroscopy
ncRNAs	Noncoding RNAs
NMR	Nuclear magnetic resonance
OUT	Operational taxonomic unit
PCA	Principle component analysis
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphism
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
SCSU	Sole carbon-source utilization
SIP	Stable-isotope probing
SPE	Solid-phase extraction
SSCP	Single-stranded conformation polymorphism
ssDNA	Single-stranded DNA

T-RFLP Terminal restriction fragment length polymorphism

18.1 Introduction

Microorganisms (such as viruses, bacteria, fungi, protozoa, algae, and nematodes) can be found in almost every environment ranging from the boiling hot springs to coldest regions of the Arctic including depths of the ocean. They exist in a very large number, being an important component of the plant (particularly the rhizosphere) and soil ecosystems (Barua et al. 2017). Microbes interact with each other and the abiotic environment, thereby creating a micro-niche that significantly play very crucial role in different biogeochemical cycling of elements (Wall and Virginia 1999), and organic compounds (Barua et al. 2017), which positively influence the above ground ecosystems. Various types of culture media have been successfully developed for selective isolation of microbes from different environments in the laboratory. However, the inability to simulate the required microbial growth conditions such as the necessary nutrients/substrates, surfaces, optimal temperature, pressure and atmospheric gas composition, the required symbionts, regulation of waste products from their own metabolism, etc. can make it very difficult or impossible to isolate these microbes (Barua et al. 2017). The molecular-based approaches came to light in the late twentieth century, propelling the study of genetic materials such as the DNA and RNA. Lane et al in (1985) proposed the direct cloning of DNA from the environment by the use of polymerase chain reaction (PCR) to explore the diversity of ribosomal RNA (rRNA) sequences. Early metagenome studies centered on 16S rRNA were short and conserved (i.e., identical sequences in DNA and/or RNA that remains relatively unchanged in the phylogenetic tree) within a species. The direct evaluation of many rRNA sequences revealed that many 16S rRNA sequences do not belong to any known culturable species indicating that many microbes were not isolated. Hence, the facts that the culture-dependent techniques identify less than 1% of the microbial species in a sample (Hugenholtz et al. 1998) in combination with other factors make this method obsolete for effective study of microbial samples.

The classical molecular methods such as the nucleic acid reassociation, hybridization, and genetic fingerprinting methods were highlighted in this chapter. Due to the high cost of the emerging novel techniques for studying microbial samples, these classical molecular methods can be used as prescreening techniques before applying the expensive methods to further identify and differentiate microbial communities (Johnston-Monje and Lopez Mejia 2020). Furthermore, this chapter presents a highlight of the culture methods, the advantages, and disadvantages. Most importantly, it discussed the classical molecular techniques with emphasis on the latest information on novel methods in microbial ecological research, the omics approach, as well as the use of a combination of "omics" approach for effective microbial study.

18.2 Overview of the Traditional Culture-Based Techniques

The metabolic activities of microbes such as energy source utilization (e.g., carbon or nitrogen) coupled with other requirements for growth were used by early microbiologists to characterize and differentiate microbes. The traditional culture-based techniques for microbial analysis can be performed using (a) liquid culture medium and (b) solid culture medium.

18.2.1 Liquid Culture Medium

Historically, liquid culture was the first medium used for microbial cultivation and isolation (Bonnet et al. 2019). Subsequently, the need to simulate the microbial natural environment through optimization of their specific growth requirements and conditions led to the development of general purpose and specific liquid culture media such as nutrient broths. The liquid culture media increase the chance of isolation of bacteria from mixed samples than fungi (Bhadange et al. 2013). It can serve as preincubation media prior to inoculation on solid media, where it particularly helps to revitalize and activate damaged or dormant cells ready for transfer to a new nutrient environment (Austin 2017). Liquid medium also serves as a rich reservoir for environmentally induced expression of specific genes (Çelen et al. 2018). Serial dilution of mixed samples in liquid media helps in eventual isolation of pure cultures on solid media, as well as in reducing the microbial population to levels that are macroscopically convenient for enumeration.

18.2.2 Solid Culture Medium

The plate count method is one of the ways of enumerating microbial cells. After inoculating a sample on a typical microbiological media, viable cells in the sample will grow to form a visible colony, and the number of colonies on the plate can be counted if appropriate dilutions are plated out. The plate count method is very cheap and can provide information about viable and culturable microbial population as only the viable cells will grow; dead cells will not grow to form colonies. The inability to select the suitable microbiological media and growth conditions limits the use of this traditional method (Tabacchioni et al. 2000). Additionally, it is time consuming as it takes up to a day before results are obtained. The major drawback is the inability to detect unculturable microbes.

The community-level profiling system also known as the sole-carbon-source utilization (SCSU) is another culture-dependent technique. It was originally invented to identify pure cultures of bacteria up to the species level reflected by their resultant

metabolic properties. This technique evaluates microbial population based on their functional properties. The resulting data are then analyzed with the aid of multivariate techniques to compare their metabolic properties (Preston-Mafham et al. 2002). Notably, some microbes have fast growth, whereas others are slow growers, and some may utilize the secondary metabolites to grow during incubation. Therefore, this assay may not be used for organisms that take longer time to grow. The major advantages include the ability to distinguish between communities of microbe, its reproducibility, and simplicity, as well as the large data that describe the metabolic characteristics of the microbial communities (Zak et al. 1994). However, SCSU technique does not reflect the metabolic diversity of microbes in situ, and the carbon sources may not be a representative sample of those present in the environment. In addition, this method favors only fast-growing and culturable microbial community, thus restricting its applications (Yao et al. 2000; Garland and Mills 1991).

18.3 Classical Culture-Independent Molecular Techniques

18.3.1 Nucleic Acid Reassociation and Hybridization

Nucleic acid hybridization is dependent on the specificity of base-pairing of nucleic acids to generate a signal through a labeled probe. This fundamental principle of the technique has been applied in the detection and quantification of DNA/RNA on solid phase blots, cytogenetic localization of DNA/RNA on cells, as well as comparative gene expression analysis of purified DNA (Kim et al. 2015; van Dekken and Bauman 1988). The nucleic acid hybridization approach requires the extraction and purification of total DNA from environmental samples, denaturation of the double-helical structure through heating, followed by slow cooling of the DNA bulk as the complementary strands reanneal. It requires target oligonucleotide or polynucleotide probe designed from known sequences. The probe ranges in specificity from domain to species, including markers that can either be florescent marker (such as fluorescein and rhodamine) or a radioactive label (usually a molecule probe incorporated with isotopes such as ^{32}P, ^{35}S, ^{125}I, and ^{3}H). Although the radioactive probe has the advantage of high sensitivity in detection, however, due to safety measures for operators and the cost of disposing radioactive wastes, the non-radioactive probes are often preferred in molecular biology assays. It is important to note that the rate of reassociation is dependent on the similarity of the sequences. Decreased rate of DNA reassociation is as a result of increase in the diversity of the DNA sequence.

This technique can be used to differentiate microbial species based on the ability of the PCR products (amplicons) to anneal at a specific range of temperature. It is routinely used as the bedrock for studying microbial taxonomic and genomic relationships (Subhash and Lee 2018). For optimal DNA reassociation, hybridization should be performed at 55 °C, while for others adapted to stringent temperatures, reassociation could occur at a temperature up to 70 °C, and it has been shown

that this DNA–DNA hybridization technique could reliably identify some bacteria up to the species level (Carvalho et al. 2007). Related studies have proposed novel species of *Yersinia* and *Paracandidimonas* based on combined data of DNA–DNA reassociation, phenotypic, and phylogenetic identification techniques (Murros-Kontiainen et al. 2011). Subsequent to denaturation, extraction and purification of the genomic DNA is carried out using specific isolation kits (Zhang et al. 2018; Yao et al. 2019). Earlier, the DNA–DNA hybridization using DNA macro-array was proposed as an emerging tool that is simple, easy, and modifiable to a high-throughput DNA hybridization technique that could result in a data matrix with high discriminatory ability between genomes (Ramisse et al. 2003). Later, the development in the genomic identification by DNA–DNA hybridization led to the application of microarrays including those based on open reading frames, and others relying on the random DNA fragments. It was more efficient than the microarray, allowing the use of larger number of probes. Further improvement in these techniques have resulted in the utility of microarrays in both identification and determination of relatedness between species of microorganisms, as already demonstrated with the community genome microarray hybridization (Wu et al. 2008). In determining the species relatedness, the DNA–DNA association cutoff value was generally delineated at $<70\%$ and was established as the threshold value at or below which separation into different species is taxonomically recommended, because high level of relatedness is presumptive at or above 70% hybridization (Christensen et al. 2011; Niu et al. 2018). With recent advances in bioinformatics, the wet-lab-based experimentally determined DNA–DNA hybridization can be alternatively performed by comparing genomes in silico, using web-based software (Auch et al. 2010; Niu et al. 2018).

This technique is not influenced by PCR biases, and its potential to enable one to study DNA or RNA in situ offers an extra advantage. However, it may not detect sequences with low copy numbers, and its lack of high sensitivity as well as its dependence on lysing and extraction efficiency are obvious limitations.

18.3.2 Genetic Fingerprinting Methods

DNA fingerprinting can be described as a molecular tool that enables comparative analysis of fragments of DNA in consortium of samples so as ascertain the level of biodiversity and relationship (Fraher et al. 2012). These methods include but are not limited to amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP), single-stranded conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and ribosomal intergenic spacer analysis (RISA).

18.3.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

In this technique, the conserved region of the 16S rRNA gene is enzymatically amplified with universal primers or primers specific to the genus or species using the PCR technique. A tetra-cutter restriction enzyme is used to digest the amplicons to obtain the species analyzed, followed by the separation of the restricted fragments on the agarose or polyacrylamide gel. The genotype or strain type is determined as the profile bands emerge on the gel (Tiedje et al. 1999). The ARDRA technique is similar to the RFLP. In the past, the ARDRA approach was a useful tool in selection of clone libraries, as well as in the determination of phylogenic groups in a diverse microbial community through strain typing. Tingli et al. (2018) recently used it to prescreen samples before the use of more expensive high-throughput sequencing technique. A combination of ARDRA approach and high throughput sequencing was used to determine the time point at which the colistin sulfate showed the greatest negative effect on soil bacteria before using the high-throughput technique to identify more bacteria with higher accuracy (Tingli et al. 2018). Sánchez et al. (2019) used the ARDRA technique to characterize lactic acid bacteria (LAB) from Amazonian Peruvian fruit using *Mse*I, *Hae*III, and *Alu*I restriction enzymes. While the later revealed four restriction profiles, the first two enzymes showed three restriction profiles. From their findings, it was evident that the restriction enzyme *Alu*1 better differentiated the LAB isolates up to four species. Some restriction enzymes are better when compared to some others in terms of efficient digestion and differentiation of gene segments of microbes. Therefore, successful ARDRA technique is dependent on the right selection of restriction enzymes. It is very important to note that restriction enzymes with similar recognition sequences (isoschizomers) create difficulty in analysis.

The advantages of ADRA technique include the following: (1) ability to distinguish isolates from different environments up to the species level (Dec et al. 2016); (2) it can identify microbial communities undergoing structural changes. However, microbial diversity cannot be measured and phylogenetic clusters within a community fingerprinting profile cannot be identified, which implies that intra-specific variability cannot be detected with this technique (Sánchez et al. 2019; Kingston et al. 2010). Aside from these demerits, the major limitations of this technique include time consumption, and it is labor intensive. Additionally, its applicability is best in environments with low complexity. Also, in ARDRA, different bands can belong to the same group of organisms.

18.3.4 Restriction Fragment Length Polymorphism (RFLP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The RFLP is similar to the amplified 16S ribosomal DNA restriction analysis (ARDRA) technique where the DNA from the environmental sample is digested using the restriction enzyme followed by separation of fragments by electrophoreses on agarose gel, as well as detecting the fragments by southern blot hybridization to a labeled probe (Liswara et al. 2020). RFLP probes are maintained in suitable bacterial vectors, which makes it more convenient to isolate the DNA fragment when the need arises. The RFLP technique can show co-dominant alleles. However, in conducting research involving poorly studied groups of wild species or orphan crops, significant capital is needed for the development of suitable probes for such poorly studied groups as the probes may not yet be available. In addition to this setback, RFLP technique requires large quantity of DNA and is not amenable to automation.

In T-RFLP, the primers are labeled (usually with fluorescent dyes), cut by the restriction enzymes, and visualized in electropherograms. The multivariate statistics can be used to analyze T-RFLP fingerprints to compare microbial populations. Using tools such as MiCA and TRiFLe, the individual terminal restriction fragment sizes can be compared to sequence databases to predict their taxonomic level (Shyu et al. 2007; Junier et al. 2008). TRFLP is the most commonly used tool by plant biologists. Its advantages include high speed, reproducibility, as well as high resolution. Walitang et al. (2018) used this approach to study the community structure and diversity of bacterial endophytes of rice with varying tolerance to salt stress. With T-RFLP, the authors were able to determine the shift in bacterial diversity and dominance resulting from their physiological adaptation to salt stress. Also, the T-RFLP technique has been applied in biocontrol studies (Wan et al. 2018), plant-associated fungi (Penuelas et al. 2012), study of plant mycobiomes (Johnston-Monje et al. 2017), among others. Although the T-RFLP technique is seen as one of the outdated methods of studying microbial diversity, it is still a useful tool employed by researchers to economically prescreen samples before the application of more expensive methods such as the high-throughput sequencing (HTS) to cross-validate their results (Johnston-Monje and Lopez Mejia 2020).

18.3.5 Single-Stranded Conformation Polymorphism (SSCP)

The SSCP is also known as the single-stranded chain polymorphism. This technique detects the conformational difference of single-stranded DNA (ssDNA) sequence of same length as a result of the differences in the sequences. Through gel electrophoresis, a single nucleotide change or point mutation in a double-stranded DNA molecule cannot be differentiated because the physical properties of the double-helical structure of the DNA are almost the same for both alleles. Interestingly, after

denaturation, two ssDNA of the same length with distinct sequences can undergo a characteristic three-dimensional folding, thereby assuming a unique conformational state, which can be differentiated by gel electrophoresis (Orita et al. 1989). This technique is commonly used in the study of viruses especially mutating strains (Kubo et al. 2009). Presently, the PCR with universal primers are used to generate the target amplicons, and the amplicons denatured through heating followed by digestion with lambda exonuclease. The digested amplicons are run on non-denaturing acrylamide gel. The 3-D structure of the single-stranded DNA is determined by its nucleotide sequence, and this has a strong impact on its electrophoretic mobility pattern. Alteration of a single base in a 300-base sequence of ssDNA can affect the migration pattern (Schmalenberger and Tebbe 2014). In SSCP, maintaining a constant temperature ensures reproducibility of results as gels run with the same samples at different temperatures will produce different results (Kaczanowski et al. 2001). The SSCP is not only applicable in virus particle study but have also been applied in a recent study to profile rhizosphere populations of bacteria (Lamizadeh et al. 2019), as well as to study plant-associated fungal populations (Alguacil et al. 2009).

18.3.6 Denaturing Gradient Gel Electrophoresis (DGGE)

In DGGE, DNA fragment of up to 500 bp are chemically denatured using progressively increasing gradients of DNA chemical denaturants (usually a mixture of formamide and urea). The forward primer used during the DGGE process is tagged with 30–50 bp of GC-rich nucleotide sequence to ensure complete separation of the double-stranded amplicons during electrophoresis. As the amplicons pass through the polyacrylamide gel containing gradients of DNA denaturants, it separates the double-helix DNA fragment during the electrophoresis until it reaches its denaturizing threshold where its movement through the gel is halted to form a band. Amplicons that belong to different taxonomic level have different levels of migration through the gel. This implies that specific taxon has its unique denaturizing threshold. Hence, the difference in the denaturizing threshold is as a result of variation of base sequence within the amplified DNA molecules. To identify a particular microbial taxonomic level, the bands from the polyacrylamide gel are excised, re-amplified, and then sequenced. The resulting bands from the DGGE gel can be visualized by staining with DNA dyes such as ethidium bromide, GelRed, or SYBR Safe, followed by the photographing of stained band, which can be digitized for further community profile analysis. DGGE of the 16S rDNA had been applied to profile bacterial phyllospheres (Cid et al. 2017). Additionally, the DGGE of the 18S rDNA approach has been applied to study plant-associated mycobiomes (Chen et al. 2012; Gao et al. 2012; Jeewon et al. 2018). It is employed to economically prescreen samples before using the more expensive and sensitive HTS (Johnston-Monje and Lopez Mejia 2020).

The major benefit of using the DGGE technique is that one can observe spatial and time-based changes in microbial community structure and can vividly reveal microbial species present in a sample (Malik et al. 2008). On the other hand, DNA bands with the same melting point can pose a problem in taxonomic identification. Sequence heterogeneity between multiple rRNA operons of a bacterium can lead to several bands in DGGE and may result in the overestimation of the microbial diversity present in the sample. Additionally, inability to cast gels with consistent and progressive gradients of denaturant may make reproducibility of results very difficult in different experiments (Yang et al. 2010).

18.3.7 Ribosomal Intergenic Spacer Analysis (RISA)

The RISA technique is commonly known as community fingerprinting. It is a way of conducting microbial community analysis of environmental samples or to evaluate treatment impacts. The PCR is used to amplify the intergenic spacer region (ISR) or non-transcribed spacer (Sigler and Zeyer 2002; Borneman and Triplett 1997). Oligonucleotide primers complementary to the conserved regions are used to generate RISA fragments of most dominant community in the environmental sample. Significant heterogeneity in length and nucleotide sequence of the ISR determines the taxonomic value. The automated version of RISA is known as ARISA, and the latter is said to be a good choice for community fingerprinting because the nucleotide sequence and length of the ITS regions of bacteria and fungi have greater variability when compared to the coding regions such as the rDNA (Johnston-Monje and Lopez Mejia 2020). The RISA technique was employed to discover novel copper-tolerant bacterial endophytes (Cubaka et al. 2010), as well as in the study of microbial rhizosphere community (Khan et al. 2017). The demerits of this approach are similar to that experienced in a conventional PCR such as quality and concentration of DNA, annealing time, as well as mismatch of primer.

18.4 Modern Molecular Methods of Studying Microbial Communities

18.4.1 Stable-Isotope Probing Techniques

This technique is used in ecological studies to detect elemental fluxes of nutrients in metabolically active microorganisms while linking their metabolic function to their taxonomic identity (Vogt et al. 2016). Generally, this technique makes use of stable isotopes to identify and characterize metabolically active microbes in an environment. The SIP approach involves incubating an environmental sample to a substrate enriched with heavy stable isotopes (^{13}C, ^{15}N, ^{3}H, and ^{18}O). When these substrates

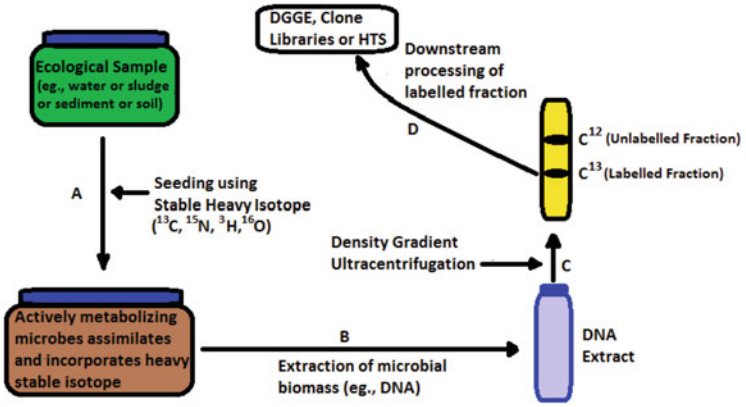

Fig. 18.1 The major processes in SIP technique

get assimilated by microbes, they form part of the microbial biomass (like the DNA, RNA, lipid, and proteins). Because the heavy stable isotopes are labeled (isotopically), they act as biomarkers of metabolically active community present in a particular environmental sample. Upon assimilation and incorporation in active microbial biomass, the biomarker can be recovered and subjected to analysis using techniques such as fingerprinting, microarray, omics approach (such as the metagenomics), or the next-generation sequencing to determine the active microbial community present (Uhlik et al. 2013) (Fig. 18.1).

The target microbial biomass where the isotopically labeled biomarker was recovered from determines the category of SIP in an ecological study. When the biomass where the biomarker was recovered is a DNA, it is categorized as the DNA-SIP. The same is applicable to others like the RNA-SIP, as well as the protein-SIP. Among these SIP categories, the DNA-SIP with the aid of 13C-based heavy stable isotopic label has been the most widely employed technique to identify active microbial populations with their group metabolic function in an environmental sample (Radajewski et al. 2002; Neufeld et al. 2008; Zhang et al. 2016; Angel et al. 2018; Ziels et al. 2018). For example, the DNA-SIP approach can help to identify which organisms are actively consuming new product of photosynthesis (Radajewski et al. 2002). After the SIP-experiment, the DNA recovered from this process can be subjected to many downstream analyses (such as DGGE, clone libraries, and HTS analysis) to obtain important genetic information including those related to the organisms functional and ribosomal genes involved in metabolic activities, which make it more advantageous. Presently, it is not only feasible to have a combination of high-throughput sequencing together with SIP to characterize complex microbial communities in the environment but also researchers have been able to identify the rate at which heavy stable isotopes get incorporated in thousands of microbial communities (Youngblut et al. 2018). In DNA-SIP technique, the incubation process can be performed in the laboratory setting using a laboratory microcosms built using samples collected from the environment (i.e., in vitro

DNA-SIP-based method) (Wald et al. 2015; Paes et al. 2015) or in situ by direct seeding of labeled isotopes (artificial enrichment) in the soil (Liou et al. 2008; Key et al. 2013). Exposure time and the concentration of the substrate are extremely important. Additionally, excessive labeling must be avoided as there are tendencies to distribute the isotopes to other trophic levels as a result of cross-feeding and trophic interactions (Neufeld et al. 2007). The inclusion of control (i.e., the set-up experiment containing unlabeled substrate) helps to checkmate and confirm that the recovered isotopically labeled DNA resulted from the assimilated labeled substrate. Neufeld et al. (2007) and Dunford and Neufeld (2010) outlined the DNA-SIP protocol and the technical considerations involved in DNA-SIP experiment.

The use of RNA as a biomarker in RNA-SIP technique has been projected to be superior as a result of notable properties such as higher synthesis rate, the ability to reflect cellular activity, responds better to environmental conditions, as well as replication-dependent turnover in the cell (Manefield et al. 2002; Whiteley et al. 2007). Because the RNA-SIP method requires a rigorous process for gradient evaluation, it is technically more demanding than the DNA-SIP. However, in terms of gradient media, cesium chloride gradient medium is not appropriate for the RNA-SIP technique because both precipitates at the same buoyant density. As a result, the cesium trifluoroacetate provides a better alternative to obtain the required gradient formation (Rickwood 1992; Manefield et al. 2002). The RNA-SIP approach has been employed to identify active microbial community in different environmental samples including the rhizosphere of rice (Lu and Conrad 2005), bioreactor sludge (Manefield et al. 2002), and in grassland soil (Rangel-Castro et al. 2005). Among the categories of SIP technique, the protein-SIP-based method is more sensitive than the nucleotide-based SIP approach (such as RNA-SIP or DNA-SIP technique), detecting as low as $<1\%$ of ^{13}C incorporated in the protein unlike the former that requires relatively higher level of incorporated ^{13}C label of about 20% (Taubert et al. 2011). The nano-secondary ionization mass spectrometry and high-resolution mass spectrometry are used to detect the incorporated heavy isotope in the protein followed by calculation of the relative isotopic abundance by analyzing the distribution of various isotopes of the peptides.

The ability to apply the SIP approach in different environments such as the soil, water, and sediments without any basic information of the microbial community responsible for the degradation activity makes the SIP advantageous. On the other hand, the SIP approach is expensive, labor intensive, and may pose technical difficulties. Moreover, the stable heavy isotopes can be expensive, and the timeframe to implement it is dependent on the SIP approach used. Researchers should be cautious when analyzing the results of SIP experiment.

18.4.2 Quantitative PCR

This technique was developed to determine the quantity of target DNA or cDNA nucleic acid. The quantitative PCR is also known as the real-time PCR. Extracting

the DNA is an extremely important step in analyzing genetic materials from environmental sources. Depending on the source of the sample (for example, water), the sample can be processed by first concentrating biomass. It is then filtered, followed by extraction of DNA and characterization of the genetic material using targeted approach (such as qPCR technique) or non-targeted approach (such as metagenomics) (Manaia et al. 2018).

Quantitative analysis of PCR products is dependent on the knowledge of the four major phases in the PCR amplification curve (i.e., linear ground phase, early exponential phase, log-linear phase, and plateau phase). In the log-linear phase, amplification is not detectable; the amplicons are only detected in the early exponential phase where the quantification of the nucleic acid must be done at this stage (Saunders 2004). At log-linear phase, there is low PCR efficiency and depletion of reagent components. In contrast to the normal PCR process where there is end-point detection of PCR products (amplicons) at the plateau stage, in the qPCR, there is real time detection of products at the early exponential phase. This is because the data from the plateau phase cannot accurately quantify the amount of template nucleic acid used before the process began. In qPCR, when significant and specific amplification occurs, a point will reach where the fluorescence signal will rise above the ground level; this is known as the RT-PCR threshold. The cycle number at which the fluorescence signal crosses the threshold is known as the threshold cycle.

Protocol on the PCR conditions has been described by Salonen et al. (2010), Rinttila et al. (2004), and Jian et al. (2020). In order to monitor DNA amplification real-time, a fluorescence detection system is integrated into the thermocycler to track the progress of the reaction during each cycle. To measure the accumulation of PCR amplicons, one can use either a fluorescent dye-based detection (Rinttila et al. 2004) or a fluorescent probe-based detection (Cardullo et al. 1988). The fluorescent dye-based detection provides DNA detection in a non-sequence-specific manner, as the fluorescence signal may originate from non-specific PCR amplification. Due to the tendency for non-specific amplification of DNA in this approach, preliminary analysis can be conducted to show that only the target PCR product is obtained from the PCR conditions used. Additionally, a melt curve analysis may be performed after RT-PCR to identify non-specific amplification (Rinttila et al. 2004). In fluorescent probe-based detection, there is specific detection of DNA sequences using fluorescent-labeled oligonucleotide probes. Such probes include the TaqMan probes which are labeled with a fluorophore at the 5′ end and a quencher at the 3′end. During the elongation process, the enzyme (Taq DNA polymerase) reaches the 5′end of the probe. The 5′-3′exonuclease activity of the polymerase cleaves off the fluorophore resulting in increased fluorescence. Another type of probe is the fluorescence resonance energy transfer (FRET) probe, which binds to the PCR amplicon in a sequence-specific manner. Other fluorescein reporter probes include molecular beacons, scorpions probes, etc. Fluorescein dye includes SYBR Green. The 16 S rRNA, 5.8S rRNA, inter-transcribed spacer region/gene, as well as functional genes such as the pmoA, norS, amoA, and dsrA unique to ammonia oxidizers, methane, and sulfate oxidizers have been manufactured and applied in quantification of

microbial communities such as bacteria and fungi (Fierer et al. 2005; Foti et al. 2007).

Quantification of nucleic acid using the real-time PCR approach can be either absolute quantification or relative quantification. In absolute quantification, the amount of target nucleic acid is reported as a copy number or a concentration. And it requires a standard curve already generated from external standards with known copy number or concentrations of an exact nucleic acid sequence or gene (Handy et al. 2006). In relative quantification of nucleic acid, the amount of target nucleic acid is reportedly compared to the abundance of another gene.

An efficiency of 1 implies that the amount of PCR product doubles at each cycle (an ideal PCR experiment). An efficiency of <90% is derived from a real PCR analysis as a result of limitations in the experiment. Lowered efficiency could be a pointer to one or more of these flaws, which includes inaccurate sample or reagent preparation, the presence of PCR inhibitors, low enzyme quality, which resulted in poor activity, and flaws in primer or probe design.

The qPCR technique has been employed to quantify microbial genes extracted from environmental water sample (Rocha and Manaia 2020), for quantitative microbial profiling (Jian et al. 2020), as well as in quantifying pathogenic and autochthonous bacteria in fecal sample (Rinttila et al. 2004).

18.5 "Omics" Approaches to Studying Microbial Sample

18.5.1 Genomics

These involve techniques for DNA studies which either target single locus or even whole genome analysis (Redenšek et al. 2018). Latest DNA array technologies include the functional DNA array, phylogenetic oligonucleotide arrays, and the next-generation sequencing technologies.

18.5.1.1 Functional DNA Array

Functional DNA or gene arrays (FGAs) are designed with probes for the housekeeping genes coding for the constitutive enzymes of microbes. The critical step in designing the probe is the confirmation of the identified genes of interest by aligning them with the seed sequences coding for known enzymes involved in the cell's basic biological processes (Van Nostrand et al. 2012; Tu et al. 2014). Unlike the regular PCR, the technique overrides the need for sequence amplification and well adapted for multiple analyses of functional genes present in diverse DNA samples, especially unknown consortia of samples recovered from the environment (Nikolaki and Tsiamis 2013). Because of their specificity for the conserved functional DNA sequences, they are very useful in microbial identification from consortium of mixed samples and thus have been successfully applied in detection of virulence

genes, thereby allowing the differentiation of pathogenic strains from nonpathogenic strains of microbial species (Jaing et al. 2008; Lee et al. 2013). The past decades witnessed the advances in functional gene arrays with the development of GeoChips 1.0 and 2.0 technologies. Currently, there are three array tools based on the FGAs techniques for comprehensive analysis of functional genes. These include the GeoChips 3.0, 4.0, and 5.0. They are popular for their high sensitivity, specificity, and ease of use in the biodiversity and composition study of microbial functional genes associated with environmental stress responses, metal cycling in nature, and other biochemical and pathophysiological functions in their human hosts (Nikolaki and Tsiamis 2013; Shi et al. 2019). Particularly, Geo 5.0 which also offers the additional advantage of quantitative insights of the functional genes is already utilized in impact assessment of structure of underground water pollution to the aquatic microbial community (Shi et al. 2019). The technique has also been applied in biodiversity analyses of metal, oil, and pesticide contaminated sites (Van Nostrand et al. 2012).

18.5.1.2 Phylogenetic Oligonucleotide Arrays

PhyloChip microarray identifies and quantifies specific component of a microbial community in a complex environment such as the gut, ground water, or soil, based on the 16S rRNA signature gene loci, and also, identify and classify the physiology and phylogeny of the microbial samples (Schatz et al. 2010). It can be used to track different fecal contaminants of human and animal origins in water and simultaneously study their phylogenetic relationship (Dubinsky et al. 2016). PhyloTrac is a computational tool developed to comprehensively analyze the PhyloChip array data, which has comparative advantages of normalization, accurate operational taxonomic unit (OTU) quantification, multiple and integrated visualization, and analytics (Schatz et al. 2010). Comparatively, the use of high-density microarrays is more economical and less laborious for the study of microbial communities in complex environments than the conventional PCR, and because of its specificity to rRNA gene signature, it could be used to differentiate between the active and inactive components of such natural microbial community (DeAngelis et al. 2011). With reference to their capacity to accommodate oligonucleotide probes, two types of PhyloChip were developed, the low-density and the high-density PhyloChip arrays, each of which has its peculiar comparative advantage. While the high-density is popular for its capacity to analyze larger number of probes, the low-density is more convenient for analysis of smaller number of samples and requires workable number of reference organisms, thereby increasing the specificity and sensitivity of the approach, which is not cost-effective to achieve with the equivalent high-density technique (Loy et al. 2011). Some modifications were evidently adapted to increase the sensitivity of a special oligonucleotide PhyloChip system, by incorporating PCR-amplified 16S rRNA sequence from a reference organism before their hybridization with the microarray oligonucleotide probe (Loy et al. 2005).

18.5.1.3 Next-Generation Sequencing

DNA sequencing technologies are core and constantly evolving high-throughput techniques in the field of genomics and bioinformatics. Illumina is one of the most widely used sequencing by DNA synthesis approach, and cheaper variants such as MiniSeq and MiSeq Illumina have emerged for lower and mid-scale applications at relatively shorter time interval (Ravi et al. 2018). On the other hand, HiSeq, NextSeq, and NovaSeq were adapted for larger genomic projects.

In order to address certain constraints in the second generation including the generation of short-reads that result in disjointed DNA assemblies, the third-generation sequencing technologies were introduced as they can sequence longer primary DNA reads and full-length transcripts of simple and complex DNA configurations with higher accuracy using direct or synthetic long-read technologies without prior need for fragment amplification (Levy and Myers 2016). Unlike the earlier sequencing protocols, these technologies are based on the single nucleic acid molecule (real-time) and nucleic acid sequencing (Mignardi and Nilsson 2014), which following their high sensitivities can capture (real-time) broad spectrum of changes in DNA or RNA sequences and can be interrogated with very minimal bias (Behjati and Tarpey 2013). PacBio platforms including its latest RSII-Seq technology can generate long reads and are also regarded as a perfect instrument to complete assembly of genomes (van Dijk et al. 2014). Similarly, nanopore technology, MinION is a small, long-read sequencing device that relies on the conductivity of the nanopore as DNAs are introduced to identify nucleotide bases (Besser et al. 2018). With in situ sequencing of single-cell RNA (scRNA) (Ke et al. 2016) and messenger RNA (mRNA) (Mignardi and Nilsson 2014), the prospects of genomic wide applications of fourth-generation technologies are not out of sight.

Single-molecule real-time (SMRT) sequencing infers and records the sample DNA epigenetic alterations as interpulse duration, hence making it potentially useful in diagnosis of microbial and infectious diseases, especially when its long-read quality is facilitated with error editing algorithms (Ardui et al. 2018). More affordable variants of SMRT evolved as hybrids in which accurate short reads combine effectively with the longer reading length for direct detection of epigenetic methylation (Rhoads and Au 2015). In order to assemble larger genomes including de novo genomes of eukaryotes, a probabilistic, locality-sensitive hashing assembly tool MHAP (MinHash alignment process) was developed to overlap noisy long reads (Berlin et al. 2015). Compared to other long-read data assembling algorithms, a higher contiguous and accurate FALCON and FALCON-Unzip algorithms were later invented for SMRT sequencing data assembly that would also facilitate deeper understanding heterozygous genome variants and haplotype structures (Chin et al. 2016) (Table 18.1).

The next-generation sequencing approach has been recently applied to reveal the presence of *Sulfurovum* spp. as the dominating microbial community in the sediment–seawater interface of a deep-sea cold seep (Qing-Lei et al. 2020), genomics of viral population (Posada-Cespedes et al. 2017), transmission clusters and

Table 18.1 The operating principles of next-generation sequencing technologies, and their advantages and disadvantages

NGS technologies	Sequencing principle	Advantages	Disadvantages	Generation
Roche 454	Pyrosequencing	Cost-effective and faster, low error rate/high throughput	Requires relatively high start-up costs, must be conducted in a large scale, running identical bases/homopolymer regions (such as TTTTT) can be difficult to distinguish	Second generation
Illumina's Solexa	Sequencing by synthesis	High accuracy, cheaper cost per base, many data can be run	Running data can take time (many days), short read length (50-150 bp), high cost, difficult for De novo assembly	Second generation
ABI's SOLiD technology	Sequencing by ligation	Relatively accurate, 5500XL can be used to run independent lanes, high throughput, low cost per base	Short reads, relative long time run, high cost of instrument, two-base encoding, and sequential ligation may result to error propagation across reads	Second generation
Life technologies ion torrent technology	Ion torrent semiconductor sequencing by synthesis	Cheaper (start-up cost), high accuracy, faster (less than 3 hrs) depending on the machine and chip used, medium/low cost per base, 10–1000 Mb of data per run (scalable)	Approximately 100–200 bp of read lengths (short read length), insertions and deletions (indels) errors, homopolymer repeats ($>$ 6 bp) is difficult to enumerate and may increase error rates, through put id lower compared to other HTS technologies.	Second generation
PacBio SMRT	Sequencing by synthesis (DNA polymerase)	Ability to run very long read lengths (above 10 kb), can utilize a single molecule as template	Expensive (start-up cost), medium/high cost per base, low accuracy, depends on DNA polymerase	Third generation
Nanopore	Electronic signal sequencing (exonuclease)	Portable, single molecule of DNA or RNA can be sequenced without PCR amplification of sequencing fragments, relatively low-cost genotyping, can process samples rapidly displaying results in real-time	Depends on exonuclease, low resolution due to the rapid movement (1 to 5 μs) of DNA through the nanopore	Third generation

outbreaks (Grubaugh et al. 2019), evolutionary biology of viruses (Geoghegan and Holmes 2018), phylogenetic and phylodynamic analysis (Beale et al. 2017), as well as in analysis of bacterial communities to capture significant higher diversity of bacteria in groundwater ecosystems with more accurate and detailed results (Karczewski et al. 2017).

18.5.2 Transcriptomics

These are high-throughput methods and technologies used to analyze the expression levels of both coding RNAs (cRNAs), and non-coding RNAs (ncRNAs) of a microbial sample to understand their roles in physiological conditions (Redenšek et al. 2018). The type of the sample or the source of the microbial gene for expression studies is one of the key considerations in evaluating the outcome of transcriptomic studies. This is because it was found that a host cell genetically responds to pathogenic bacteria in a different manner as it would to a food-borne bacteria (Kjellin et al. 2019). The importance of ncRNomics is related to the modulatory roles of microRNAs and longer noncoding NRA in gene transcription and protein translation. RNA sequencing (RNA-seq) and microarray methods were common transcriptomic techniques routinely used, with efficiently similar outcomes. For transcriptomic analysis, RNA is normally extracted in a homogenizer after adding the appropriate reagent, usually a brand of Invitrogen. Alternatively, RNA extraction kit to which Invitrogen is added can be used, followed by DNA digestion on column. This is then followed by high-throughput RNA sequencing or microarray studies including tiling array and gene expression array (Toledo-Arana et al. 2009). Usually, the RNA yield is evaluated with a nanodrop, while the agarose gel electrophoresis identifies the quality, and also, a poly (A) extraction can be applied to target the isolation of the mRNA from the total RNA extracted depending on the study interest (Kjellin et al. 2019). A high-throughput technique with the ability of complete transcript length sequencing, SMRT (single-molecule real time), has recently made huge impact in transcriptomics, with additional potential of enhancing new gene discoveries including identification of new annotated isoforms (Rhoads and Au 2015). Single-cell RNA sequencing is an emerging interest in transcriptomics, with the evolution of the fourth-generation sequencing technologies such as ISS (in situ sequencing) and the smFISH (single-molecule fluorescent in situ hybridization), which enables direct sequencing of spatially resolved nucleic acids in cells and/or fixed tissues (Ke et al. 2016).

18.5.3 Proteomic Approaches

Proteomics comprise a wide array of molecular biology and biochemistry techniques, integrated with statistical and computer software for identification and

quantification of microbial protein profiles, whose expressions are controlled by the interactions between the organism and its immediate microenvironment (Costa and Franco 2017). Protein profiles are traditionally evaluated based on various chromatographic techniques such as the two-dimensional gel electrophoresis (Redenšek et al. 2018). Studies of protein structure, posttranslational modification, expression, and function currently involve the use of advanced and high-throughput technologies such as the protein arrays, mass spectrometry, and NMR spectroscopy (Aslam et al. 2017). These later two technologies are especially very essential tools in metabolomics (Breindel et al. 2019).

18.5.3.1 Mass-Spectrometry-Based Proteomic Technologies

These represent the major technique for protein profiling and quantification, especially on a large-scale, wherein proteins are identified according to their specific mass-charge ratio that reflects their individual molecular weights following efficient separation of the complex protein structures (Zhang et al. 2014; Aslam et al. 2017). This technology basically requires an ion source to convert the protein samples into gas-phase ions, a mass analyzer that uses electric or magnetic current to separate the ions based on their mass-charge values, and a detector or ion sensor (Matthiesen and Bunkenborg 2013; Aslam et al. 2017). The introduction of the highly sensitive soft ionization generating tools, the matrix-assisted laser desorption/ionization (MALDI) that works on liquid chromatographic separated samples, and the electrospray ionization (ESI) after HPLC separation are very significant innovations in proteomics. The ESI is especially useful for multiplex quantitative analysis of a complex protein, which the MALDI is a nondestructive sample technique, which is a very powerful tool for direct solid analysis and possible discovery of biomarkers (Calderón-Celis et al. 2018). Besides, the quantification of the core protein structures, phosphorylation, or acetylation posttranslational modification studies involve extra enrichment step using immobilized-metal affinity chromatography (IMAC) among other techniques (Hashimoto et al. 2019).

18.5.3.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

The application of NMR in proteomics has enabled the study of protein conformational changes, which is naturally dynamic including their 3D structures (Kwan et al. 2011). Also, while mass spectroscopy (MS) techniques are comparatively more sensitive, NMR spectroscopy is more direct and does not involve sample preparation steps (Emwas 2015). NMR techniques have found relevance in the studies of protein–protein interactions (interactomics) and also gene and product interactions to understand the global influence or impact of such interaction on the microbial cell's homeostasis. In-cell NMR spectroscopy enables high atomic level resolution of protein molecules, which requires in vitro stabilization of the target sample through X-ray crystallographic or other similar techniques and is widely applicable

for detecting the protein-protein structural and functional interactions (Breindel et al. 2019). The high-pressure NMR spectroscopy facilitates systematic study of high-energy and stably folded protein structural conformations (Akasaka 2015). Furthermore, in metabolomics, high-resolution liquid-state NMR was found very applicable in the detection of water-soluble and water-insoluble metabolites in liquid and solid food samples (Ralli et al. 2018).

18.5.3.3 Protein Array

Protein arrays are highly sensitive and specific high-throughput techniques for multiplex real-time functional and analytical studies of protein interactions (Hinchliffe et al. 2016). Latest innovations in protein arrays include next-generation protein arrays, Quantum Dot indirect immunofluorescence, lectin arrays, and ArrayCAM protein array imager (Srivastava 2016). Nucleic acid programmable protein arrays (NPPA) is a related high-throughput, cost-effective platform that relies on query and target proteins synthesized in vitro, for the study of protein-protein interaction, without requiring prior protein purification (Tang et al. 2017). Infrared imaging could be used for analyzing high-density protein microarrays data of the protein secondary structure, posttranslational modifications, and can provide an alternative tool to the regular fluorescence and enzyme probes (De Meutter et al. 2017).

18.6 Metabolomics

Metabolomes are the end products of cellular processes, which represent a complete set of metabolites in a biological cell, tissue, organ, or organism (Jordan et al. 2009). Metabolomics reveal a direct "functional readout of the physiological state" of an organism. It measures quantitatively the metabolic response of a living organism to genetic modifications and pathophysiological stimuli.

Environmental metabolomics study may be performed on prokaryotic and eukaryotic microorganism with the *Saccharomyces cerevisiae* and *Escherichia coli* used as model organisms for eukaryotic and prokaryotic microbes, respectively (Kim et al. 2015; Reed et al. 2017). Environmental metabolomic project maybe in situ (field-based) or artificially subjected to environmental-controlled stressors in a laboratory setting (laboratory-based) (Campillo et al. 2019; Davis et al. 2016). To ensure proper replication of experiment for statistical analysis, both filed and laboratory-based metabolomic studies should have a meticulous number of samples planned and type of control/reference site exposures and environmental stressor exposures as there are natural variations in biological samples from each treatment group (Simmons et al. 2015). In laboratory-based metabolomic study, environmental stressors such as temperature, light, and diet are optimized and the microbial

populations are set under strict control in order to reduce perturbation of the metabolome (Kovacevic and Simpson 2020).

Following sample collection, metabolites are extracted by the addition of internal standards and derivatization. It is important to note that the sample selected must be compatible with the proposed analytical method. Hence, applying distinct methods to extract analytes from metabolomic sample is generally recommended (Mushtag et al. 2013). Metabolomic sample is made up of analytes with high degree of complex mixtures but when separated makes the complex mixture simpler before other downstream detection processes.

Different extraction methods include liquid–liquid extraction, supercritical phase extraction, solid-phase extraction (SPE), microwave-assisted extraction or solid-phase micro-extraction (SPME) method. The extraction method to be use is dependent on the proposed type of analytical procedure. However, in order to extract a wide range of analytes or compounds, solid phases or solvents that have the ability to extract with broad spectrum of compounds must be chosen in order to cover a wide range of compounds. Non-targeted metabolomics analysis is nonbiased because with the aid of analytical instrument (like the NMR spectroscopy or high-resolution MS) and metabolomics databases, one can comfortably identify many metabolites (Bingol 2018). On the other hand, if the extraction is targeting a specific group of compounds, then the conditions of extraction must be selectively tailored to favor the extraction of the targeted group. Hence, a targeted metabolomic analysis identifies a preplanned set of metabolites that are chosen from the intended biological sample to be analyzed or from software databases of metabolite libraries (Bingol 2018). A targeted metabolomic analysis usually employs the MS approach as the most preferred method (Emwas 2015; Mullard et al. 2015).

The metabolites can be quantified using liquid chromatography or gas chromatography in combination with MS and/or NMR spectroscopy prior to mass detection of analyte (Beale et al. 2018; Sasaki et al. 2018). But for metabolomic studies, the NMR and mass spectrometry remain the two best methods of analyzing metabolomic samples. Researchers have supported the combination of direct infusion mass spectrometry (DIMS) with high-resolution mass analyzers such as Orbitrap mass analyzers or Fourier transform-ion cyclotron resonance (FT-ICR) mass analyzers as a result of its fast analyses of metabolomic samples and its non-targeted method of analysis capturing broad spectrum of metabolites (Ghaste et al. 2016; Southam et al. 2017; Taylor et al. 2016). But when this technique is employed without sample separation, it gives rise to ion suppression and complexity of the mass spectra, which makes it disadvantageous (Ghaste et al. 2016).

The outcome of using these methods generates big data that can be analyzed using multivariate analysis such as principle component analysis (PCA), partial least squares or multi-tables analysis. With these methods, metabolites with different levels of expression can be identified and distinguished between samples (Lucio 2009). The PCA and the hierarchical cluster analyses are normally used to explore the characteristics, as well as comparing sample features. Following metabolite identification and quantification, databases (online) such as MetaboAnalyst 4.0 comprises of different tools for interpretation of NMR spectroscopy and MS data

and analyzing the metabolic pathways can link the metabolites to their functions (Chong et al. 2018). This may help explain the anomalies in the levels of metabolite when linked to the metabolic pathways that maybe affected (Chong et al. 2018; Kanehisa et al. 2016). In addition, the Kyoto Encyclopedia of Gene and Genomes pathway database can provide information on many metabolic pathways including the individual metabolites in each step (Kanehisa et al. 2016).

The advantages of MS-based approach include its ability to detect and quantify metabolites even at very low concentrations (femtomolar to attomolar range) coupled with high resolution ability. Despite these advantages, the MS approach is not without downsides as only quickly ionizing metabolites can be detected. Additionally, analytes or metabolites with equal eluting time (coeluting compounds) may experience ion suppression and ion enhancement, which engenders difficulties in reproducing results (Engskog et al. 2016). Contaminated ion source of the mass analyzer can alter the retention time of the metabolites during the stationary phase separation leading to instability in mass detection (Zelena et al. 2009).

Using NMR spectroscopy as an analyte detection method has the advantage of being able to identify and structurally reveal unknown metabolites, as well as the potential to differentiate isomeric compounds (Bingol and Bruschweiler 2015). There is no need to separate samples during NMR spectroscopy. However, the NMR spectroscopy has low sensitivity and can only identify metabolites that are present in larger quantities usually in concentrations >1 μM.

18.7 Multi-Omics Approach

Following the recent improvements in various omics technologies, researchers have realized that one omics approach may not completely answer a research question. Studying different omics processes provide a more holistic view and helps to better address a research question (Fig. 18.2).

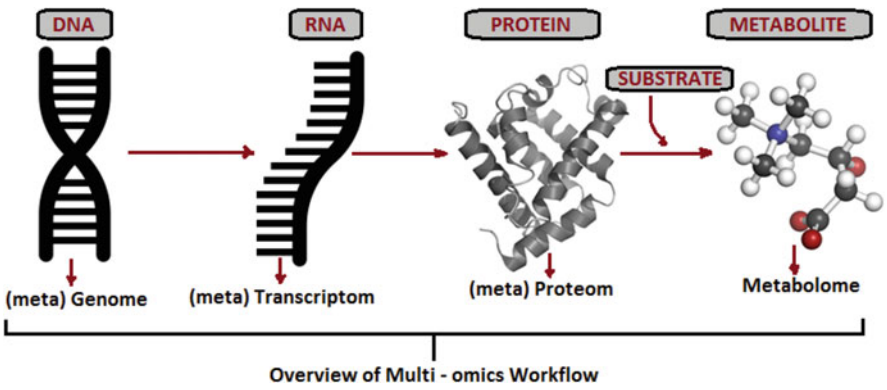

Fig. 18.2 Overview of the multi-omics approaches

A very large number of data is produced from multi-omics technology. Due to easy access to big datasets from omics researches, integrating multiple omics approach becomes very difficult because the experimental designs and analytical tools used for genomics, proteomics, transcriptomics, or metabolomics studies may not be compatible when integrating or comparing multi-omics approaches (Pinu et al. 2019). For instance, transcriptomics and proteomics approach are highly quantitative but the qualitative method used for these techniques are not suitable for metabolomics (Pinu et al. 2019).

To ensure quality and more integrated omics research, it is important to be familiar with metabolomics approach because the process of taking samples, handling, and processing them can provide useful guidelines for use in other omics technologies. Additionally, the experimental design, analysis of data, and its integration as employed in metabolomics studies are very much compactible with other omics technologies (such as genomics, transcriptomics, and proteomics research) (Strom 2019; Pinu et al. 2019).

18.7.1 Keys to Designing an Experiment for Better Integration of Multiple Omics Approach

Before starting a multi-omics study, it is important to conduct a review of published literatures available on this subject so as to have fore-knowledge and be able to answer specific hypothesis-testing questions as described by Pinu et al. (2019). In fact, it is important to note the scope and restrictions of the study, the perturbations to be included in the study and how best to control and measure them, the dose(s)/time (s) points needed, as well as the omics platforms that will yield the most value. Additionally, factoring in how best to replicate the experiment, how to analyze biological samples (individually or pooled) and know the fundamental reasons/bases for pooling or not pooling a biological sample, ultimately, time factor and availability of resources required to carry out the experiment must be put into consideration (Pinu et al. 2019).

The compatibility of omics processes must also be considered. This is because in some cases, using the same sample to generate multi-omics data may not be recommendable as treatments given to sample may interfere with subsequent downstream processes. For example, using formalin to fix paraffin-embedded tissues is suitable for genomic studies but not with transcriptomic studies because samples treated with formalin and used for transcriptomic studies still end up having the RNA degraded (Strom 2019), whereas in proteomics, it leads to the cross-linking of proteins (O'Rourke and Padula 2016) and as well interferes with mass spectrometry analysis, consequently altering proteomics and metabolomics experiments. Although recent improvements in MS technology can quantitatively profile formalin-fixed paraffin-embedded tissues (FFPE) but analyzing FFPE still remains

a concern to researchers intending to carry out a multi-omics experiment (Coscia et al. 2018; Mertins et al. 2016).

To avoid downstream consequences during analysis and interpretation of data, the requirements for collecting biological samples, processing them, as well as storage should be considered. Broadhurst and Kell (2006) outlined statistical approaches required to prevent false discoveries in omics experiments including metabolomic studies and how to compute and analyze metabolomics data (Ren et al. 2015). In exploratory data analysis, the principal component analysis (PCA) is commonly employed; however, the projection pursuit (PP) technique can even perform better than PCA but fails to yield useful information when the sample size is smaller than the number of variables. Interestingly, Hou and Wentzell (2014) produced a regularized projection pursuit (RPP) approach that addressed this problem.

Each omics approach has a required number of sample size, and it is important that the sample size is sufficient to give good statistical power to the generated data (Pinu et al. 2019). In general, studies requiring high precision or accuracy in analytical approach that can be achieved using fully quantitative techniques (such as targeted metabolomics and proteomics studies) usually need small number of samples. On the other hand, non-quantitative metabolomics studies (such as untargeted metabolomics and proteomics studies) may require larger samples (Pinu et al. 2019).

Strom (2019) also outlined the protocol for storing bio-samples in order to maintain their biological integrity prior to omics analysis. In the protocol, it is evident that following the metabolomic approach to handling bio-samples is effective for other omics technologies. For instance, to halt ongoing metabolic activities and avoid enzymatic degradation, bio-samples are quickly processed and stored at -80 °C, which also preserves the integrity of RNA and the protein component of the sample for transcriptomics and proteomics studies (Strom 2019).

18.7.2 Approaches for Analysis and Interpretation of Multi-Omics Data/Data Integration

This is one of the major challenges in multi-omics analysis as each omics technology yields large amount of data-rich sets where integrating them becomes a problem. At this point, multi-omics now becomes the bottleneck in the entire workflow, thus needing a multidisciplinary team of data scientists, microbiologists, biostatisticians, biochemists and others to critically examine the experimental design of the study, as well as to validate the quality of data generated from the omics analysis before subsequent downstream integration of results (Beale et al. 2016).

The three approaches for analyzing and integrating multi-omics data are as follows:

1. The post-data analysis integration technique

2. The integrated data analysis technique
3. The model-based integration technique

In post-data analysis integration approach, the omics datasets are independently analyzed and integrated to synthesize a universal model metabolic pathway in post-analysis activity (Beale et al. 2016). This technique has been employed in a number of studies such as assessing biological wastewater treatment systems/sewage sludge (Beale et al. 2016), evaluating the resistance of microorganisms in deep sea sediments after an oil spill (Kimes et al. 2013), and characterizing the permafrost microbial ecosystem (Hultman et al. 2015). In integrated data approach, specialized data integration tools are used to merge the outputs of different omics datasets before analysis and interpretation (Kuo et al. 2013). It does not depend on human interpretation, but the identical features of each omics data are statistically derived. The model-based approach is mostly employed in a well-defined system. This implies that one need to be fully informed of the system in question so as to be able to compare new results from the experiment to already established modeled predictions. One can get more information from preexisting data from genomics, transcriptomics, and/or metabolomics study on the system in question. Although this method is said to be unachievable, but researchers have recorded compelling achievements using the model-based approach. For instance, Tian et al. (2013) identified major glycogen regulators in *Escherichia coli* using simulations from hybrid functional Petri net model. Also, this approach has been employed by Noecker et al. 2016 to merge taxonomic and metabolomic data to determine the effects of the ecology of microbes on its metabolic variation. Moreover, having a previously published work and metabolomic data on this subject positively contributed to the success of the research and many others (Noecker et al. 2016; Srinivasan et al. 2015). This also stresses the importance of metagenomics studies in integrating multi-omics studies. For the systems modeling approach to be feasible, one needs a comprehensive and correct quantitative reference data about the genome of the organism of interest, the transcriptome, the metabolome, or the proteome. Consequently, only well-defined systems (such as known model organisms) or one with already made quantitative multi-omics data that are accurate can be relevant for systems modeling technique (Pinu et al. 2019).

There are a number of databases and software used in integration of multi-omics datasets irrespective of the data integration approach employed. Some are species-specific, whereas others are multispecies data base. Although some software domain are context-specific (example for medical and clinical omics use or specific plant species or model organisms), there are also general multi-species databases that include the yeast metabolome data (Ramirez-Gaona et al. 2017), which is a metabolite database that can be employed in metabolomics studies and the *E. coli* metabolome database (Sajed et al. 2016), which is an annotated metabolomics and metabolite pathway database. Additionally, there are multi-species collection of resources for genes and proteins (e.g., GenBank, the biorepository housing nucleotide sequences and the protein transitions of many organisms). Since it is an open-access proteomics database, it is publicly available for easy access in proteomics

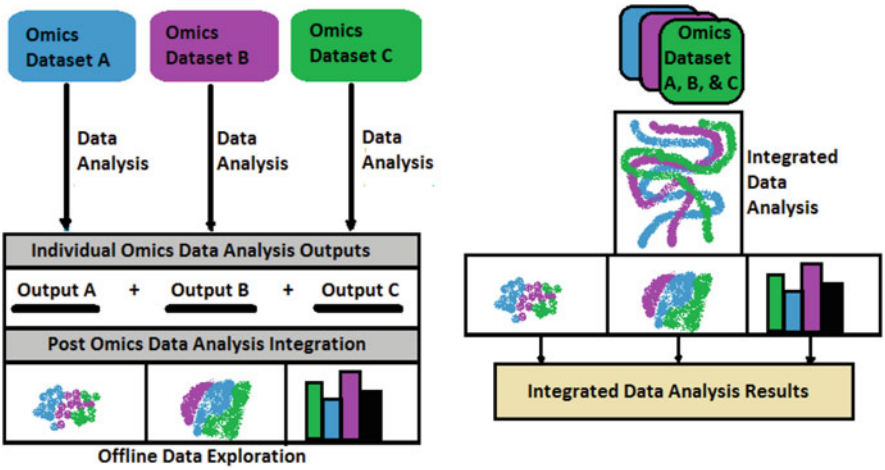

Fig. 18.3 Pictorial representation of data integration approaches

studies (Benson et al. 2013). Further details about databases and software used for the integration of multi-omics data can be found in the paper by Pinu et al. (2019) (Fig. 18.3).

18.7.2.1 Pitfalls in Multi-Omics Integration and Future Perspectives

Multi-omics integration is very difficult/nearly not feasible when the available data are from multiple sources and qualitative in nature. Majority of the omics datasets are very noisy and greatly vary. This makes reproducibility and comparison of data very difficult (Dihazi et al. 2018; Perez-Riverol et al. 2017). Some omics approach can produce data that are highly reproducible, accurate, and qualitative in nature (such as DNA sequence data), whereas most of the data from metabolomics studies are highly qualitative in nature with poor reproducibility (Schloss 2018; Tabb et al. 2010). The nature of the omics data is as a result of the measurement approach employed during the study. For instance, in proteomic studies, not using a well-defined peptide reference standards or standard peptides that are isotopically labeled to identify and quantify proteins will result in having a qualitative data. Similarly, most researchers conducting microarray or transcriptome studies do not use generally acceptable reference materials with well-defined number of transcripts but use qualitative microarray technologies which yield qualitative data at the end of the omics analysis. Likewise, in metagenomics studies where researchers do not always identify microorganisms using the standardized 16S-RNA or standard Operational Taxonomic Unit (OUT) definitions. Additionally, more than 80% of metagenomics data published are qualitative in nature with very few numbers of researchers employing a well-defined metabolite reference standard or multiple reaction monitoring or isotopically labeled metabolite standards. Consequently, comparing results

from one metabolomic or proteomics approach to another varies significantly (Schloss 2018; Tabb et al. 2010). Although, using reference standards with standardized omics approach can help checkmate the problems associated with omics techniques; however, inter-laboratory differences in storing samples, extracting, and handling protocols can still pose a problem (Pinu et al. 2019). "Precisely measured, quantitative data that is calibrated to standard reference materials, checked against authentic standards, assessed with quality controls and measured with universal standard operating protocols (SOPs) is what is needed for robust, reproducible multi-omics integration" (Pinu et al. 2019). Such data are not impossible to obtain as several omics' studies have successfully generated high-quality quantitative data including transcriptomics (Wilhelm and Landry 2009), as well as metagenomics research (Nayfach and Pollard 2016). With availability of quantitative data, multi-omics integration becomes feasible and ring trails (i.e., intra- and inter-laboratory comparisons can be done).

One may find it difficult to interpret signals or may even misinterpret it during molecular analysis if no meta-data are available (Pinu et al. 2019). In the absence of meta-data, the researcher can collect a very large number of datasets (bearing in mind the cost of collecting and processing large datasets) in order to reduce sources of variation (Zeevi et al. 2015).

Another key factor affecting multi-omics integration is limited knowledge or information of multi-omics integrating tools and software. Not that the software are not available and accessible as there are numerous tools of high-quality designed for multi-omics integration as listed by Pinu et al. (2019), many researchers may not be aware of such tools and their functionality. Some omics researchers may be aware of some other tools but may decide to stick to what they are familiar with probably due to user-friendliness. Moreover, some can pose technical difficulties either during installation or as a result of system incompatibility or non-user-friendliness. Pinu et al. (2019) suggested a central repository that can catalog these software tools and their functionality for easy access.

The situation where a database requires different input formats (e.g., non-standard format) and gives an output format that is incompatible with other software/programs (known as lack of interoperability), consequently requiring the researcher to convert one program output to suit another program input for analysis, is another major setback in multi-omics integration. To potentially address this problem, a findable, accessible, interoperable, and re-usable (FAIR) data standard for bioinformatics software is recommended (Wilkinson et al. 2016).

Other limitations of multi-omics integration are insufficient open access biological pathway for better integration of omics data. This is because some biological pathways (e.g. KEGG, MetaCyc and Reactome) (Kanehisa 2002; Caspi et al. 2010; Fabregat et al. 2018) were designed and developed even before the advent of some omics technologies such as the metabolomics and proteomics and as such not comprehensive enough to incorporate novel information associated with the new omics approach. In addition, many other important pathways may not be inclusive; examples are drug action and drug/xenobiotic metabolism pathway, protein signaling pathway, gene activation pathway, and so on (Pinu et al. 2019). Some pathway

databases may contain some of these metabolic pathways but with restricted access (closed-access) (e.g., Ingenuity Pathway Analysis system). Up-to-date user-friendly software, open-access with comprehensive machine-readable features, would enhance the integration of omics data. Further limitations to multi-omics technology have been outlined by Pinu et al. (2019).

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Part III
Agricultural Biotechnology

Chapter 19
Rhizobium Diversity Is the Key to Efficient Interplay with *Phaseolus vulgaris*. Case of Study of Southern Ecuador

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Contents

Abstract Plants acquire different combined forms of nitrogen by addition of ammonia and/or nitrate fertilizer or manure to the soil, during organic matter decomposition, by the conversion of nitrogen into different compounds, or by biological nitrogen fixation (BNF). Diverse soil bacteria collectively called rhizobia are capable to fix N_2 from the atmosphere through symbiosis with legume plants. The N_2 fixed by the legume crops represents a renewable source of nitrogen for agricultural soils, turning symbiotic nitrogen fixation (SNF) in a natural process of significant importance in world agriculture. Within the legumes carrying out this

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process, common bean (*Phaseolus vulgaris* L.) constitutes a staple, being the most important grain legume worldwide, especially for developing countries. However, *P. vulgaris* is a low nitrogen fixer compared with other legumes, mainly attributed to the presence of high but inefficient diversity of indigenous rhizobia in soil, increasing the promiscuity of bean genotypes and lack of response under field conditions. Rhizobia diversity has been extensively studied. Polyphasic taxonomy and recently the average nucleotide identity approach have allowed to discover about 117 so-called *Rhizobium* species and the real genetic differences of microsymbionts in ecosystems around the world. Nevertheless, phylogenomic, ecological, and population genetic criteria to delineate biologically meaningful species in interplay with their host are still needed. Therefore, understanding genotypic variabilities between bean genotypes and *Rhizobium* strains contributes to achieve an efficient interaction, increase plant parameters, nitrogen fixation, and yields of common bean. Here, we discuss about the most recent studies on *Rhizobium* diversity linked to *P. vulgaris* in the American continent as the center of origin/diversification and outside this continent. The abiotic and biotic factors mediate the efficiency of the interaction, with special emphasis in the promiscuity of common bean as a constraint to achieve high nitrogen fixation rates and we show a case of study at southern Ecuador where genotypic variability among local bean genotypes and native *Rhizobium* strains was assessed to seek the efficiency of symbiosis based on its diversity.

Keywords *Phaseolus vulgaris* · Diazotrophs · Interaction · Diversity · Nitrogen fixation

19.1 Introduction

Legumes are one of the most widespread plants worldwide. These provide a wide range of nutritional factors, being an important source of protein-rich food and feed, oil, fiber, minerals, and vitamins (Pandey et al. 2016). The contributions of these plants to ecosystem services, especially in agroecosystems, are also of particular relevance, such as soil fertility by contributing nitrogen (N) through atmospheric N_2 fixation (Martínez-Romero 2003); improve the structure and increase soil organic carbon status (Wobeng et al. 2020); reduce the incidence of pest and diseases in cultivations (Daryanto et al. 2020), and increase the overall productivity and economic benefits of the production systems (Preissel et al. 2015).

Because N is the most necessary element for plant development, legumes become essential crops for its incorporation into agroecosystems (Misra et al. 2020). The N_2 fixed by the legume crops represents a renewable source of N for agricultural soils, turning symbiotic nitrogen fixation (SNF) in a natural process of significant importance in world agriculture. Globally, legumes in symbiosis with soil rhizobia are reported to fix 20–22 Tg of N each year in agricultural production systems (Herridge et al. 2008). Table 19.1 shows the biological systems capable of fixing N, the rates of

Table 19.1 A comparative study of the different N_2 fixers

N_2 fixers	Type	Family	Important species	N_2 fixed (kg N ha^{-1})	Recommended crops
Rhizobium	Symbiotic	Rhizobiaceae	*R. meliloti* *R. leguminosarum* *R. ciceri* *R. etli* *R. tropici* *R. fredii*	50–100	Pulse legumes like chickpea, red gram, pea, lentil, black gram, etc., oil-seed legumes like soybean, groundnut and forage legumes like berseem, and Lucerne
Azotobacter	Free living	Azotobacteriaceae	*A. chroococcum,* *A. vinelandii,* *A. beijerinckii,* *A. insignis* *A. macrocytogenes*	20	Rice, wheat, barley, oat, sunflowers, maize, line, beetroot, tobacco, tea, coffee, and coconuts
Cyanobacteria	Symbiotic, Free living and Symbiotic-Associative		*Nostoc sp.,* *Anabaena sp.,* *Gloeotrichia sp.,* *Tolypothrix sp.,* *Aulosira sp.,* *Aphanotheca sp.*	20–30	Submerged rice, barley, oats, tomato, radish, cotton, chilli, sugarcane, maize, lettuce
Azolla	Symbiotic-Associative	Salviniaceae	*A. caroliniana,* *A. microphylla,* *A. filiculoides* *A. Mexicana*	30–50	Submerged rice with maximum temperature
Azospirillum	Symbiotic-Associative	Spirilaceae	*A. lipoferum,* *A. brasilense,* *A. amazonense,* *A. halopraeferens,* *A. brasilense,* *A. trakense*	20–40	Maize, sugarcane, sorghum, pearl millet

Source: Kumar et al. (2018), cited by Misra et al. (2020)

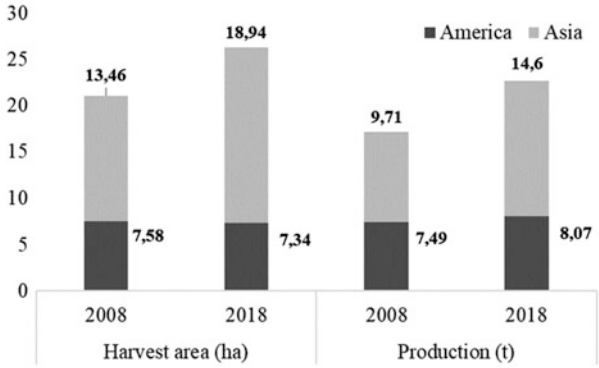

Fig. 19.1 Comparison of dry bean harvest area and production in the last decade. FAO (2020, data 2018). http://www.faostat.fao.org/

fixation, and the crops in which this important process can be carried out, highlighting legumes with an average fixation rate from 50 to 100 kg N ha^{-1}.

Among legumes, common bean (*Phaseolus vulgaris* L.) is a staple for human nutrition and one of the main sources of protein and calories in the world, mainly for developing countries (Los et al. 2018). Bean harvest areas and productions have been increasing significantly during the last decade by 22.7% and 28% respectively globally. These indicators have special relevance in Asia and America (Fig. 19.1), which are the largest producers worldwide. The total harvest area in Asia raised 28.9%, with a production of 33.5%. While in America, the total harvest area decreased in 3%, but production grew in 7.1% (FAO 2020. http://www.faostat.fao.org/, assessed on June 18, 2020). Despite its importance, the bean crop is characterized by low productivity (882.3 kg ha^{-1}) especially because of poor cropping practices, such as the inadequate supply of fertilizers and pests and diseases control (Vieira et al. 2010).

Surprisingly in America, the center of origin of this pulse and with a wide tradition of cultivation, although production has increased, the lands destined to this crop are reducing. Therefore, stimulating crop production and yields is crucial for many countries in this region. However, sustainable agricultural practices should be addressed to reduce partial or total dependence of N fertilizers and its ecological and economical costs.

As a legume, beans can carry out biological nitrogen fixation (BNF) through symbiosis with root nodule inducing soil bacteria collectively called rhizobia, allowing plants to grow in N-deficient soils (López-Guerrero et al. 2012; Sánchez et al. 2014). *Rhizobium* is a gram-negative symbiotic bacterium that colonizes the roots of leguminous plants forming root nodules, which helps in ammonia production (Mahdi et al. 2010). SNF by root nodules of legumes contributes far more to the N economy of natural communities and to the fertility of soils than the asymbiotic systems (Table 19.1) (Gunnabo et al. 2019). N-fixing root nodules can fix 100–200 times more N_2 than free-living bacteria, because of the capability of nodules to continue to fix N_2 for long periods of time (Tanveer et al. 2019).

They utilize the photosynthetic products of plants as a carbon source and, in return, fix atmospheric N_2 for their host (Misra et al. 2020). BNF becomes the

cheapest and the most environmentally correct form to provide N to plants and the most intensively studied model of beneficial plant–microbe interaction (Vieira et al. 2010). They are considered to be the most competent biofertilizer for legumes in terms of the amount of N fixed (Jehangir et al. 2017). For instance, 75% of the total N in plant was derived from SNF by faba bean; 62–94% by soybean, groundnut, pea, and lentil; 54–58% by cowpea, chickpea, and pigeonpea; and 39% by common bean (Dwivedi et al. 2015). However, in some legumes like *P. vulgaris*, poor nodulation or lack of response to inoculation in field conditions has been attributed to the (1) presence of a high but inefficient population of indigenous common bean rhizobia in soil and in seeds (Andrade et al. 2002), (2) genetic instability of selected strains (Satyanarayana et al. 2018), and (3) sensitivity of the symbiosis to environmental stresses, such as high temperatures, soil dryness, and low soil fertility (Graham and Ranalli 1997; Hungria and Vargas 2000).

Rhizobia biodiversity nodulating *P. vulgaris* is one of the most important constraints to achieve a proper interplay between bacteria and legume. This wide diversity of microsymbiont leads to the promiscuity of common bean and to reduce nodulation and N fixation performance in plants (Peix et al. 2015). Only in the American continent, a total of 11 new *Rhizobium* species have been isolated from *P. vulgaris* so far (Shamseldin and Velázquez 2020). The wide rhizobia biodiversity has been classified by polyphasic taxonomy, including 16S ribosomal RNA gene sequencing, multilocus sequence analysis (MLS), biochemical properties, and phenotypic features (Shamseldin et al. 2017). Currently, the average nucleotide identity (ANI) based on nodulation host range concerning reference strains (Ormeño-Orrillo et al. 2015) is used as an alternative approach (González et al. 2019). However, although ANI is indicative of genomic clusters, phylogenomic, ecological, and population genetic criteria to delineate biologically meaningful species are still needed (Vinuesa et al. 2018).

Although is a fact that the ability of *P. vulgaris* to be nodulated by different symbiovars linked to species primarily isolated from nodules of this legume and also by symbiovars linked to species initially isolated from nodules of other legumes allows *P. vulgaris* to establish N-fixing symbiosis in very different ecosystems around the world (Shamseldin and Velázquez 2020), a few studies about the diversity of rhizobia establishing symbiosis with *P. vulgaris* have been published. To date, only two reviews have been published in the present century (Martínez-Romero 2003; Shamseldin and Velázquez 2020). Therefore, understanding rhizobia biodiversity can trigger the efficiency of the symbiotic process. Here we discuss the genotypic variability among bean genotypes and Rhizobium species, as an important role to enhance nodulation, plant parameters, N fixation, and grain yields. We show a case study in southern Ecuador that ranges from microsymbionts molecular analysis, through the search for efficiency under controlled conditions, to determine the response under field conditions.

19.2 Understanding *Rhizobium* Diversity and Distribution to Improve Interplay with *Phaseolus vulgaris*

The family Rhizobiaceae gather the seven genera *Rhizobium*, *Neorhizobium*, *Allorhizobium*, *Agrobacterium*, *Ensifer* (syn. *Sinorhizobium*), *Shinella*, and *Ciceribacter*. However, several so-called *Rhizobium* species do not exhibit robust phylogenetic positions (Mousavi et al. 2015). The current classification of *Rhizobium* species is mostly based on phenotypic features, 16S ribosomal RNA gene sequencing, multilocus sequence analysis (MLS) of housekeeping genes, DNA: DNA hybridization (DDH), and average nucleotide identity (ANI) values (González et al. 2019). To date, this genus consists of 117 described species, of which 18 had been isolated from common bean root nodules almost worldwide (Tong et al. 2018), but particularly in the Mesoamerican and Andean centers of common bean origin/diversification (Fig. 19.2).

Common bean forms N-fixing symbioses promiscuously with bacteria belonging to different genera of alpha and beta Proteobacteria (Michiels et al. 1998; Peix et al. 2015). Within alpha-Proteobacteria, the species and symbiovars nodulating *P. vulgaris* belong to the genera *Rhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Pararhizobium* (formerly *Rhizobium*), and *Bradyrhizobium* (Mousavi et al. 2015), while from the beta-Proteobacteria can be nodulated by species from genera *Burkholderia* (currently *Paraburkholderia*) (Talbi et al. 2010; Martínez-Aguilar et al. 2013; Dall'Agnol et al. 2017) and *Cupriavidus* (da Silva et al. 2012).

19.2.1 Rhizobia Strains Identification Linked to P. vulgaris *in the American Continent*

When analyzing the biodiversity of *Rhizobium* species nodulating common bean, the American continent and specifically Center and South America play a preponderant role. A total of 11 new *Rhizobium* species have been isolated from *P. vulgaris* in this continent: *Rhizobium etli* (sv. *phaseoli*), *Rhizobium acidisoli* (sv. *phaseoli*), *Rhizobium hidalgonense* (sv. *phaseoli*), *Rhizobium esperanzae* (sv. *phaseoli*), and *Rhizobium mesoamericanum* (sv. *phaseoli*) in Mexico; *Rhizobium phaseoli* (sv. *phaseoli*) in the USA, *Rhizobium freirei* (sv. *tropici*), *Rhizobium leucaenae* (sv. *tropici*), and *Rhizobium paranaense* (sv. unnamed) in Brazil; *Rhizobium tropici* (sv. *tropici*) in Colombia, and *Rhizobium ecuadorense* (sv. *phaseoli*) in Ecuador. Interestingly, only *R. phaseoli*, *R. etli*, and *R. tropici* are considered indigenous of the American continent, just like their symbiovars: *phaseoli* and *tropici* (Shamseldin and Velázquez 2020). Other species, on the other hand, have not been reported in other nearby American countries such as *R. acidisoli, Rhizobium anhuiense, Rhizobium mesoamericanum, R. hidalgonense,* and *Rhizobium ecuadorenses*. Finally, *R. hidalgonense* and *R. ecuadorense* have been found outside the American continent, in Croatia, Southeast Europe (Rajnovic et al. 2019).

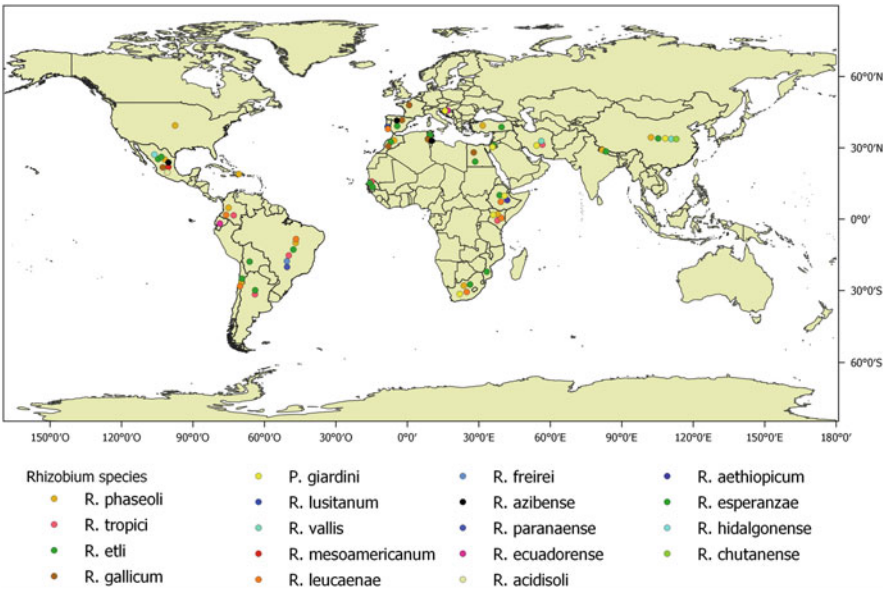

Fig. 19.2 Species distribution of *Rhizobium* originally isolated from *P. vulgaris*

Rhizobium etli and *Rhizobium phaseoli* were some of the first isolates from common beans in the continent, together with *Rhizobium tropici*. However, *R. phaseoli* was reclassified as biovar (currently symbiovar) of *R. leguminosarum* (Jordan 1982) and then of *Rhizobium etli* (formerly *R. leguminosarum* type I strains) (Eardly et al. 1992; Segovia et al. 1991). Currently, *R. phaseoli* is recognized as an independent species from *R. leguminosarum* and *R. etli* mainly because of the presence of divergent core genes (Ramírez-Bahena et al. 2008). *Rhizobium tropici*, similarly to *R. etli*, was previously classified as *Rhizobium leguminosarum* (type II) (Martínez-Romero et al. 1991). It was the first description of a rhizobial species to include sequences from 16 s rRNA gene and also the first species to carry the symbiovar *tropici*. In contrast to *R. leguminosarum* sv. *phaseoli* type II, *R. tropici* strains tolerate high levels of acidity and high temperatures in culture, and are also symbiotically more stable (Ormeño-Orrillo et al. 2012). Interest in the evolution of *R. tropici* results from its close genetic resemblance with Agrobacteria. *R. tropici* and some *Agrobacterium* strains resemble one another in morphology, growth rate, tolerance to pH, DNA-DNA hybridization, and in the 16S and 23S rRNA sequences (Martínez-Romero 1994), which indicates the possibility of discovering the linkage between symbiosis and pathogenicity (Gomes et al. 2012).

Formerly, the species *R. tropici* was designated as having two subgroups, A and B. Nevertheless, Ribeiro et al. (2013) proposed the reclassification of *R. tropici* type A strains as a novel species: *Rhizobium leucaenae* sv. *tropici*. The species *R. tropici* and *R. leucaenae* found in Colombia and Brazil were also found in soils of other American countries such as Argentina (Aguilar et al. 2001) and Chile (Baginsky

et al. 2015), supporting the possibility of co-evolution between *P. vulgaris* and its microsymbionts in the centers of host genetic diversification. Similarly, the distribution of species *R. etli* and *R. phaseoli* supports this hypothesis (Shamseldin and Velázquez 2020). In addition to the distribution of these bacteria, *R. phaseoli, R. etli, R. tropici*, and *R. leucaenae* were also found outside the American continent in Europe (García-Fraile et al. 2010; Valverde et al. 2011), Africa (Diouf et al. 2000; Mhamdi et al. 2002; Shamseldin and Werner 2005; Aserse et al. 2012; Zinga et al. 2017), and Asia (Adhikari et al. 2013; Cao et al. 2014; Chen 2019).

During the last decade, reports of new *Rhizobium* species isolated from common bean in America were linked to symbiovar *phaseoli* such as *Rhizobium acidisoli* (Román-Ponce et al. 2016), *Rhizobium hidalgonense* (Yan et al. 2017), *Rhizobium esperanzae* (Cordeiro et al. 2017) in Mexico, and *Rhizobium ecuadorenses* in Ecuador (Ribeiro et al. 2015). These last two species have been reclassified from the clade containing *R. phaseoli/R. etli/R. leguminosarum*, using mainly the approach based on nodulation host range and genomic ANI. *R. esperanzae* and *R. ecuadorense* have been reported to have higher similarity with *R. etli* on the 16S rRNA gene sequence phylogeny and phylogeny of nifH analysis (Ribeiro et al. 2015; Cordeiro et al. 2017). Similarly, *R. acidisoli* and *R. hidalgonense* have identical 16S rRNA genes to other *Rhizobium* species. For instance, the phylogeny of 16S rRNA gene sequences of *R. acidisoli* suggests it is most closely related to *Rhizobium anhuiense* (99.7% similarity) (Román-Ponce et al. 2016), and *R. hidalgonense* was found to be similar to *R. acidisoli* using 16S rRNA and nifH gene sequences (Yan et al. 2017).

19.2.2 Microsymbionts Beyond America

The first described nodulating common beans outside of America were *Rhizobium gallicum* (sv. *gallicum* and *phaseoli*), *Rhizobium giardinii* (sv. *giardinii* and *phaseoli*), and *Rhizobium lusitanum* (sv. *tropici* and *phaseoli*) in Europe. However, only *R. lusitanum*, isolate in Portugal, is considered indigenous of this continent (Valverde et al. 2011). Since then, *R. gallicum* has been found in American countries (Amarger et al. 1997; Mhamdi et al. 1999; Sessitsch et al. 1997), and *R. giardinii* has also been found in Asian and African soils (Herrera-Cervera et al. 1999; Mhamdi et al. 2002; Aserse et al. 2012; Wang et al. 2016; Rouhrazi et al. 2016). The phylogenetic analysis of these bacteria shows that *R. gallicum* has similarities with *R. etli* (98%) and recent evidence suggests that the NodC of the pSym of *R. etli* is distributed in some strains of *R. gallicum* (Verástegui-Valdés et al. 2014). On the other hand, 16 s rRNA gene sequence analysis places *R. giardinii* on a lineage independent of *Rhizobium* (Amarger et al. 1997), prompting Mousavi et al. (2015) to place *Rhizobium giardinii* in the new genus *Pararhizobium*.

In Asia, the microsymbionts of *P. vulgaris* were directly introduced from American countries to the China region through *P. vulgaris* seeds. This hypothesis is supported by the high similarities in the symbiotic genes (nodC and nifH) between

the Chinese and American *R. etli* populations (Cao et al. 2014). Indeed, two new species isolated in this continent carry the symbiovar *phaseoli*: *Rhizobium vallis* (Wang et al. 2011), reported also in Iran (Rouhrazi et al. 2016), and *Rhizobium chutanense* (Huo et al. 2019). According to 16S rRNA sequence analyses, *R. vallis* showed the most similarity with *Rhizobium lusitanum* (99.1% sequence similarity) and *Rhizobium rhizogenes* (99.0%) strains, although DNA–DNA relatedness values were very lower (Wang et al. 2011). Also using 16S rRNA analyses, *Rhizobium chutanense* was found to be most similar to *R. ecuadorense, R. fabae, R. pisi,* and *R. esperanzae* (Huo et al. 2019).

African diversity of nodulating rhizobia *of P. vulgaris* has their origins with the introduction of American and European strains (Mhamdi et al. 1999; Aserse et al. 2012; Zinga et al. 2017; Kawaka et al. 2018). In the last decade, two new species within the genus *Rhizobium* have been isolated in this continent: *Rhizobium azibense* sv. *gallicum* (Mnasri et al. 2014) and *Rhizobium aethiopicum* sv. *phaseoli* (Aserse et al. 2017), although the first specie has strains belonging to North Tunisia, Spain, and Mexico (Silva et al. 2005). *R. azibense* was previously unsigned as *Rhizobium gallicum*, based on 16S rRNA, *recA*, and *atpD* gene sequences (Mnasri et al. 2014), but phylogenetic analysis based on *recA, atpD, dnaK*, and *thrC* sequences showed that it is distinguished from a group closely related to *Rhizobium gallicum*. On the other hand, the closest phylogenetic reference (combined *recA* and *glnII* sequences) of *Rhizobium aethiopicum* were strains of *R. etli* (94% similarity) and *Rhizobium bangladeshense* (93%) (Aserse et al. 2017). Lastly, the distribution of *R. azibense* and *R. aethiopicum* is still unknown, given that these bacteria have not been reported in other countries, though recently *R. azibense* together with *R. bangladeshense* were found nodulating in legumes in Bangladesh-South Asia (Tanim et al. 2019).

19.3 The Efficiency of *Rhizobium*–Bean Interaction Mediated by Biotic and Abiotic Factors

Several factors directly influence the efficiency of symbiotic plant–*Rhizobium* interactions, particularly in field conditions. Among them, (1) compatible strain and host selection, (2) saprophytic competence, (3) root hair attachment and competition for infection, (4) growth characteristics of bacteria within infection threads as well as (5) effects of plant physiology, and (6) the abiotic environment (Terpolilli et al. 2012). The first of the factors, related to the strains type and host selection, is one of the most significant to achieve the desired effect of N_2 fixer bacteria application.

Rhizobia are soilborne bacteria with a key role in the SNF process as symbiotic partners of legumes. *Rhizobium* spp. populations reside in soils and are in constant evolution, being able to adapt to different habitats (Martínez-Romero 2003). The colonization success and symbiotic N_2 fixation depends on their attachment abilities to biotic and abiotic substrates (Wielbo et al. 2015). Wang and Chen (2004) described how *Rhizobium* colonize new environments, facing two different

problems. In the first place, *Rhizobium* needs to establish a population in the local community among groups of indigenous bacteria, and there exists a competition between space and nutrients. In the second place, is the adaptation of *bacteria* to the soil environmental factors, such as humidity, salt content, pH, temperature, and symbiotic partners, which are determinant for the establishment of rhizobia in a specific environment (Misra et al. 2020).

Bean cultivation is considerably varied by soil type, land use pattern, and genotype. It is demonstrated that root exudates act as substrates in soils and signaling molecules, which are required for establishing plant–bacteria interactions (Kour et al. 2019). The successful root colonization is an important and initial step in the interaction of beneficial bacteria with plants (Hungria and Mendes 2015). In order to acquire beneficial effects from the microbial communities, the effective colonization of the plant root area is crucial (Yadav et al. 2015).

Successful root colonization by a bacterium is the result of interactions with physical, chemical, and biological characters of the environment as well as properties of the bacterium itself. A clear understanding of the bacterial colonization process toward the plant is required (Dutta et al. 2014). Between these bacterial colonizers, some genera of rhizobia are cataloged as effective colonizers. They synthesize plant growth hormones and can make organic and inorganic phosphates soluble (Avis et al. 2008).

Many rhizobacteria like rhizobia possessing the enzyme ACC deaminase catalyze the conversion of ACC to ammonia and α-ketobutyrate, which indirectly decrease the ethylene concentration in plants under drought stress (Glick 2012). By facilitating the development of longer roots, these rhizobacteria may enhance the survival of seedlings, which help in combating the effect of stress ethylene (Zahir et al. 2009). The root elongation plants under drought stress can allow a better access to water and uptake of nutrients (Misra et al. 2020). Ethylene is also known to compromise the nodule formation and N fixation in legume (Sapre et al. 2019). *Rhizobium* with ACC deaminase activity can diminish the deleterious effect of ethylene under drought stress by increasing the nodulation and N fixation in its symbiotic legume partner (Belimov et al. 2009).

Salinity of the agriculture soil is a significant issue all over the world and it is also a determinant environmental factor for reduction of growth and yield of agricultural crops (Misra et al. 2020). The use of plant growth-promoting rhizobacteria (PGPR), as well as those described for the genus *Rhizobium*, can stimulates mechanisms of action for the amelioration of salt stress, and to reduce the application of chemical fertilizers and pesticides in the agricultural fields and improve soil health (Yadav and Saxena 2018). ACC deaminase producing *Rhizobium* strains can improve the growth and quality of mung beans under salinity stress (Ahmad et al. 2012). Bacteria that are tolerant to stress have better nodulation ability and greater ability for N fixation of legumes to grow and survive under stressed conditions. Rhizobial populations vary in their tolerance to major environmental factors. Together, the expression of all these features can improve the efficiency of the rhizobia–legume symbiosis (Naveed et al. 2015).

19.3.1 Promiscuity as a Biotic Constraint for Achieving a High Rate of N Fixation in Common Bean

Common bean is nodulated by different *Rhizobium* species and symbiovars, allowing this pulse to establish N-fixing symbiosis in very different ecosystems around the world (Shamseldin and Velázquez 2020). However, among the biotic factors restricting the *Rhizobium*–bean interplay, the microsymbionts biodiversity in the soil and in nodules is crucial. As reported previously, *P. vulgaris* is a promiscuous legume in its interaction with *Rhizobium* species and symbiovars (Michiels et al. 1998; Pérez-Ramírez et al. 1998). Indeed, *P. vulgaris* has been considered as a promiscuous host because it can be nodulated by several divergent nodC symbiovars (Peix et al. 2015). Also, in the laboratory under axenic conditions, *P. vulgaris* is nodulated by many more *Rhizobium* species than in agricultural fields. In addition, most of these species belong to the genus *Rhizobium* such as *R. calliandrae, R. grahamii, R. jaguaris, R. leguminosarum, R. mayense, R. mongolense, R. miluonense, R. multihospitium, R. rhizogenes, R. yanglingense, R. sophorae, R. sophoriradicis,* and *R. laguerreae sv. viciae* (Jiao et al. 2015; Flores-Félix et al. 2019).

Although promiscuity could be an important feature for plant breeding, in *P. vulgaris* is one of the most important constraints to achieve high rates of N fixation by this pulse (Dwivedi et al. 2015). Symbiotic interactions between common bean and its microsymbionts are not always equally effective in the N fixation (Peix et al. 2015). For example, compared with other *Rhizobium* species, the strains of *R. tropici, R. freirei*, and *R. paranaense* show higher tolerance to environmental stress and high efficiency in N_2 fixation, so are thus considered for use in commercial inoculants, especially in countries where they were discovered (Martínez-Romero et al. 1991; Zurdo-Piñeiro et al. 2004; Gomes et al. 2015; Mwenda et al. 2018; Ipsilantis et al. 2019; Elizalde-Díaz et al. 2019). Moreover, *R. etli* and *R. tropici* strains showed different efficiency on different plant genotypes, which affect the stability of the performance of inoculants (Gunnabo et al. 2019).

19.4 Seeking Efficiency of *Rhizobium* Species Based on Its Biodiversity

Since bean is a poor N fixer compared to other grain legumes (de Sá et al. 1993; Naveed et al. 2015; Yadav et al. 2020), inoculation of bean lines or genotypes with *Rhizobium* strains well suited to different agroecological regions with high capacities to fix atmospheric N is required. However, the response of the crop and inoculation may not be satisfactory in cases in which highly competitive native *Rhizobium* populations are present in the soil, restricting root colonization by the inoculant strain (Hungria and Vargas 2000; Brito et al. 2015; Hungria and Mendes 2015; da Conceição et al. 2018). Therefore, to recommend a *Rhizobium* strain with high

Table 19.2 Summary of results on the relationships among nodule number, nodule weight, % N_2 in shoot, total N_2 fixed, nitrogenase activity, root weight, shoot weight, harvest index, seed yield, and 100-seed weight in common bean

Number and type of germplasm	Trait combination	Correlation coefficient	References
50 Iranian germplasm	Grain yield, 100-seed weight, and harvest index with nodule number, N% in shoot, and total N_2 fixed	0.208[a]–0.584[a]	Reza Golparvar (2012)
	Nodule number with total N% in shoot and total N_2 fixed	0.466[a]–0.517[a]	
	Total N% in shoot and total N_2 fixed	0.671[a]	
47 Andean, Meso-American gene pool	Root and nodule weight linearly correlated with mg N fixed per plant	0.71[a]–0.74[a]	Vadez et al. (1999)
8 cultivars	Shoot weight and mg N fixed per plant	0.46[a]	Westermann and Kolar (1978)
	Acetylene reduction activity (mmol C_2H_4 per plant) and mg N fixed per plant	0.38[a]–0.54[a]	
	Nodule weight with mg N_2 fixed per plant	0.84[a]	
	Plant weight and seed yield with mg N_2 fixed per plant	0.55[a]–0.74[a]	

Source: Dwivedi et al. (2015)
[a]Weight, refers to dry weight of the sample

agronomic performance, previous several tests are necessary to confirm its competitiveness (Leite et al. 2018).

Although the competitive effect associated with rhizobia promiscuity in common bean is well known, it would be important to take this feature into account when searching effective strains in symbiosis with bean genotypes. This is a fact that makes the *Rhizobium* biodiversity in this crop should not only be approached as a negative aspect but also as an opportunity to obtain adequate genotypic variability among *Rhizobium* strain × bean genotypes.

To test the effectiveness of *Rhizobium* inoculation in common bean, several types of researches have been developed under laboratory, greenhouse, and field conditions, where the strain–-bean genotype interactions play an important role to seek the proper genotypic variability to enhance plant parameters and yields. In this regard, Dwivedi et al. (2015) reported that genotype × environment and genotype × *Rhizobium* strain interactions are a prerequisite to identifying germplasm and *Rhizobium* strains for effective symbiosis in legumes. Correlations between these factors can enhance plant parameters related to an effective symbiosis, such as nodule number, nodule weight, root and shoot weight, N_2 fixation, and yields in bean plants (Table 19.2).

The infectivity of *Rhizobium* species nodulating common bean has been assessed in different studies. Mhamdi et al. (2002) have reported nine groups of rhizobia isolated from *P. vulgaris* in Tunisia. They delineated: *Rhizobium gallicum biovar* (bv.) *gallicum, Rhizobium leguminosarum bv. phaseoli* and *bv. viciae, Rhizobium*

etli bv. phaseoli, Rhizobium giardinii bv. giardinii, and four groups related to species of the genus *Sinorhizobium, Sinorhizobium meliloti, Sinorhizobium medicae*, and *Sinorhizobium fredii*. Among the isolates assigned to *R. leguminosarum*, two-thirds were ineffective in nitrogen fixation with *P. vulgaris* and harbored a symbiotic gene typical of the biovar *viciae*. The *S. fredii*-like isolates did not nodulate soybean plants but formed numerous effective nodules on *P. vulgaris.*

In Africa, the increasing interest in the use of rhizobia as biofertilizers in smallholder agricultural farming systems has prompted the identification of a large number of tropical rhizobia strains and led to studies on their diversity. Besides in Tunisia, Koskey et al. (2018) obtained 41 *Rhizobium* isolated from the root nodules of MAC 13 and MAC 64 climbing beans in agro-ecological zones of Eastern Kenya. The analysis of molecular variance based on restriction digestion of 16S rRNA genes showed that the largest proportion of significant ($p < 0.05$) genetic variation was distributed within the rhizobia population (97.5%) than among rhizobia populations (1.5%). The high degree of morphological and genotypic diversity of rhizobia within Eastern Kenya shows that the region harbors novel rhizobia strains worth exploiting to obtain strains efficient in biological N fixation with *P. vulgaris*.

Near this continent, in Spain, Mulas et al. (2011) analyzed the rhizobia present in nodules of the variety "Riñón," in order to select native rhizobial strains to be used as biofertilizers. The analysis of *rrs* and housekeeping genes of the strains showed that they belong to two phylogenetic groups within *Rhizobium leguminosarum*. Strains LCS0306 from group I and LBM1123 from group II were the best N fixers among all strains isolated and were selected for field experiments. The field research showed that the biofertilization of common bean with native and selected rhizobial strains can completely replace the fertilization with chemical N fertilizers.

Approaching the American continent, Díaz-Alcántara et al. (2014) analyzed phylogenetic relationships with rhizobia isolated from the American and European countries linked by trade routes since the discovery of America. In this study, effective rhizobial strains nodulating common bean were isolated in the Dominican Republic. A total of 25 isolates were obtained from nodules, concluding that the strains of *R. phaseoli* sv. phaseoli, as well as those from *R. etli* sv. *phaseoli*, are indigenous to mainland America from where they were introduced along with *P. vulgaris* seeds. The results of this study showed that the analysis of *P. vulgaris* endosymbionts present in the islands located between America and Spain is important for biogeographical studies of these rhizobia, as well as for increasing the knowledge of the coevolution of *Rhizobium–Phaseolus vulgaris* symbiosis.

In Mesoamerican and Andean region, the center of origin and diversification of *P. vulgaris*, various studies based on microsymbiont diversity have been conducted (Segovia et al. 1991; Souza et al. 1994; Hungria and Vargas 2000; Martínez-Romero 2003; Aguilar et al. 2004; López-López et al. 2010; Santos et al. 2011; Servín-Garcidueñas et al. 2012; Ribeiro et al. 2013; Verástegui-Valdés et al. 2014; Torres-Gutiérrez et al. 2017; Tong et al. 2018; Ramírez-Puebla et al. 2019). In Ecuador, an Andean region from which common bean originates (Rodiño et al. 2010), few rhizobia identification studies have been carried out, despite potentially being an important source of rhizobial diversity, which is a key determinant of common bean

productivity (Baginsky et al. 2015). Ribeiro et al. (2015) determined the taxonomic affiliations of isolated strains from Ecuadorian soil (*Rhizobium ecuadorense*) previously reported by Bernal and Graham (2001), showing that all the Ecuadorian isolates corresponded to three novel lineages from the *Rhizobium etli* group that fall into the *R. phaseoli/R. etli/R. leguminosarum* clade. One of these lineages, with representatives isolated mostly from Ecuador, seems to be a dominant lineage associated with beans from that northern and central region (Ribeiro et al. 2013).

The few previous studies from the Ecuadorian Andean region have focused only on determining the phylogenetic potential of symbionts (Bernal and Graham 2001; Ribeiro et al. 2013). However, the effect of rhizobia on plant phenotypic parameters and the variability among isolated strains are rarely studied under laboratory, greenhouse, or field conditions. Therefore, it is important to assess the phenotypic parameters of isolated strains, such as nodule formation and plant biomass production. The determination of these parameters is not only necessary to elucidate the capability of isolated strains to grow under different environmental conditions but also to understand how bacterial inoculation enhances plant growth and yields (Torres-Gutiérrez et al. 2017).

19.4.1 Genotypic Variability Among Local Bean Genotypes and Native Rhizobium *Strains. Case of Study of Southern Ecuador*

19.4.1.1 *Rhizobium* Biodiversity at Southern Ecuador

Serial experiments were performed to unravel *Rhizobium* biodiversity and its genotypic variability with local bean genotypes. Here we describe the first study published by Torres-Gutiérrez et al. (2017), in which they show the genetic diversity of native *Rhizobium* strains from the southern region of Ecuador.

For *Rhizobium* isolation, firstly, sampling was performed in nine municipalities of Loja province in the southern region of Ecuador. Global positioning system data were recorded at each collection point and altitudinal levels (meters over sea level) were determined (Table 19.3).

In each of the sampling areas, roots nodules of *P. vulgaris* were taken randomly and stored in moisturized Ziploc® bags to prevent drying. The isolation methodology proposed by Sánchez et al. (2014) was used with minimal modification. Briefly, individual nodules were dissected from the roots using a flame sterilized scalpel and tweezers and were washed thoroughly in distilled water using a sieve to remove all traces of soil. Subsequently, nodules were transferred to a sterile Petri dish and surface disinfected by immersion in 10 mL of 3% NaClO for 3 min, followed by immersion in 0.1% $HgCl_2$ for 2–5 min. Finally, the nodules were washed profusely with sterile distilled water. For bacteria molecular identification, isolated colonies were grown overnight in Yeast Extract-Mannitol (YM) medium at 30 °C with shaking at 250 rpm in a shaker incubator (Techine TS1500, USA). DNA extraction

Table 19.3 Georeferentiation of sampling sites at southern Ecuador

Municipality	Samples	Georeferentiation			Soil type	Altitudinal levels[b]
		S	W	Height[a] (m.o.s.l)		
Pindal	4	04°07′06″	80° 06′32″	800	Inceptisols	1
Paltas	3	04°02′46″	79°46′78″	940	Entisols	
Catamayo	4	04°05′99″	79°18′29″	1078	Inceptisols	2
Calvas	2	04°26′56″	79°35′52″	1193	Inceptisols	
Sosoranga	3	04°19′50‴	79°47´ 35″	1549	Entisols	
Gonzanamá	3	04°07′87″	79°25′50″	1680	Inceptisols	
Loja	8	03°56′86″	79°12′48″	2120	Entisols	3
Celica	2	04°05′95″	79°57′78″	2029	Inceptisols	
Saraguro	5	03°36′56″	79°15′26″	2691	Inceptisols	

[a]Average height of the sampling sites in meters over sea level (m.o.s.l.)
[b]Altitudinal level 1: from 800 to 940 m.o.s.l., altitudinal level 2: from 1078 to 1680 m.o.s.l., altitudinal level 3: from 2120 to 2691 m.o.s.l

was performed using a ChargeSwitch® gDNA Mini Bacteria Kit (InvitrogenTM, USA), according to the manufacturer's instructions. DNA quality was checked by&spi2;quantification in NanoDrop (NanoDrop 2000, Thermo Scientific, USA) and electrophoresis in a 1% agarose gel (1 g agarose in 100 mL TBE buffer). The&spi2;16S rRNA gene of isolates was amplified with the conserved primers: ARI C/T (5'CTGGCTCAGGAC/TGAACGCTG3') and pH (5'AAGGAGGTGA-TCCAGCCGCA3') (Clermont et al. 2009), which amplify almost the full length of the 16S rRNA gene (1500 bp). The PCR-amplified 16S rDNA fragments were purified using a PureLink® PCR Purification Kit (InvitrogenTM, USA) and the sequence analysis was performed using an Applied Biosystems 3100 DNA Sequencer. Sequence assembly was performed with BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). The closest related sequences were identified using the FASTA program and compared with those available in the GenBank database.

The 16S rDNA assay (Table 19.4) demonstrated the presence of nine species of *Rhizobium* among the 20 isolated strains, including *Rhizobium tropici, R. etli, R. etli* bv. *mimosae*, *R. leguminosarum*, *R. leguminosarum* bv. *viciae, R. mesoamericanum, R. undicola* and two unclassified species, *Rhizobium* sp. and uncultured *Rhizobium* sp. These results show the wide *Rhizobium* diversity at southern Ecuador, as well as the prevalence of *R. tropici*, which nodulates *P. vulgaris* in this region.

Table 19.4 Genetic diversity of *Rhizobium* strains at southern Ecuador

Strain code	Sampled municipality	Altitudinal levels[a]	Accession no.	Closets to FASTA hit	Sequence identity (%)
NAR1	Paltas	1	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
PIN1	Pindal	1	JQ797311.1	*Rhizobium etli* str. ECRI 15	100
PIN3	Pindal	1	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
TAB1	Calvas	2	EF555479.1	*Rhizobium* sp. rf033	98
COL1	Calvas	2	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
COL6	Calvas	2	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
TAM1	Catamayo	2	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
CB1	Catamayo	2	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
NAM1	Gonzanamá	2	JX122134.1	*Rhizobium mesoamericanum* str.	100
SOS1	Sosoranga	2	KM672515.1	*Rhizobium undicola* str. MR68	100
SOS4	Sosoranga	2	KC172298.1	*Rhizobium uncultured*. Clone DM6-85	100
LP1	Célica	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
VP1	Loja	3	CP006986.1	*Rhizobium etli* bv. *mimosae* str. IE4771	99
VP2	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
RC2	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
TUR1	Loja	3	KP027679.1	*Rhizobium leguminosarum* str. KSM-004	100
RAI1	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
Q2	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
Z1	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
Z3	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100

[a]Altitudinal level 1: from 800 to 940 m.o.s.l., altitudinal level 2: from 1078 to 1680 m.o.s.l., altitudinal level 3: from 2120 to 2691 m.o.s.l

19.4.1.2 Authentication of *Rhizobium* Isolates and N Fixation under Greenhouse Assay

All isolates were assessed to determine their capability to promote nodule number, biomass production, and N content of *P. vulgaris* in pot experiments under greenhouse conditions. For the experiment, a complete randomized experimental design with ten replicates was performed. The inoculation with the wild-type strain *Rhizobium etli* CNPAF512 (obtained from the culture collection of the Centre for Microorganism and Plant Genetic of Catholic University of Leuven, Belgium) and a treatment without inoculation were the controls.

Certified *P. vulgaris* cv. Mantequilla seeds were obtained at Loja Market. Seeds were surface disinfected as described previously by Vlassak et al. (1998) and pre-germinated for 2 days on moist filter paper in the dark at 28 °C. One pre-germinated seedling was planted per pot. Bean plants were harvested at 21 days after inoculation (DAI) to determine nodule number, nodule dry weight (mg), and total nitrogen content in the shoots (% total N). The best response strains were grouped by Ward's method, using Euclidean distance, taking into account the results of nodulation, dry weight of nodules, and N fixation.

Figure 19.3 (panel A) shows that at 21 days after inoculation, all isolates were able to nodulate the host plant. The nodule number and nodule biomass were variable among the isolates, but most of them yielded significantly higher values than the controls, primarily for nodule number, highlighting the potential of native strains to nodulate a local beans cultivar. The inoculation with *R. leguminosarum* bv. *viciae* COL6, *R. etli* bv. *mimosae* VP1, and *R. mesoamericanum* NAM1 was statistically significant among the treatments. However, no significant difference was observed among them and with *R. tropici* (NAR1), *R. undicola* (SOS1), *R. tropici* (LP1), *R. tropici* (VP2), *R. leguminosarum* bv. *viciae* (Q2), and *R. leguminosarum* bv. *viciae* (Z1). These nine bacterial isolates belonged to the group with the best nodule formation in bean plants (group A) forming an average of 75 nodules per plant. Following this group, a total of ten isolates (*R. etli* PIN1, *R. leguminosarum* PIN3, *R.* sp. TAB1, *R. leguminosarum* bv. *viciae* COL1, *R. tropici* TAM1, *R. tropici* CB1, *R.* uncultured SOS4, *R. tropici* RC2, *R. tropici* RAI1, and *R. leguminosarum* bv. *viciae* Z3) were clustered in the second most important group (B) for nodulation, and only one isolate (*R. leguminosarum* TUR1) and wild type strain CNPAF512 were included in group C, having the lowest nodules number. Although no significant differences were shown among several native strains and *R. etli* wild type strain CNPAF512, the low responses by the reference strain could be associated with the fact of adaptation to a new environment. Thies et al. (1991) state that native rhizobia are generally more competitive than introduced strains. Finally, as expected, the control treatment was unable to nodulate the host plant.

In panel B, the nodular biomass showed significant differences among the isolates. A group A, with a total of seven isolates (R. tropici RC2, *Rhizobium mesoamericanum* NAM1, *R. tropici* LP1, *R. leguminosarum* bv. *viciae* Z1, R. sp. TAB1, *R. leguminosarum* PIN3, and *R. leguminosarum* bv. *viciae* COL6) exhibited

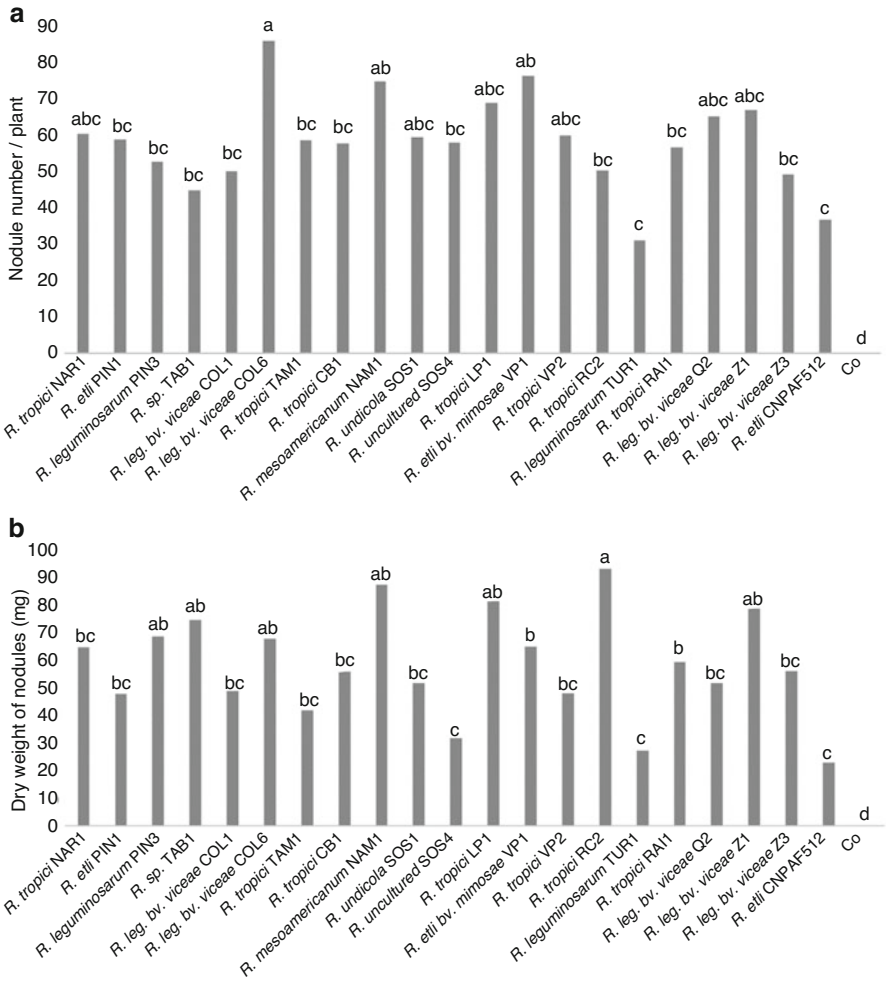

Fig. 19.3 Nodule formation (**a**) and nodular biomass (**b**) by strain. Plants were inoculated with the isolates showed in each column. Co: plant no inoculated. Letters on bars indicate significant differences between the mean ranges according to the Kruskal–Wallis/Mann–Whitney test for $p \leq 0.05$ $n = 10$

the most significant results, with nodule dry weight values ranging from 70 to 92 mg. The largest group (group B) of strains (11 isolates) had moderate nodule dry weights, with values from 38 to 65 mg, and the low values (group C) belonged to *Rhizobium* uncultured SOS4, *R. leguminosarum* TUR 1, and the wild-type strain CNPAF512. The results for these two strains were consistent with the nodule number. The results obtained with wild-type strain, as well as for TUR1, could be related to their erratic interaction with *P. vulgaris* cv. Mantequilla. *R. etli* CNPAF512 was isolated from Mesoamerican soils and has been shown to be effective with the common bean

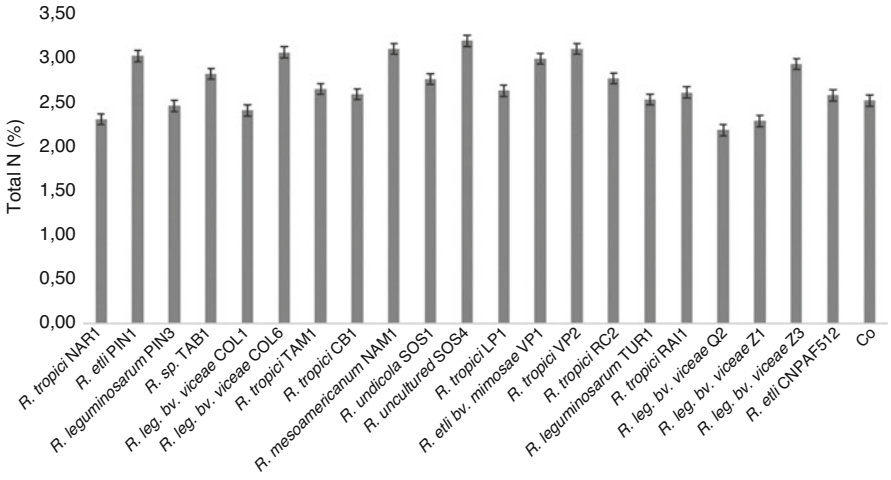

Fig. 19.4 Nitrogen fixation by *Phaseolus vulgaris* cv. Mantequilla inoculated with *Rhizobium* strain and non-inoculated treatments. Values are average of total nitrogen expressed in percentage from ten replicates. For the analysis, shoot from each treatment was dried and processed by Kjeldahl method

(Remans et al. 2007), but it may not be as efficient at promoting nodule formation and nodule biomass for Andean cultivars. Several studies have focused on the lack of response of wild-type strains in common beans. Mostasso et al. (2002) and Hungria et al. (2003) demonstrated the low activity of the reference strain as CIAT899 when used to inoculate *P. vulgaris*.

Despite the high diversity of morphological, genetic, and nodulation parameters, N fixation was rather homogenous for most of the treatments assessed (Fig. 19.4). The native strains, including uncultured *Rhizobium* sp. SOS4, *R. tropici* VP2, *R. leguminosarum* bv. *viciae* Z3, R. *leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1, and *R. etli* PIN1, yielded the highest shoot N content. Results obtained using COL6 and NAM1 were expected for N fixation, due to the performance observed for the nodulation parameters. Voisin et al. (2003) and Yadegari and Rahmani (2010) reported that with the inoculation of efficient *Rhizobium* strains, the amount of N_2 symbiotically fixed by common beans is increased and is strongly correlated with the number of nodules and nodular biomass.

These results allowed the selection of the best strains according to their response to nodulation, nodular biomass, and N fixation (Fig. 19.5) to assess their efficiency in further researches under field conditions.

The results shown by native strains from southern Ecuador are in agreement with other previous studies (Slattery et al. 2004; Figueiredo et al. 2008; Peoples et al. 2009; Mulas et al. 2011; Karaca and Uyanöz 2012; Kawaka et al. 2014), which have been conducted with the purpose to select efficient *Rhizobium* strains to enhance nodulation, N fixation, and growth of common bean genotypes.

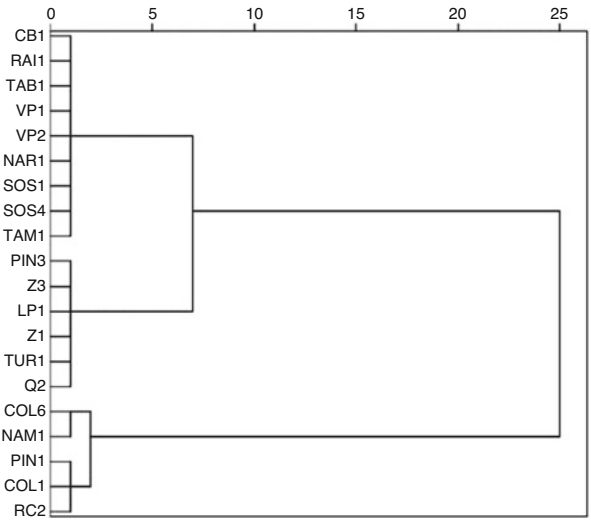

Fig. 19.5 Dendrogram constructed using Ward's method for the selection of *Rhizobium* strains

19.5 Conclusions and Perspectives

The rhizobia diversity plays an important role in establishing an effective and efficient symbiotic relationship with *Phaseolus vulgaris*. Through this exhaustive review, the biogeographic and genetic distribution of microsymbionts capable to nodulate common bean globally has been shown. Although there is a net of *Rhizobium* genetic distribution worldwide, we show that most of these strains belong to symbiovars indigenous from America, such as *phaseoli* and *tropici*, where this legume has its origin center. It is evident that biotic factors like the promiscuity can effectively limit beneficial interactions, being a constraint for stimulation of nodule formation and nitrogen fixation on *P. vulgaris*. Compatible interactions, as shown in the case of study in southern Ecuador, help to understand the genotypic variability between *Rhizobium* strains and local bean genotypes. Despite the amount of scientific information regarding the diversity of diazotrophic bacteria, more studies are needed focusing on the application of effective interplay under different agroclimatic conditions. The goal should be directed to achieve plant growth stimulation and to increase yields under field conditions with biofertilizers application and thus the reduction and/or elimination of nitrogenous fertilizers to carry out sustainable agricultural processes.

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Chapter 20
Algae as Environmental Biotechnological Tool for Monitoring Health of Aquatic Ecosystem

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Contents

Abstract Increased pressure on the freshwater ecosystems caused by human activities is a communal concern. Assessing the status of the ecosystem is necessary for the existence of aquatic ecosystems. Inspecting fluctuations in algal communities provides an understanding of the changes occurring in the system. Information of algal population dynamics can help to construct operative management strategies for aquatic ecosystems. Commonly used biomonitoring approaches include presence/absence of algal groups, diversity indices, multimetric approaches and use of phytoplankton functional groups. Among these techniques, multimetric approaches

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and functional groups are most frequently used to estimate the health of aquatic ecosystem.

Keywords Algae · Assemblages · Functional groups · Biomonitoring · Freshwater

20.1 Introduction

Freshwater comprises only about 2.8% of the total water available on the earth in which 0.61% is groundwater, 0.009% in the form of freshwater lakes and 0.0001% as rivers; is water available for human usage? Overpopulation, development of industries and urban expansion have increased the demand for water, causing increased risk to freshwater ecosystems. Implementations of comprehensive management strategies are required for sustainable development of these vulnerable aquatic ecosystems.

Algae occupy almost all aquatic and moist terrestrial habitats even they are living on the surface of plants and animals (Hoffmann 1989; Round et al. 1990). The lifecycle of algal communities in the aquatic environment is significantly varied with some occurring periodically, acting for a short period and then fading.

Sunshine affects certain algae in such a way that they multiply rapidly in a few days or within a short span of time resulting in a bloom that spreads over a large part of the water body. As a result of this, the supplies of the chemicals dwindle and the huge population gradually ceases to make provisions for another group of algae.

The quantity of nutrition available within the water will decide the growth patterns of plant life. Therefore, the nutritional requirements of algae differ markedly from one group to another and depending on the availability of the physical and chemical factors the different groups of algae arise.

As algae serve as primary producers of aquatic ecosystem, hence they have desirable characters to be considered as biological indicators of the ecosystem. The health of aquatic ecosystem can be assessed using algae, as they act as primary producers of aquatic food web and they are sensitive to enormous numbers of pollutants which possibly are the reasons for deterioration of the ecosystem. The phytoplanktons are highly sensitive; they respond rapidly to minute changes in the physico-chemical characteristics of the ecosystem and cause notable change in the entire food web of aquatic ecosystems.

Environmental biotechnology is the study of the use of microorganisms and their byproducts to protect from environmental pollution through the process of bioremediation and biomonitoring of solid, liquid and gaseous inhospitable surroundings.

Microalgae are largely used as environmental biotechnological tool in aquatic monitoring programs and to remove toxic pollutants through bioremediation technique (Wan-Loy 2012). Generally, sensitive species are used as indicators and tolerant species for bioremediation of contaminants.

20.2 Biomonitoring

Biomonitoring is defined as the act of observing and evaluating the progressive or retrogressive changes within an ecosystem brought about by the external/internal factors (Markert et al. 1999).

The origin of the trophic system of the lake was based on the amount and type of plankton available in the aquatic ecosystem. The use of a variety of indices and quotients of phytoplankton is significantly contributed to monitor the health of the ecosystem (Rawson 1956).

The trophic state of the aquatic ecosystem is indicated by the presence or absence of certain algal groups. The colonial blue-green algae develop very dense algal blooms during summer, predominantly indicating high nutrient content available in the ecosystem. Such algae are considered as a key component of various trophic indices. Some unicellular cyanobacteria are indicative of clean water to moderately polluted water.

Favism of chlorophycean members to peculiar habitats is used to get the information on environmental condition prevailing in the aquatic ecosystem. The processes like man-made eutrophication, contamination by metals and acidification are indicated by the over dominance of filamentous Chlorophyceae (Cattaneo et al. 1995).

Ambient conditions of ecosystem decide the diversity and distribution of algal species. Algae present in waters with increased amount of inorganic compounds (*Goniochloris fallax*), humic waters (*Botrydiopsis* spp., *Tribonema minus* spp.), water with high concentration of calcium (*Mischococcus* spp., *Ophiocytium* spp.), organically rich water (*Chlorosaccus* spp.), brine water (*Vaucheria prolifera*, *Tetraedriella* spp.) and acid bogs (*Botrydiopsis* spp., *Centritractus* spp.) have been recorded by various researchers (Bellinger and Sigee 2010).

Chrysophytes are extensively used for assessing the contemporary aquatic environment due to their varied ecological preferences. These tiny plants contain siliceous cell wall, which remains as empty frustule after the death of organism for several years in the benthic layer. This property is copiously used for sediment analysis to reveal the historical environmental conditions of aquatic ecosystem.

In several parts of the world, many assessment methods have been established by using algae. Commonly, three different approaches that have been used to monitor ecosystem health are (1) The Q index (Borics et al. 2007), Trophic Index of Potamoplankton [TIP] (Mischke and Behrendt 2007), Pollution Tolerance Index [PTI] (Kentucky Department for Environmental Protection Division 2002), Trophic Diatom Index [TDI] (Kelly and Whitton 1995) and Pollution Sensitivity Index [PSI] (Kelly et al. 1995a, b) have been used to understand the community composition, preferences and tolerances of species within the community. (2) Algal diversity in the ecosystem is represented as a common indicator of ecosystem integrity. (3) Index of Biotic Integrity [IBI] developed by Karr (1981) using multimetric approach is a combination of first two methods used to assess the overall impact on the ecosystem.

The use of multimetric indices is considered ideal by several researchers for examining the risk assessment and management of ecosystem and has been developed for different provinces (Dong et al. 2015; Birk et al. 2012; Bae et al. 2010; Zalack et al. 2010; Zhu and Chang 2008).

20.2.1 Diatoms

Diatoms are dispersed in almost all aquatic habitats including marine and freshwater ecosystems. They are considered to be a good tool for environmental monitoring as they are more responsive to pollutants (Dixit et al. 1992). Diatoms commonly found in polluted waters are represented in Fig. 20.1. The accumulation of diatoms on the substratum is appropriate for assessment of water quality (Hill et al. 2000; Stewart et al. 1999; Stevenson and Pan 1999; Rott et al. 1998; Prygiel and Coste 1993; Dixit et al. 1992; Rushforth and Brock 1991; Round 1991; Rott 1991; Sabater et al. 1988; Patrick 1977). The diatoms are taxonomically diverse group, possess short life cycle and are copiously sensitive to ecological features (Stevenson 1984). Indices of diatom community structure have been used to evaluate the impact of pollution on lotic waters by many researchers (Archibald 1972; Patrick 1973; Stevenson 1984; Friedrich et al. 1992; Podani 1992; Ho and Peng 1997; Stewart et al. 1999; Hill et al. 2000). The sensitivity of benthic diatoms to pollution was used to estimate the degree of water pollution by calculating an index by Descy (1979). Diatom indices

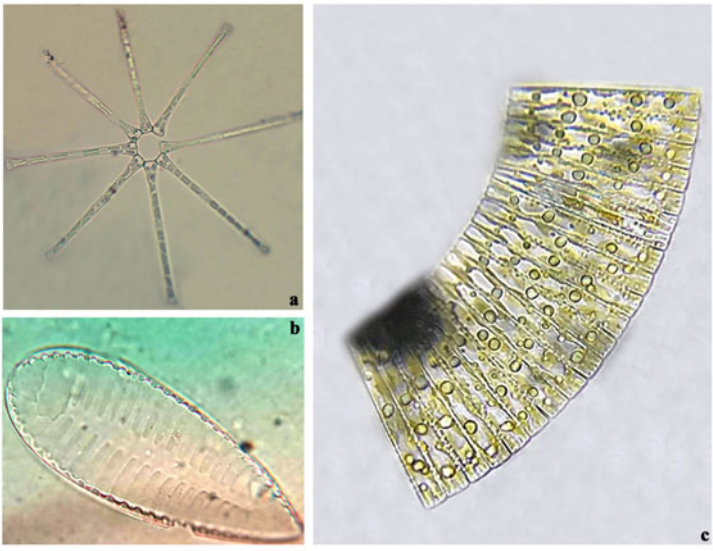

Fig. 20.1 Common genera of diatoms found in polluted aquatic habitats: (**a**) *Asterionella formosa*, (**b**) *Surirella* sp., (**c**) *Meridion* sp.

are directly linked with organic pollution, ionic strength and eutrophication (Prygiel and Coste 1993; Kelly et al. 1995a). The taxonomic diversity of diatoms is useful to estimate the change in an ecosystem and it has been recommended to consider as a standard mean in biological monitoring (McCormick and Cairns Jr 1994). For assessing the degree of pollution in lotic system, relative abundance and differences in specific sensitivity of diatom species to pollution are reliable (Maznah and Mansor 2002).

20.2.2 Desmids

Desmids are hardly or not to be expected in polluted waters and the reason for the absence of desmids in polluted waters is attributed to the fact that under nutrient-rich conditions they cannot grow as fast as other algae, so lose in competition. They are generally more common and diverse in oligotrophic lakes and ponds (Gerrath 1993). Ecologically, desmids are highly sensitive microorganisms and are used as a reliable tool in aquatic conservation management especially in those cases where macro-organisms fail (Coesel 1983, 2001). High concentration of dissolved oxygen favours the growth of desmids (Rao 1955). Scarcity of desmids in water is due to the eutrophic nature of water (Van-Oye 1934). Presence of blue-green algae has an antagonistic effect on the abundance of desmids (Seenayya 1971). Greater number of desmids make their presence when the nitrates and phosphates are in low concentrations (Pearsall 1932), whereas higher potassium has positive influences on the growth of desmids (Zafar 1967). Desmids indicate acidic nature of water; neutral-to-alkaline pH does not support distribution of desmids (Ngearnpat and Peerapornpisal 2007).

20.2.3 Chlorococcales

Members of Chlorococcales algae are known to prefer inorganic nutrients with alkaline pH (7–9.5) and moderately high temperature (Howland 1931; Gonzalves and Joshi 1946; Zafar 1967). Philipose (1967) found that Chlorococcales thrived well in water rich in NO_3 than phosphates. Chlorococcales thrive well in hard water and rich growth of Chlorococcales is a sign of pollution. Species of *Scenedesmus* and *Pediastrum* (Fig. 20.2) are well known for their adaptability to organic pollution (Busing 1998). Eutrophic ecosystems support a large number of Chlorococcales during summer season (Rott 1984).

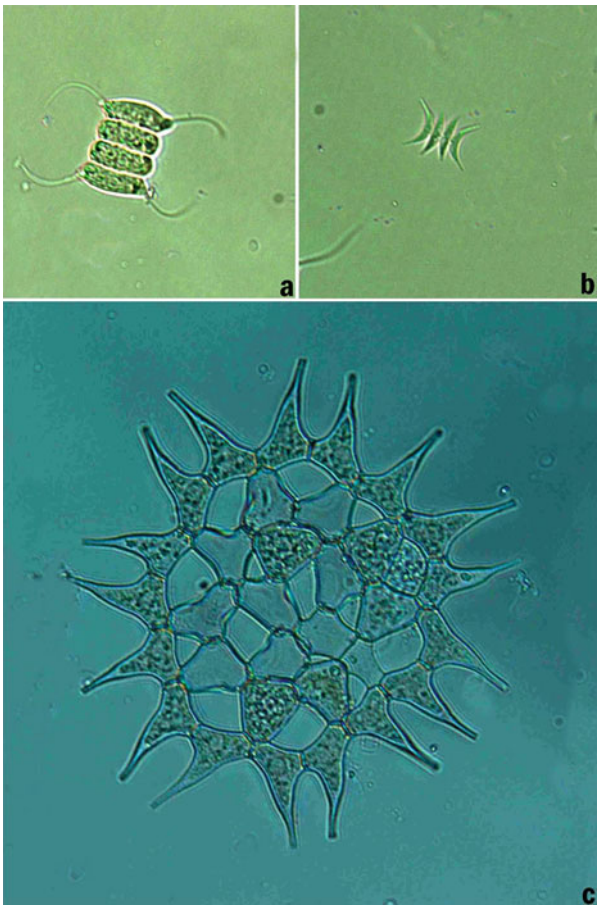

Fig. 20.2 Common members of Chlorococcales found in organically polluted waters: (**a**) *Scenedesmus quadricauda*, (**b**) *Scenedesmus dimorphus*, (**c**) *Pediastrum simplex*

20.2.4 Euglenophyceae

High concentrations of phosphates and low concentrations of oxygen support the growth of Euglenaceae (Singh 1960; Munawar 1970). The greater concentrations of inorganic elements such as carbon dioxide, phosphates, nitrates, iron, albuminoid ammonia and oxidable carbon as well as higher temperature and lesser concentrations of dissolved oxygen in water are most favourable for the growth of Euglenaceae members (Hosmani 2012). However, *Euglena* and *Phacus* are species with greater ecological amplitude to their occurrence in aquatic systems exhibiting varying levels of pollution load (Tiwari and Shukla 2007). Occasional presence of *Phacus* is a simple and direct indication of higher pollution load in the ecosystem, because species of *Euglena* and *Phacus*, in general, are considered to be dominant, tolerant genera of polluted waters.

20.2.5 Cyanophyceae

Cyanophyceae are ubiquitous organisms in water and occur as permanent blooms in some of the waters and in others as seasonal outbursts of the genera like *Microcystis* (Fig. 20.3) releasing a neurotoxin microcystin causing the death of aquatic organisms, but can also occur casually in insignificant numbers. Decrease in levels of nitrates and phosphates is responsible for making the way for increase in blue-green algal populations (Pearsall 1932; Philipose 1960). Increase in these two factors favours an abundance of forms other than Cyanophyceae that overshadow these and cause their decrease (Munawar 1970). High concentrations of calcium, neutral to low pH and low concentrations of dissolved oxygen support an abundance of certain species of Cyanophyceae (Ganapati 1940; Rao 1955). Lakes that become eutrophied primarily because of an excess of phosphate are typically characterized by a shift towards the dominance of phytoplankton by Cyanobacteria.

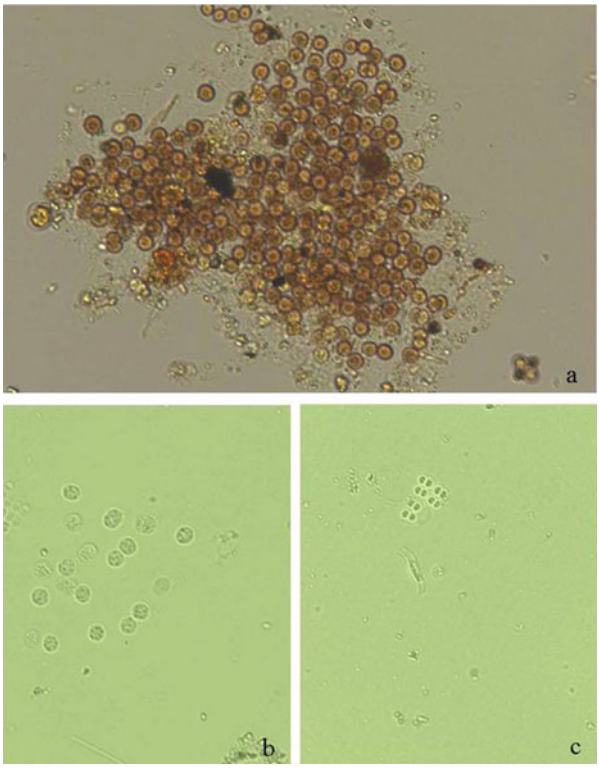

Fig. 20.3 Representatives of Cyanophyceae in eutrophic waters (**a**) *Microcystis crassa,* (**b**) *Chroococcus dispersus*, (**c**) *Merismopedia glauca*

20.2.6 Algal Assemblage

The algal communities with their component species are more appropriate indicators for habitat conditions in an ecosystem. Such kinds of biological assemblages are greatly helpful in environmental monitoring of any ecosystem which get affected by natural and anthropogenic pressure. Any disturbance either natural or man-made plays a significant role in changing the structure of algal communities in ecosystem. The relationship between algal assemblages and nutrient concentration has the strongest association with each other in comparison with other aquatic organisms in an ecosystem (Whitton and Kelly 1995). Hence algal composition, community structure with their vital activity has been used to assess the anthropogenic effect on aquatic ecosystems (McCormick and Cairns Jr 1994). Due to reliability and comfort of procedure, the use of algae in biomonitoring lentic and lotic ecosystems is increasing (Whitton et al. 1991; Whitton and Rott 1996; Prygiel et al. 1999).

In comparison with biomass and productivity descriptors, the seasonal succession of phytoplankton gives better insights and hence is used to predict the long-term environmental changes in the ecosystem (Moline and Prezelin 1996). Studies on polluted system with reference to various pollutants have thrown light on the effectiveness of plankton as bioindicators. Algae are sensitive to pollution or other events and are therefore commonly used for monitoring environmental contamination (Wu 1999). Algae are a noteworthy for assessing water quality and pollution.

Algal community analysis is important to analyse the trophic status of aquatic systems. The phytoplankton composition serves as a better indicator of trophic state and ecological condition of aquatic systems (Stoyneva 1998). Monthly chemical analysis of water and numerical analysis of phytoplankton are part of routine monitoring surveys (Busing 1998). However, limnological studies bring the account of seasonal changes in planktons and its species composition and are expressively referred to recognize, resist the eutrophication in aquatic ecosystems. Phytoplankton analysis enables us to learn the peculiarities of sensitive communities in very sensitive environments in general. Frequent disorganization of system is common in the aquatic ecosystem due to very sensitive and delicate community structure (Reynolds and Irish 1997).

Long-term study of seasonal changes in phytoplankton is directly related to variations in water parameters which are useful in establishing general ecological ideas and finding the solutions. The changes in species composition of algal communities are well responded to environmental variation (Naselli-Flores and Barone 2000). Study of aquatic communities and the factors affecting its stabilities is one of the central challenges of ecology since water bodies have always been of great importance for mankind as sources of water and food from fisheries (Kamenir et al. 2006). In Ecology the study of patterns of community structure, underlying control mechanisms and resilience when subjected to ever-growing external climatic and anthropogenic impacts is significant from both theoretical and practical point of view (Odum 1971; Begon et al. 1996). The metabolic function of aquatic system depends

on the temporal variation in the structure and function of phytoplankton community (Calijuri et al. 2002).

Phytoplankton community structure and species type indicate some of the crucial environmental developments in aquatic systems. Water pollution may be measured through chemical and biological methods (Sweeting 1994). Aquatic organisms are exclusively dependent on water and are more established as bioindicators than terrestrial organisms. The chemical analysis of water sample is more expensive because it needs sophisticated modern tools. Therefore, biological analysis of water sample is widely accepted to assess the structural or functional integrity of ecosystems (Norris and Thoms 1999). For prolonged monitoring of succession process, the algal communities are found to be an ideal group because the group has diverse requirements and tolerances to a wide range of water parameters (Hughes et al. 1999). Water parameters may disturb the structure of community, cell structure and biological activity of the organisms. Diverse species of algae dominance indicate eutrophication in the tropical and Mediterranean regions (Fernandez-Pinas et al. 1991).

Q index $\left[Q = \sum_{i=1}^{n} piF \right]$ developed by Padisak et al. (2006) categorizes lentic water bodies into five grades, excellent, good, medium, tolerable and bad, required by Water Framework Directive for assessing the ecological status of the lakes. The phytoplankton assemblage index (Q) developed for WFD ecological status assessment includes the relative share (*pi*, where $pi = ni/N$; *ni* biomass of the *i*-th functional group; *N*: total biomass) of functional groups in total biomass and a factor number (F) established for the *i*-th functional group in the given lake type.

Physico-chemical and biological conditions of the aquatic habitat govern the development of phytoplankton present at the basal trophic level of food web on which entire aquatic life depends. The change in phytoplankton affects the structure of higher trophic levels in the ecosystem; hence, assessing phytoplankton assemblage provides holistic information essential for understanding the extent of disturbance and planning necessary action for the conservation of precious water resources as well as wildlife in relation to such systems.

20.2.7 Phytoplankton Functional Group

The chance of appearance of a species is predicted in a better way by using algal assemblages. A single planktonic collection may contain several taxa, including plentiful species as well as species introduced artificially by human interference. The aquatic environment becomes favourable to some species and they start growing in number, but the number of other species drops as the same condition will not be inspiring them.

The pattern of 'vegetation recognition' is encouraged by considering planktonic assemblages in freshwaters. Rather than considering phylogeny of the species,

common adaptive features of the species were taken into consideration for the formation of functional groups by Reynolds et al. (2002). Initially, only 14 functional groups (coda) were recognized by Reynolds (1980); additional information on ecologies of many planktonic species resulted in subdivision of some groups and creation of additional coda (Reynolds 1984; Padisak et al. 2009). This method simplifies the long taxonomic classification into functional groups (coda) of phytoplankton based on the ecological features of planktonic species (Naselli-Flores and Barone 2011); however, it requires the algal taxonomic skills to identify the phytoplankton to species level and assign them to diverse coda.

20.2.8 Morpho-Functional Groups

Morpho-Functional Group classification proposed by Salmaso and Padisak (2007), updated by Tolotti et al. (2012), includes 33 groups. The measures for establishing morpho-functional groups comprise external features, gelatinous covering, existence of flagella, carbon metabolism and taxonomy of phytoplankton demarcated by Weithoff (2003). It is a comparatively simple method to assess the ecological status of aquatic body based on morphological traits influencing the functional and ecological features (Allende et al. 2019).

20.2.9 Morphologically Based Functional Groups

The classification anticipated by Kruk et al. (2010) categorizes planktonic algae into only seven groups purely based on morphological characters like volume, maximum linear dimension, surface: volume ratio and presence/absence of flagella, siliceous structure, mucilage and aerotopes, which influence the autecology of phytoplankton. This method is the simplest among all other methods discussed in this chapter and can be easily used by the person with little expertise in the phytoplankton taxonomy.

20.3 Conclusion

As algae are primary producers of aquatic food web, they are suitable to be used as bioindicators to assess and predict the effect of pollutants on every trophic level of the ecosystem. Some algae are indicators of organic pollution whereas few are indicators of oligotrophic nature of the water. In traditional methods, presence/absence approach is used for assessing the trophic state of the aquatic ecosystem, like the presence of groups Chlorococcales and Cyanophyceae was used as indicators of eutrophic waters, whereas the presence of desmids as indicators of mostly oligotrophic acidic waters. Long-term monitoring of aquatic ecosystem using the

comprehensive approaches like algal assemblage, use of multimetric indices and functional grouping for monitoring is necessary to identify the cumulative effect of the physico-chemical and biological changes in an ecosystem and plan better management strategies for conservation and restoration of ecosystem. Functional Groups of Phytoplankton, Morpho-Functional Group (MFG) and Morphologically Based Functional Group (MBFG) classifications are now being used extensively throughout the world in biomonitoring programs as they provide a better understanding of the ecosystem health in comparison to traditional presence/absence approaches.

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Chapter 21
Contribution of the Environmental Biotechnology to the Sustainability of the Coffee Processing Industry in Developing Countries

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Abstract The global demand of coffee beverages stimulates high levels of production in coffee-growing countries. Contradictorily, this does not contribute to eradicate the imbalances in social benefits, economic growth, and environmental impact that are identified when analyzing the different stages of this agroindustry. This phenomenon is palpable in the primary processing or benefit of the coffee fruit, in which the green coffee bean is obtained. The benefit (wet, dry, or semi-dry) is developed in agricultural areas mainly of developing countries, where the financial resources available for the implementation of cleaner production strategies and effective waste treatment are limited. As a consequence, both liquid and solid wastes become a source of severe pollution of surrounding ecosystems and deterioration of farmers' living conditions. This chapter addresses the alternatives to transform coffee wastes into value products through bioprocesses, with a focus on circular economy and its contribution to the sustainability concept in developing countries. It was confirmed that the particularities of the scenarios in which coffee production and trade are developed make it complex to generalize alternatives of waste treatment or use. Furthermore, a production model that meets the multiple factors involved is also very complex but not difficult to be achieved. Sustainability initiatives and the role of biotechnology, with emphasis in anaerobic digestion, for the coffee production are analyzed. A holistic approach to solve the problems that persist in the process and the coffee value chain is exposed. It was concluded that it is necessary to incorporate into the concept of products of excellence and high quality standards in the coffee agroindustry the requirement of production conditions in which the waste streams are reused or returned to nature with non-aggressive characteristics for the environment. Biophysical indicators play an important role in making it possible.

Keywords Anaerobic digestion · Bioprocesses · Circular economy · Coffee processing · Sustainability

21.1 Introduction

The current environmental panorama is driven by the industrialization of natural resources under linear production schemes in which nature was only conceived as a subject of exploitation to support consumer production and market models (Lucas 2010). The transition toward more sustainable models, where the use of resources is optimized and the waste generated is minimized, represents huge challenges. The

development of new technologies and the use of alternative energy sources to reduce the dependence on oil, natural gas, and coal are main targets to be accomplished. Hence, interest arises toward the wastes of agro-industrial activities, mainly from crops. This biomass is generated in large volumes, becoming a source of contamination when disposed in the medium without prior treatment (Silva-Martínez et al. 2020).

For instance, the coffee production chain illustrates this phenomenon. Coffee processing is one of the most polluting because it generates wastes and wastewaters at all stages from harvest to coffee drink. Conversely, this agroindustry constitutes one of the main contributors to economic growth and jobs in many countries (Dadi et al. 2019). The economic, environmental, and social impact of this process is evident. More than 90% of coffee production takes place in developing countries, while its consumption is greater in developed countries, with industrialized economies (Wahyudi et al. 2020).

According to the Food and Agriculture Organization (FAO) of the United Nations, Latin America contributes 57.6% of the world coffee production, followed by Asia with 27.5% and Africa with 14% (FAO 2019). The list of the main producers and exporters reported by the FAO and the International Coffee Organization (ICO) includes countries from Latin America, Asia, and Africa undoubtedly led by Brazil, Vietnam, and Colombia (ICO 2020). As a consequence, the sources of waste generation are found in developing regions which are characterized by limited access to postharvest technologies. Moreover, there is a lack of controls in the quantification, classification, and final disposal of waste, and the regulations often are of low requirements in comparison with the standards of developed countries. Reports demonstrate that these wastes are generally mismanaged (Alemayehu et al. 2019; Wahyudi et al. 2020). On the other hand, environmental biotechnology applied to biodegradable wastes is one of the options to prevent, stop, and reverse the degradation of the environment. Several attempts have been made to achieve proper alternatives of coffee waste management as valuable chemical compounds recovery (Rajesh Banu et al. 2020; Villa Montoya et al. 2019), food supplements (Neves et al. 2019), compost, anaerobic digestion (Dadi et al. 2019), and other bioconversion processes (Atabani et al. 2019). The benefit/cost ratio or technical troubleshooting undermines the application on an industrial scale. To the best of our knowledge, only few efforts on sustainable and circular economy proposal have been assessed (Figueroa and Omar 2016). This chapter is aimed to analyze the scenarios in which the production and trade of coffee is developed as well as the efforts for its waste management as a starting point to assess how close is this agroindustry to being considered as sustainable.

21.2 Solid Coffee Wastes: Problem or Opportunity?

Coffee wastes characteristics, quantity, and final disposal are subjected to the coffee grain processing system. At least three methods are used to extract the coffee grain from the benefit stage: semi-dry, dry and wet (Wintgens 2004), although the wet

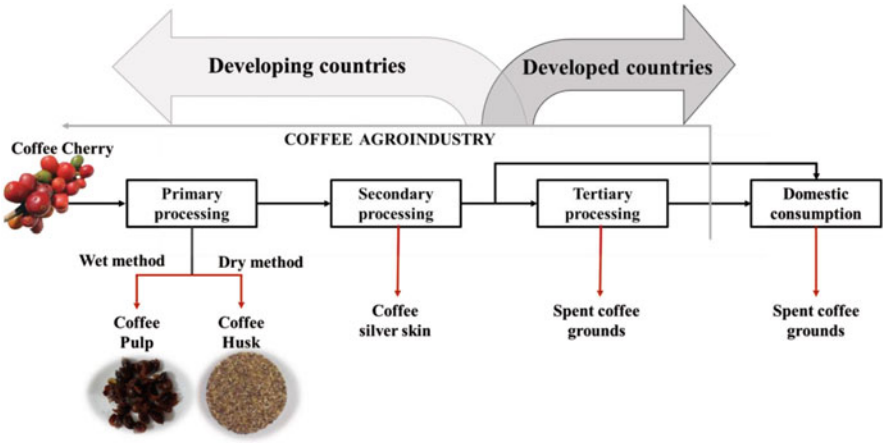

Fig. 21.1 Waste streams from the coffee production and consumption stages

method is the most useful (Haddis and Devi 2008; Ijanu et al. 2020; Murthy and Madhava Naidu 2012). Figure 21.1 illustrates the process and the corresponding waste streams.

The selection of the method depends on the cultivated species and the water resource availability in the region in which the coffee is cultivated and harvested. In the dry method, the fruits are exposed to the sun for 3–4 weeks to remove the external skin and pulp (Murthy and Madhava Naidu 2012). The solid waste that is generated is known as husk. This method has the advantage of being technologically simpler than the wet method, reducing water consumption, and consequently the wastewaters generation during the process. Nevertheless, scientific reports emphasize that the reduction of water in the process reduces the aromatic quality of the coffee grain (Gonzalez-Rios et al. 2007), affecting the final coffee product (Chanakya and De Alwis 2004). The wet method consists in a mechanical separation of the pulp and skin of the fresh cherry coffee, assisted by water as a transport media. Subsequently, the grain is washed prior to fermentation to remove the mucilage attached to the grain (Murthy and Madhava Naidu 2012).

In Ethiopia, it is estimated that more than 240,000 tons of coffee husks are disposed annually to the environment as a result of the fruit processing by the dry method (Shemekite et al. 2014). The polyphenol content in these residues, approximately 9%, might inhibit the growth of the plant root and lead to an intensification in greenhouse gas emissions (GHG) through spontaneous anaerobic decomposition. Another study revealed that in the Walleme River, in Southern Ethiopia, the wastewaters of a wet coffee processing plant alter the quality of the receptor water bodies. This is reflected in outranged values of total soluble and dissolved solids, chemical oxygen demand (COD), biochemical oxygen demand (BOD), ammonia, and total nitrogen which are above the maximum limits allowed by the Ethiopian Environmental Protection Agency and World Health Organization, as well as acid pH values

Fig. 21.2 Coffee processing: semi-dry (**a–c**) and wet method (**d–i**)

up to 5.55 determined downstream (Jini and Minuta 2017). This shows that regardless of the method used, a considerable degree of contamination occurs in the environment.

The information reported in the literature concerning the process mass balance is very varied. It is based on sampling that differs considerably in origin, variety of the coffee plants, time of harvest, use and type of fertilizers, and production models. Therefore, establishing flow diagrams of the methods and reporting accurate data on the amounts and composition of wastes is a challenge. Figure 21.2 illustrates the stages of the benefit process by the semi-dry (a-c) and wet (d-i) methods. The common factors in both are the use of water to facilitate pulping and the generation of abundant coffee pulp as the main solid waste. Although the infrastructure is different, both methods are developed in mountain ecosystems and there is no documentation of a waste management strategy.

The wet benefit, being highly water demanding, is often located near rivers acting as wastewater receptor bodies, in regions with few environmental regulations or little control. Approximately, a quantity of 15–20 L of water is required to process 1 kg of

Table 21.1 Chemical characterization of the coffee pulp and husk

Parameter (%)	Pulp				Coffee husk	
	a	*b*	*c*	*d*	*d*	*e*
Dry matter	84.58	–	86.8 ± 6.3	92.30 ± 0.05	93.50 ± 0.04	–
Organic dry matter	–	–	90.7 ± 3.0	–	–	–
Volatile solid	–	–	–	88.30 ± 0.16	92.80 ± 0.05	–
Ash	–	15.4	–	11.69 ± 0.16	7.15 ± 0.05	–
Cellulose	20.7	23	14.7 ± 1.6	31.60 ± 1.74	32.00 ± 1.52	24.5
Hemicelluloses	3.6	20	10.2 ± 0.4	8.50 ± 1.42	14.50 ± 1.04	29.7
Lignin	14.3	22	10.1 ± 3.7	15.50 ± 1.64	17.50 ± 1.55	23.7
Sugar	9.7	–	14.8 ± 5.3	–	–	–
Fat	–	–	1.3	0.99 ± 0.07	1.45 ± 0.03	–

a: Dias et al. (2015), *b*: Gurram et al. (2015), *c*: Pleissner et al. (2016), *d*: Chala et al. (2018a, b), *e*: Rajesh Banu et al. (2020).

coffee bean (Ijanu et al. 2020), which generates a large volume of high strength wastewater due to its tannin, alkaloid (caffeine), pectin, protein, and sugar content. The high content of organic matter is evidenced in COD values of 50 g/L and BOD values up to 20 g/L. That is the main reason of its rapid fermentation, producing organic acids and diminishing pH values up to 3.5 (Alemayehu et al. 2019). Furthermore, the wastewater contains approximately 388 mg/L of phenolic compounds. These characteristics lead to eutrophication, increased turbidity, and bad odors in the waterways and disable their uses for domestic, recreational, and commercial purposes. Regarding the solid coffee wastes generated in the wet benefit process, its volume is also considerable based on the fact that the green bean, the main product of the process, represents only 18% of the total weight of cherry coffee, so 82% of the fruit is considered waste (Adams and Ghaly 2007). The fraction corresponding to the solid coffee waste includes pulp (43%), mucilage (12%), and parchment (6.1%) (Ijanu et al. 2020). The polluting capacity of the solid waste in rivers and ecosystems adjacent to the benefit centers is compared to that described for wastewater. It is recognized that the increase in total soluble solids, total ammonia, and total nitrogen content in rivers are related to the presence of pulp and mucilage proteins discharged with wastewaters (Jini and Minuta 2017). As a consequence, the efforts to implement adequate waste management systems, as well as the investigations, are focused on the biological waste treatment, mainly by anaerobic digestion, the aspect that will be detailed afterwards (Alemayehu et al. 2019; Fia et al. 2012; Haddis and Devi 2008; Ijanu et al. 2020; Rossmann et al. 2012).

The coffee waste characterization is varied due to the analytical methods used (gravimetric, spectrophotometric, or the solvent used for extraction), the methodology of harvesting and conservation, as well as the fruit maturity degree as explained above. Some of these factors are not totally described in the literature. For instance, the total content of polyphenols and the antioxidant capacity of the pulp extract differ between coffee varieties (Geremu et al. 2016). Table 21.1 summarizes the

characterization of the solid coffee waste and demonstrates the potential use of these wastes in bioconversion processes.

In addition, these wastes are characterized by their content of toxic compounds for ecosystems. The pulp has 1.8–8.6%, 1.25–1.3%, and 10.7% of tannins, caffeine, and chlorogenic acid, respectively. In the same order, the levels of these compounds in the husk are 4.5–9.3%, 1.2%, and 12.59% (Janissen and Huynh 2018).

In general, waste biomass from agroindustry is considered an alternative energy source, but in some rural regions, it is the only choice. The high carbon content of these wastes is an advantageous property for the bioenergy production. To this end, George et al. (2019) reported a high carbon content in the coffee husk, approximately 41.82% based on dry weight. Protásio et al. (2013) determined the energy potential of the coffee husk and parchment, characterized by a carbon content of $46.5 \pm 0.38\%$ on a dry basis. The estimated lower and higher caloric values as well as the energy density values allow establishing advantageous equivalence ratios with respect to fossil fuels. The authors calculated that with 1 m^3 of these wastes, it is possible to substitute: 100 L of fuel oil, 119 L of oil, 1221 L of diesel or 135 L of gasoline for the same amount of energy.

Thus, coffee wastes are as problem as our ineffectiveness of looking for an opportunity with integral solutions.

21.3 The Environmental Biotechnology and the Solid Coffee Wastes

21.3.1 Animal Food and Biotechnological By-Products

Coffee pulp and husk are classified as lignocellulosic residuals, which have the disadvantage of being not easily degraded by microorganisms. The presence of antiphysiological and antinutritional compounds make them not suitable substrates for bioconversion process (Raphael and Velmourougane 2011). Furthermore, the use of appreciated chemicals present in such wastes for applications at the food industry, animal feeding, and pharmaceutical industries requires the rapid selection of the most valuable waste streams and their early conservation to avoid contaminations that hinder their use for those purposes (Mata et al. 2018). For bioconversion processes, pretreatments are required in order to promote the release of sugars. Thus, high demands for energy and/or the use of chemical products are a crucial point for the economic and environmental viability of the process (Moreno et al. 2019). Consequently, technological bioconversion alternatives for the exploitation of these agricultural wastes should take these aspects into consideration.

A wide number of researches with coffee wastes use pretreatments methods to obtain high value by-products. Chemicals such as chlorogenic acid (Torres-Mancera et al. 2013), enzyme b-glucosidase (Dias et al. 2015), hydrogen, alcohols, and volatile fatty acids (Villa Montoya et al. 2019), single cell protein (Ubaidillah and

Muzakhar 2019), or cellulose fiber (El Achaby et al. 2019) are obtained from coffee pulp and husk in processes that involve pretreatment and the rejection of the liquid or solid fraction from the multiple extraction/purification stages.

The coffee pulp and husk have been studied as an alternative to reduce the high costs of the microbial pigments production through processes that involve numerous stages. Moreira et al. (2018) investigated the potential of these wastes as an economic culture medium to produce carotenoids with antioxidant and antimicrobial activity. The solid coffee waste was subjected to alkaline pretreatment. The solid fraction from this stage was discharged and the extract was filtered and used as fermentation media by *Rhodotorula mucilaginosa*. The recovery of carotenoids involved centrifugation, drying, maceration, successive extractions, and evaporation of the solvent solution (acetone and methanol solution) over atmospheric nitrogen gas. Finally, the pigments were solubilized in 5 mL of methanol solution (99%).

Lactic acid from hydrolyzed coffee pulp has been obtained at lab and pilot scales, using *Bacillus coagulans*. This process requires successive purification steps. A conventional method for purification uses sulfuric acid and generates large amounts of calcium carbonate precipitated. Technological alternatives such as micro and nanofiltration, softening, mono and bipolar electrodialysis, anion and cation exchange chromatography, and distillation can reduce the environmental impact (Pleissner et al. 2016). However, the mass balance of the process revealed that 67.6 kg of pure lactic acid is obtained from 1000 kg of dried coffee pulp, in addition to the costs associated with these methods. About 77% (185.6–227.5 kg) of lactic acid is lost during filtration, electrodialysis, and chromatography. The authors estimate that optimization of the purification steps would allow the recovery of 200 to 300 kg of lactic acid from 1000 kg of dry coffee pulp.

The above-described experiences are in agreement with the disadvantages of different biological treatments applied to lignocellulosic residuals (Liguori and Faraco 2016). Bacterial and fungal treatments are associated with long operating times (3–5 months), low conversion efficiency, loss of raw material rejected in different process streams, and non-tolerance of the strains to by-products. Regarding enzyme treatments, the high costs of enzymes and aspects related to recycling, immobilization, and improvement of enzyme activity are declared.

The content of chemicals such as tannins and caffeine limits the use of solid coffee waste from the wet benefit in animal feed. The coffee husk can be used as a substitute for oat hay only up to 22.5% of the total dry matter in the diet of lambs (Belan et al. 2019). Studies show that the coffee pulp ensiled with 5% of molasses, fermented for 2 months and sun-dried, can constitute up to 16% of the diet in sheep without affecting daily weight gain, feed conversion, or food intake (Salinas-Rios et al. 2015).

Extracts as additive for food can be obtained from these wastes due to their bioactive phytochemical compounds content and the low levels of antinutrients (Neves et al. 2019). To produce the extracts, it is necessary to follow stages as drying, milling, use of solvents at moderate high temperatures, ultrasound and filtration steps (Geremu et al. (2016); Heeger et al. 2017; Neves et al. 2019).

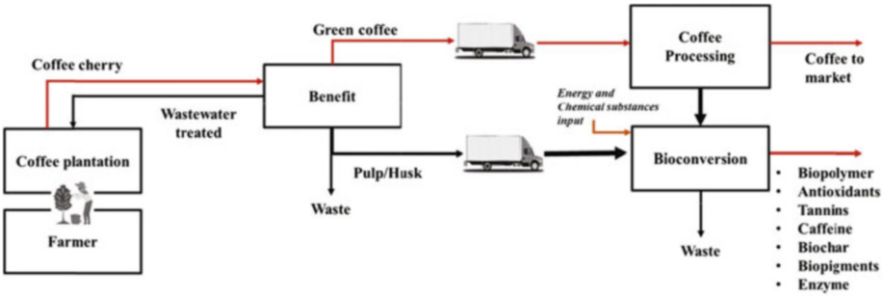

Fig. 21.3 A chain of value through product diversification of the coffee industry

In summary, the diversification of the coffee industry is necessary but the alternatives must impact positively to the environment from which takes the natural resources. A graphical visualization of this effect is shown in Fig. 21.3.

In this sense, it is mandatory to consider the following reflections:

1. The recovery of valuable products must be part of the proposal. The costs associated with the transportation and conservation of wastes impose simple, but efficient extraction processes that can be carried out in the context of local agriculture. (Magoni et al. 2018).
2. Not all "waste" streams are part of new processes, so subsequent treatment might contribute to circular economy. The characterization of the "new" wastes, for example, the solid fraction from extraction processes is a key aspect to assess the feasibility of the proposal.

21.3.2 Compost

Composting is an aerobic biological process whose practice in agricultural areas allows the treatment of organic waste to produce fertilizers and control the proliferation of environmental contamination. Nevertheless, both the husk and the pulp require an adequate composting process to diminish the severe damage caused to the soil by the application of immature compost (Dadi et al. 2019). During composting, microorganisms can degrade organic matter to produce the heat required in the process, NH_3, CO_2, organic acids, and steam.

This waste has a high C/N ratio, it does not favor the carbon degradation rate during the biological process, and therefore, the quality of the physical–chemical properties of the compost is affected. To counteract this fact, inherent in the composition of both residuals, an alternative is to use co-substrates such as cow manure and fruit/vegetable waste (Kassa et al. 2012; Shemekite et al. 2014). Vermicomposting has been shown to be suitable to improve the nutritional value and microbiological quality of compost as a fertilizer in coffee plantations (Raphael and Velmourougane 2011). The degradation efficiency achieved with this

technology is ranging between 70% and 78% with relatively long operational periods, between 112 and 165 days.

In addition to the requirements of co-substrates and operational times, it is interesting to analyze the emissions of greenhouse gases (GHG) associated with the process. A comparison carried out in three scenarios revealed that the emissions of thousands of equivalent tons of CO_2 from coffee pulp composting exceed 67% of the emissions caused by anaerobic digestion facilities. The worst scenario was the waste disposal in the open dumps (Choudhary et al. 2020).

21.3.3 Anaerobic Digestion

Anaerobic digestion (AD) and composting are not emerging technologies; however, they are two of the most evaluated alternatives for agricultural waste management provided by the environmental biotechnology. The application of both technologies is less analyzed still within the group of common management practices for the solid coffee waste as incineration and direct disposal on land or water bodies. Anaerobic digestion is a natural process in which organic matter is degraded by the action of microorganisms in the absence of molecular oxygen, to produce a gaseous stream of mainly methane and carbon dioxide, and a sludge that is used as a biofertilizer (Garfi et al. 2011, 2016). Methane provides a platform for global warming and climate change. Its production under controlled conditions is an added-value stream as it can be used to generate heat and/or energy.

The process has a positive energy balance (Chanakya and De Alwis 2004) and produces less sludge than aerobic processes. Regarding acidification caused by the emissions of NOx, SO_2, and ammonia, the potential for global warming, eutrophication and ecotoxicity, anaerobic digestion contributes to a lesser extent than compost and incineration (Lee et al. 2020). Based on research, with emphasis on solid and liquid wastes from tertiary coffee processing industry (Battista et al. 2016; Kim et al. 2017), the AD contributes to the waste treatment from all stages of the production chain.

The methane yield reported in the literature varies due to the influence of the inocula (Gu et al. 2014; Quintero et al. 2012) and the experimental methods and conditions (Chala et al. 2019). Nevertheless, they agree that the main challenges of the process are related to the waste composition. Lignin is a component of the coffee plant, difficult to digest by microbial consortia, leading to low rates of methane production. Tannins and other polyphenols, also present in the residuals, can inhibit the bioprocesses. These compounds have the ability to form hydrogen bonds with vital proteins such as enzymes (Field and Lettinga 1992), damaging the cell walls of the microorganisms responsible for the transformations of the residual (Lima et al. 2016). Finally, the unfavorable C/N ratio and the low content of micronutrients, denote a nutrient imbalance necessary for microbial growth and enzymatic activity during the anaerobic digestion process. For this reason, the use of co-substrate is reinforced (AD-Co). Regarding the design and operation of the reactor for anaerobic digestion of solid waste from the coffee benefit process, the alternative must be

Table 21.2 Simple reactor configurations for anaerobic digestion of solid coffee waste and their characteristics

	CSTR	Solid-state anaerobic reactor	Batch box
Mixing	Mechanical (intermittently or continuously)	• Digestate recirculation • Recirculation of pressurized biogas • Mixing leachates with fresh input	–
Hydraulic retention time (HRT) (days)	Long	Long	Long
Feedstocks	Mono- or Co-substrate	Ideal to lignocellulosic biomass	Commonly food, green or agricultural waste
Flow type	Completely mixed	Plug flow	Batch

Andre et al. (2018), Daniel-Gromke et al. (2018), Nguyen et al. (2019)

technologically simple, but robust, in accordance with the rural environment where it will operate. Regarding the reactor configuration, three proposals are attractive: The completely stirred tank reactor (CSTR), the solid-state anaerobic reactor and the batch box (Garage or Percolator). The main characteristics of these AD techs are summarized in Table 21.2.

Complete stirred tank reactor (CSTR) is one of the most used reactors for its versatility to operate at various levels of total solids (1–15%), easy design, and operation. These reactors have disadvantages in terms of energy requirement to maintain contact between the substrate and microbial biomass, as well as the long retention times to prevent biomass washing.

The main characteristic of the anaerobic solid-state reactor is that it operates with high levels of total solids, generally between 15 and 30%, as a consequence, less reactor volume is required. However, this leads to poor substrate–biomass contact, accumulation of volatile fatty acids (VFAs) and ammonia as main drawbacks, in addition, the dilution of the inhibitory compounds is not eased. Therefore, its implementation is less attractive.

Batch box or Garage fermenter (Daniel-Gromke et al. 2018) is a simple technology compared to those described above. This consists of a set of thermally insulated boxes, without mixing the substrate during the process. To promote homogeneous inoculation in the reactor, degradation of all material, and high methane yields, leachates are collected at the bottom and sprayed onto the fermentation substrate cyclically (Rico et al. 2020). The operation is discontinuous in terms of feeding, but it is possible to guarantee a continuous flow of biogas using multiple fermentation boxes operating at different times.

Household anaerobic reactors are the most popular devices mainly used in rural areas (Ren et al. 2018). The advantages lie in the low cost of equipment, operation, and maintenance (Nguyen et al. 2019). However, they do not operate properly when the solid content in the reactor increases, so their use is not considered for the solid residuals of the coffee benefit process.

The produced biogas can be used to generate electricity and all the lower grade waste heat from cooling and exhaust can still be used for drying coffee (Chala et al. 2018a). The methane yield from the anaerobic digestion of coffee pulp reaches 244.7 ± 6.4 L/kg_{VS}, with a methane content of 56.8%, while coffee husk reports methane yields of approximately 159.4 ± 1.8 L/kg_{VS} with 51.5% methane at mesophilic temperature (37 °C) (Chala et al. 2018a, 2019). Coffee pulp was dried naturally and cow manure was also co-digested at mesophilic temperature (35 °C). Methane content was kept constant in 60% (v/v). No reference to removal COD efficiency is mentioned (Corro et al. 2013).

For all above explained, the anaerobic digestion process leads the alternatives toward a sustainable agriculture because it encompasses a series of benefits such as: (1) the control of the proliferation of pollution sources, (2) energy saving, (3) nutrients recovery, and (4) reduces the risk of work environment and farmer living conditions deterioration.

21.4 The Role of Environmental Biotechnology Creating New Values to the Coffee Processing Industry

Agricultural and agro-industrial residues are not only a sustainability problem linked to food security, but also an economic problem, since they have a direct impact on the profitability of the entire food supply chain. The sustainable agricultural waste management focuses on three main aspects: recycling, reuse, and reduction. Nevertheless, a systematic approach is required to minimize its volume and associated impacts (Murthy et al. 2019). The United Nations (UN), the World Economic Forum (WEF), and the European Union (EU) advocate the development of global initiatives that promote the transition toward a more circular economic model. This model allows increasing resource efficiency, reducing environmental pressures (Schmidt Rivera et al. 2020) and promoting sustainable growth (Aid et al. 2017).

Coffee processing waste show potential to be exploited in the context of the bioeconomy. They are an abundant and low-cost resource that can be used through various recovery routes (Schmidt Rivera et al. 2020). Researches have been carried out into the conversion of solid waste streams (pulp, husk, and spent coffee grounds) into added-value products such as fertilizers, feed, and bioenergy (Geremu et al. 2016; Neu et al. 2016; Shakeel et al. 2014). More researched innovative schemes suggest the implementation of biorefinery concept that diversify and increase the exploitation of these wastes with better approach to a circular bioeconomy. However, the application of some bioconversion processes is only concentrated on few wastes streams as those generated during tertiary coffee bean processing (Chen and Jhou 2020; Pin et al. 2020; Schmidt Rivera et al. 2020).

The "tertiary industry" produces instant coffee as well as decaffeinate coffee. The generated wastes, liquid and solid, receive more attention than those from the postharvest stage (Atabani et al. 2019; Rajesh Banu et al. 2020; Schmidt Rivera

et al. 2020; Zabaniotou and Kamaterou 2019), probably because it is an industrialized stage of the whole process chain. The solid waste is called spent coffee grounds (SCG). This waste is similar to the one generated in establishments where coffee drinks are sold, as well as in homes. On average, a ton of green coffee generates approximately 650 kg of spent coffee grounds. At tertiary process, approximately, 2 kg of wet spent coffee ground is obtained for each kg of soluble coffee produced (Murthy and Madhava Naidu 2012).

Girotto et al. (2017) stated that this waste applies to circular bioeconomy management models since it is an adequate source to obtain biofuels and products with added value. Some international companies have promoted circular economy models to enhance the SCG (Rajesh Banu et al. 2020) and identified that the logistics of the collection system, storage spaces, and processing facilities are important variables in these models.

Some of the proposed technologies are incineration, landfill, anaerobic digestion, composting, and direct application to the land, but routes have been proposed in which biodiesel is produced as added-value product (Chen and Jhou 2020; Rajesh Banu et al. 2020). By establishing a comparison to identify the option that contributes the most to the concept of sustainability, it was demonstrated that biodiesel production, followed by composting, turned out to be the least sustainable option, with the highest negative impacts on air, water, and soil, human and ecological health in almost 70% of the evaluated categories. This was not the case for anaerobic digestion, which proved to be an alternative with a positive impact.

The findings confirm the need to investigate the waste recovery through biotechnological routes from the environmental point of view (Schmidt Rivera et al. 2020). To assess whether the biotechnology alternatives fully cover the sustainability concept dimension, the following aspects should be taken into account:

- To what extent are the problems of control and prevention of the environmental impact solved, based on the volume of treated waste?
- How do they contribute to close cycle processes while reducing contamination in coffee regions?
- Comparison of investment and operating costs.
- Are the "new" wastes generated by the biotechnological alternative identified and characterized? Is the environmental impact higher than the one caused by the originals?
- Are the biotechnological alternatives applicable to any of the coffee production chain stages?

21.4.1 How Far Is the Coffee Industry from Producing "Sustainable Coffee"?

Developing countries are the main coffee suppliers to the world market. The challenges in the coffee sector, in terms of sustainability, are social, environmental,

and economic. Some authors identify them and refer to inadequate access to financing and increased input costs aggravated by fluctuations in prices, low levels of education and knowledge, and unpredictable weather patterns that deteriorate as a result of climate change (Dietz et al. 2019).

To reverse this scenario, which threatens the relief of producers and the long-term availability of good quality coffee in an expanding market, populations involved in the culture and tradition of coffee production must have access to reasonable living standards, in terms of quality and social welfare. Furthermore, the sector must have adequate adaptation and mitigation strategies to face the increasing influence of climate change on coffee production. Certifications are an instrument created to provide these capabilities to the agricultural sector, increasing the added value of the product. These are based on the fact that consumers are motivated to pay a price premium for healthier and more environmentally friendly products that meet sustainability standards and certain precisely defined requirements and guaranteed standards (Wahyudi et al. 2020).

In the period from 2008 to 2013 in Latin America, a trend began toward the verification and international certification of sustainability in the coffee sector (Adams and Ghaly 2007; Hajjar et al. 2019; Raynolds et al. 2007; Samper and Quiñones-Ruiz 2017). In Brazil, Mexico, Colombia, Peru and Honduras, a number of voluntary environmental and social certification programs have been adopted within the coffee sector (Barham and Weber 2012; Hajjar et al. 2019). These include UTZ Certified, Fairtrade, Organic, Nespresso AAA Sustainable Quality, 4C Association, and the Sustainable Agriculture Network/Rainforest Alliance (SAN/RA) certification program. The results of the multiple evaluations on the impact of these certifications are contradictory (Ssebunya et al. 2019). While some studies identify positive effects in at least one aspect (economic, social, or environmental), some authors do not associate progress in social indicators with participation in the certification programs (Akoyi et al. 2020) or disagree when evaluating the same certification program (Dietz et al. 2018). Some findings cast doubt on the sincerity of private sustainability standards (Akoyi and Maertens 2017), based on the fact that the price that consumers pay for certified products does not translate into economic benefits for coffee farmers and there is a risk that low intensity agriculture, promoted by high imposed standards, can trap people into poverty.

Some target elements involved in the high standards are the soil or land dedicated to coffee cultivation and its agroforestry environment, the water consumed in the entire coffee product production, emissions, the social structure of the area where coffee is grown or produced, economic income, energy consumption in the production process, and waste management and the main agro-inputs used in the coffee production chain and its derivatives. These ideas identify the relationship between sustainability, circular economy, and bioeconomy from the coffee plantation to the waste treatment generated by this agroindustry (Brunerová et al. 2019).

In any case, it is essential to implement more efficient production schemes that guarantee the quality standards of the final product, in which traditional inputs, for example, chemical fertilizers are replaced by treated wastes (Ssebunya et al. 2019) increasing the profits. Faced with this complex panorama, research reveals that there

are many challenges to achieve sustainable coffee production taking into account as aggravate factor that some stages of the chain are developed in environmentally vulnerable regions with limited infrastructure (Adams and Ghaly 2007; Chanakya and De Alwis 2004; Nguyen and Sarker 2018; Onyas et al. 2018; Wintgens 2004). In Vietnam, the high levels of coffee production have a high environmental cost (Nguyen and Sarker 2018) exacerbated by unsustainable agricultural methods such as monocultures, crop waste burning, and poor management of fertilizer use and cultivation, resulting in environmental negative impacts such as deforestation and soil degradation.

The factors that constitute obstacles are technological, economic, and social. Innovation and ecology in the coffee industry are terms that involve risks, limiting investments by financial institutions (Adams and Ghaly 2007). Additionally, there are no specific tariffs for coffee certified as sustainable. The analysis of income distribution along the coffee production chain to the final consumer shows that in producing countries, it is low compared to consuming countries (Chanakya and De Alwis 2004) resulting in the decline of producer's interest in environmental aspects.

A strategy to identify opportunities to adopt cleaner production strategies and increase operational efficiency, effective use of resources and alternatives for the use of by-products is to apply established methodologies for the comprehensive evaluation of production systems. Maximizing sustainability in the sector implies going from linear to circular processes, bearing in mind that not all resource recovery alternatives are usually environmentally friendly. A correct balance of each current that is needed and delivered is what defines the real circularity of the process. Figure 21.4 represents two treatment alternatives for wastes from the coffee industry.

When biological treatment technologies are compared to evaluate symbiosis processes in the local production environment, it is observed that anaerobic digestion (Fig. 21.4b) favors the correct insertion of its product streams and that it has the potential to become the nucleus of the sustainability of the coffee industry (Aid et al. 2017; Hagman et al. 2018; Wolf et al. 2007).

Another option to approach increasingly higher levels of sustainability and that does not depend on external expensive interventions or inaccessible to most producers in the form of certifications (Hajjar et al. 2019), is the application of biophysical indicators that help drive sustainable production processes.

21.4.2 Biophysical Indicators of Sustainability Within the Coffee Production and Processing Processes

Sustainability indicators provide signals to measure progress toward goals that jointly contribute to human and ecosystem well-being (Soussana 2014). Biophysical indicators should not be confused with environmental indicators. The environmental indicator systems arise in response to the need to have adequate information for decision-making in environmental policy among other aspects. They result from the

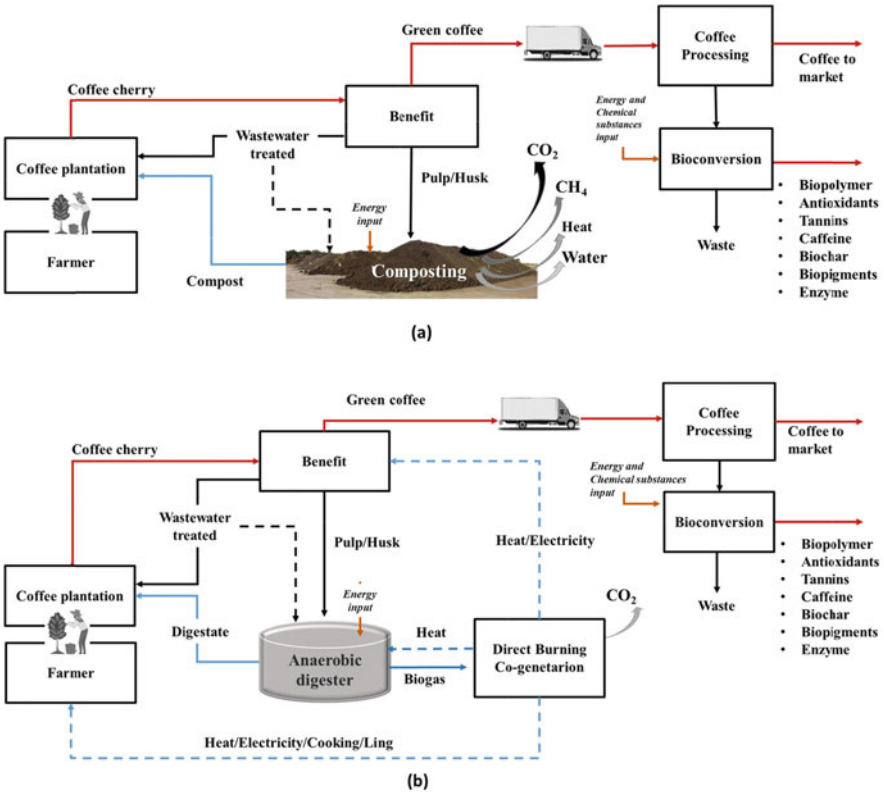

Fig. 21.4 A circular economy approach for a sustainable coffee industry. (**a**) Composting. (**b**) Anaerobic digestion

mathematical combination or aggregation of various parameters and help to reduce the large amount of existing scientific information, transforming it into a manageable number of parameters (Gibson et al. 2000; Jiménez-Herrero 2000). While environmental indicators indicate the state and variation of the environment, biophysical indicators of sustainability must also indicate the state and variation of the human system in relation to the natural system (Jiménez-Herrero 2000). Thus, an environmental indicator becomes an indicator of sustainability with the addition of time factors, limits, and objectives (Mesarovic et al. 1996).

An indicator that has been used in some Latin American territories is the product environmental footprint (PEF). This is a multicriteria environmental standard that uses the life cycle analysis (LCA) methodology applied to the coffee drink. It was established to define the environmental footprint standards of the products that would rule the European market from 2020.

Experiences in producer countries such as Peru and Colombia improve the quality of the product and the living conditions of producers with a gender, intercultural, territorial, adaptation, and mitigation of climate change approach (Suca et al. 2012).

In 2012, the declaration of sustainability of the coffee sector in Colombia was signed and the Sustainable Trade Platform was created. This project allows the farmer to produce coffee minimizing the carbon footprint. Through a comprehensive exercise, in which shade management has been technologically modified, planting more coffee trees per area, more efficient and at the same time more sustainable fertilization has been achieved. This implies that minor changes in the cultural way to do it can reverse negative environmental impact and ease the farmer labor.

Another Colombian case with an approach to sustainability from the Principles-Indicators-Criteria (PCI) method, carried out by Figueroa and Omar (2016) in the coffee industry of the Linares-Nariño municipality showed interesting results. Five dimensions were assumed to group the indicators: economic, social, environmental, cultural, and political. In general, this work concludes that the coffee agroecosystem in the San José Sector favors the farmer economy and the material sustainability of home-farms, where interactions or flows in the coffee production chain show a great contribution in the construction of community, family survival, and ecological balance. The reliability in its duration suggests that the sustainability over time of the productive units is uncertain. This is mainly due to climatic changes that limit the supply of rainwater; the recrudescence of capital income in the disposition of resources for work and material resources on farms.

21.4.2.1 Simple Biophysical Indicators Propose to Measure the Sustainability of the Coffee Industry

To apply a system of biophysical sustainability indicators to the coffee production or processing process, it is necessary to consider whether the emissions are generated and the type of emissions, body receptor for final disposal, and whether or not there was a previous treatment. Moreover, it is important to know whether there is a decrease or elimination of the organic load content from technological transformation that generates the emissions. In addition, it must be established whether it complies with certified national or international standards in order to aspire to export the product to demanding foreign markets. The energy factor has an important environmental link (Giannetti et al. 2011). Therefore, energy consumption should be considered within the value chain of the production of coffee and its derivatives.

Biophysical indicators of sustainability emerged as a response to the conceptual and theoretical insufficiency of traditional indicators, in the new context of the sustainable development paradigm. Traditional or conventional indicators are an insufficient and probably inadequate way of measuring reality, in which society implements new demands for a more balanced, fairer, and more environmentally friendly development model. The indicators must constitute a fully appropriate point of reference to guide the technological trajectory and institutional change in accordance with the challenges and objectives of the present, that is, with the quality of life and sustainable development. Likewise, the indicators must allow critical, transparent, and open analysis of decision-making processes and practices. Within

a sustainable development policy, it is necessary to configure a useful system of sustainability indicators (Pepe 2011).

It is possible to mention a group of simple biophysical indicators that could be applicable to any process of organic waste treatment at any stage of coffee production or processing. The biophysical indicators shown below will not be the only ones proposed in an in-depth analysis of a production process, but they can be assumed as a noncomplex example of what they mean and the elements that would have to be considered in order to obtain simple information on the level of sustainability of a productive system for coffee processing. This would be very important for producers, fundamentally the poorest, with fewer resources to give their own assessment of how close it is to basic levels of sustainability or how far it is from increasing them in such a way as to reduce negative impacts on the environment.

A proposal for specific biophysical sustainability indicators for the coffee production or processing industry could be the following:

$$\text{Energy autonomy} \left[\frac{\text{kWh generated}}{\text{kWh consumed}}\right]$$

This indicator assesses the amount of energy generated in the integral coffee production system, if this were the case, in relation to the unit of energy consumed by the same production system in the same period of work. All the electrical energy-consuming equipment in the technological process under evaluation must be identified and the relationship with the amount of energy generated by the same system must be found. If the indicator is greater than 1, it refers to a surplus which is desirable. It is a point of comparison between variants where energy is recovered.

$$\text{Water treatment} \left[\frac{\text{m}^3 \text{ consumed water} - \text{m}^3 \text{ treated water}}{\text{ton coffee}}\right]$$

This indicator considers the volume of water that does not receive effective treatment and the amount of processed coffee. Quantifies efforts to minimize negative impact on water bodies. It could be used to evaluate strategies to reduce water consumption by re-incorporating treated water into the process, as well as to identify water losses.

$$\text{Energy efficiency} \left[\frac{\text{kWh generated} - \text{kWh consumed}}{\text{ton coffee}}\right]$$

This indicator refers to how much electrical energy is generated in the process, considering or not the energy produced by organic waste, or for each solution proposed to satisfy the waste treatment system. In this case, this indicator offers the possibility of making a comparison in relation to the base line case that does not have an adequate waste treatment process with reference to the coffee bags produced

by the company or the tons of the leading product. The higher this ratio, the more favorable the alternative will be, due to the lower energy cost for the company.

$$\text{Air quality } \left[\text{ton } CO_2\_\text{reduced}/\text{ton coffee}\right]$$

With the intention of protecting the atmosphere, this indicator is established to estimate the ratio of equivalent CO_2 emissions avoided with the waste treatment or other bioconversion process, which are evaluated with respect to the amount of produced coffee. It is essential to calculate the avoided amount of CO_2 emitted by the system under study, or its equivalent in other gaseous compounds. This indicator will give a value that the higher it is, the better the solution is

$$\text{Thermal consumption } \left[\text{kWht consumed}/\text{ton coffee}\right]$$

This indicator refers to how much thermal energy is generated by the organic waste treatment, and a relationship is proposed with reference to the amount of produced coffee. In this case, it is interesting, since the anaerobic or incineration treatment variants allow the generation of a quantity of thermal energy that could be used in some energy need of the process itself. The higher this relationship will be, the more favorable is the solution inferring lower energy cost for the company.

Absolute indicators are also valid and should be as creative and original as they are achievable. Taking into account that the diagnosis carried out contributes to the weaknesses of the system, it could be assessed for the above-mentioned case of Colombia: number of young people incorporated into the coffee production process; number of exchanges of coffee producers in primary schools; number of billboards announcing the relevance of coffee in the region and many others. Biophysical indicator systems have a high level of uniqueness, specificity, and flexibility. The comparison that these types of indicators encourage is effective when different scenarios are managed for the same production system.

21.5 Remarks

1. Achieving excellence in the products of the coffee industry is as necessary as implementing the conditions in which waste streams are returned to nature.
2. There is a high potential for resource recovery that can contribute to a superior economic, social, and environmental balance in evaluating the impacts of the coffee process, where anaerobic digestion can play an important role as a biotechnological process.
3. To ensure the future of the coffee industry, it is essential that a conscious turn is made of this sector toward sustainability, understanding it as a holistic approach to solve the problems that persist in the production process and the value chain of this product.

4. The sustainability of the coffee industry can be promoted by obtaining international certification assumed by different agencies or organizations specialized in it, or through the application of biophysical indicator systems, which approximate the coffee production and processing to interesting values of sustainability, mainly for small producers, by increasing and adding value to the leading product.

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Chapter 22
Interactions Between Edaphoclimatic Conditions and Plant–Microbial Inoculants and Their Impacts on Plant Growth, Nutrient Uptake, and Yields

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Abstract Microorganisms play essential roles in regulating the ecosystem. Several interactions occur between plants and their associated rhizobacteria, cyanobacteria, and/or mycorrhizal fungi, which result in better plant growth. This chapter presents general aspects regarding interactions between edaphoclimatic conditions and plant–microbial inoculants and their impacts on plant growth, nutrient uptake, and yields. The different roles of the arbuscular mycorrhiza fungi (AMF) and plant-growth-promoting microbes (PGPM) are highlighted. Although the presented data demonstrated the remarkable abilities of these microorganisms, there is still much to be done on both explorations as well as the implementation of PGPM. Single microorganism inoculants, as well as formulations, have been proved to significantly increase crop production at a very low cost when compared to chemical fertilizers. Exploration that involves the understanding of the mechanism and at the same time implementation needs to take care of a great deal of optimization on field applications.

Keywords Biofertilizers · Cyanobacteria · Mycorrhiza · Rhizobacteria · Soil fertility

22.1 Introduction

Our focus in this chapter is on the impacts of edaphoclimatic conditions on plant response to inoculation with plant-growth-promoting microbes (PGPMs). These microorganisms connect roots with the soils, which allowed greater nutrient uptake. Currently, microbial inoculants for crops include three major groups: (1) arbuscular mycorrhiza fungi (AMF), (2) promoter growth plant rhizobacteria (PGPR) that include *Bacillus*, *Pseudomonas*, *Trichoderma*, and *Azospirillum*, and (3) microalgae and cyanobacteria and the symbiotic nitrogen-fixing rhizobia, which are usually not considered as PGPR.

Several interactions occur between plants and their associated rhizobacteria and/or mycorrhizal fungi, which result in better plant growth. It is known that up to 40% of photosynthetically fixed carbon is secreted into the rhizosphere by plants. These carbon materials attract microbial populations, especially those able to metabolize plant-exuded compounds and proliferate in this microbial habitat.

Microorganisms can use these compounds as substrates, resulting in an increased microbial biomass and activity around the roots, the so-called rhizosphere effect. Plants can influence bacterial gene expression, especially genes encoding plant-beneficial traits, by releasing these root exudates. Root exudation-mediated plant-associated PGPR can modulate root development and growth through the production of phytohormones (indole-3-acetic acid, IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, organic and inorganic acids, etc. The PGPR result in a reduction of the growth rate of the primary root and an increase of the number and length of lateral roots and root hairs (Etesami 2020). PGPR also modify root physiology by changing gene transcription and metabolite biosynthesis in plant cells, thereby increasing root exudations, resulting in microbial activity, and this process continues in the cycle of a plant. In this chapter, the different roles of AMF and PGPM are highlighted.

22.2 Arbuscular Mycorrhiza Fungi (AMF)

AMF are vital microorganisms for the ecosystem's balance, mainly due to mutualistic symbiosis (obligatory biotrophy) with vascular plants (Bais et al. 2006; Mosse et al. 1981; Simon et al. 1993), mainly for agricultural production and degraded areas' recovery (Munyanziza et al. 1997). These fungi, characterized by the particular form of arbuscules, spores, vesicles, and hyphae (Mosse et al. 1981), benefit plant metabolism related to carbon (C) and the acquisition and absorption of other elements, such as phosphorus (P). The physical and chemical quality of the soil is also checked when there is an increase in mycorrhization (Cardoso and Kuyper 2006; Kahiluoto et al. 2009).

According to Chen et al. (2018), AMF communities reduce soil erosion because the hyphal network and mycelium create a three-dimensional matrix that surrounds and cross-links soil particles without compacting them. Additionally, glycoproteins, such as glomalin, reduce the loss of particles and nutrients (Rillig and Mummey 2006). According to the results presented by Morris et al. (2019) , the AMF contribute to C stabilization primarily by increasing the stability of the aggregates. AMF can reduce nutrient loss from soils, such as N and P, by enlarging the nutrient interception zone and preventing nutrient loss after rain-induced leaching events (Cavagnaro et al. 2015; Jin et al. 2012; Verzeaux et al. 2017). Besides, there is the contribution of inorganic P and mineral or organic forms of N and amino acids (AA), which are taken up by specialized transporters located on the fungal membrane in the extraradical mycelium (Bonfante and Genre 2010). Cardoso et al. (2017) described that P ions react with one another, resulting in polyphosphates, which are transported by cytoplasmic streaming and reach the arbuscules.

The rhizosphere is the area of the expressive interaction in microorganisms with plants and because the AMF have the evolutionary advantage of symbiosis with a lively group of plants (Humphreys et al. 2010; van der Heijden et al. 2015). AMF are of great importance for new tools for agriculture, energy, and the environment (Uzoh

and Babalola 2018). However, there is a gap to be confronted for the widespread use of these fungi as an inoculant. Despite being so promising and profitable, large-scale adoption of the mycorrhiza inoculants has not been feasible and for this, various reasons are responsible including identification, multiplication, distribution in supply chain, and use by end user farmer. There are some limitations in adoption due to potential threat of easy contamination and limited adoption by the farmers (Sammauria et al. 2020).

According to Santos et al. (2019), inoculants are living microorganism products capable of benefiting the development of many crops; these authors reported a significant increase in the use of bacterial inoculants in the last decade and in the future. Microbial inoculants made a significant contribution to all agricultural farms and they can be easily produced by diverse companies (Sammauria et al. 2020).

On the contrary, AMF inoculants, despite their enormous potential, still do not have consistent formulations and extensive agriculture and environmental use. AMF-plant biotrophy (obligatory) is consensually a barrier for the evolution of inoculants' formulation (Herrera-Peraza et al. 2011), in addition to the application of industrial fermentations in this situation (Sudeep et al. 2020).

According to IJdo et al. (2011), between the 1990s and the 2000s, the methods to obtain the inoculant were based on a substrate, wherein host-dependent sporulation of AMF species is the determinant for inoculum production. Mass production of inoculating AMF has been studied using the methodology Airlift Bioreactor (Jolicoeur et to the., 1999), indicating methodologies to obtain pure cultures and a high-quality and low-cost inoculum methods can.

Until now, mainly in the last decade, there were consistent scientific reports using AMF via inoculants in formulations compatible with commercial fermentations. These were found to promote plant growth and the corresponding yield. In soybean production, there was significant evidence of increased productivity. Under AMF, the soybean yield ranged from (~)2100 to (~) 5100 kg ha^{-1} and under fertilization ranged from (~)2500 to (~) 4500 (Barazetti et al. 2019; Cely et al. 2016; Stoffel et al. 2020) (Fig. 22.1).

There is an increase in soybean yield under inoculation with AMF-*Rhizophagus*, which gives consistency in the dissemination of the technology to the broad fields of grain production. Others arguments are important. According to Cely et al. (2016) , AMF inoculation helped plants to take up P from fertilizers and showed high potential for use in combination with conventional fertilization. According to Barazetti et al. (2019), AMF can use different types of propagules to colonize new roots, e.g., spores and hyphae, and it may not have the same capacity to produce new units of infection, varying between species, and *Rhizophagus* may be able to colonize new roots, promoting similar colonization rates. Already, Stoffel et al. (2020) declared that *R. intraradices* increased the biomass yield, P uptake, and soybean yield under different edaphoclimatic conditions, with more marked effects on soils that originally had low or medium levels of available soil P.

Considering the high cost of mineral fertilization, the AMF inoculation used as extensive agriculture practice to supply P and other elements is important to Brazilian agribusiness. We understand that inoculant factories need to develop this

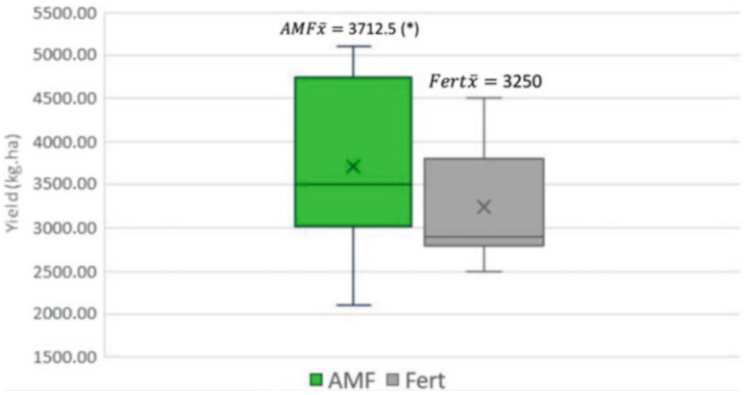

Fig. 22.1 Soybean yield (kg ha^{-1}) conducted in different Brazil regions ($n = 8$). AMF = *Rhizophagus* mycorrhiza inoculation and Fert = fertilized by similar farm conditions. (*) t-test statistically significant by 5%

technology on a large scale. Technology diffusion programs must be consistent with those of the *Bradyrhizobium* for soybean (Moretti et al. 2018).

22.3 Plant-Growth-Promoting Rhizobacteria (PGPR) for Sustainable Agriculture

The rhizosphere is an interactive zone around the roots where the biology and chemistry of the soil are influenced by the roots' release of organic compounds that can be utilized by soil microorganisms, and where the biology and chemistry of the soil are influenced by the root. Root exudates are rich mainly in amino acids, sugars, and organic acids, which serve as the primary source of nutrients and support the dynamic growth and activities of various microorganisms within the vicinity of the roots (Badri and Vivanco 2009; Huang et al. 2014).

The rhizosphere supports a large and active microbial population. Some of the interactions that occur within the rhizosphere and the rhizospheric bulk soil can be said to be either neutral, synergistic, or antagonistic (Odoh 2017; Singh 2018). The bacteria that occupy the rhizosphere are collectively termed rhizobacteria. The term plant-growth-promoting rhizobacteria (PGPR) was coined by Joe Kloepper in the late 1970s and is defined as the free-living naturally occurring soil bacteria that colonize plant roots in search for carbon source and benefit it by providing growth promotion (Katiyar et al. 2016).

Conventional agriculture plays an essential role in meeting the food demands of a growing human population, and this leads to excessive use of fertilizers. However, today agriculture demands an increase in yield with a substantial decrease in chemical fertilizer and pesticides since they not only affect the quality of food but also contribute to the degradation of the environment (Odoh 2017; Singh 2018).

Agrochemicals are considered a potential source of heavy metals that remain intact for a long time, contributing to water and soil pollution. In this context, the use of PGPR is a potential tool for sustainable agriculture and trend for the future, being an appropriate alternative in organic farming, also allowing the cultivation of poor or degraded soils (Bhat et al. 2019; Katiyar et al. 2016; Odoh 2017). The most common genera of rhizobacteria that have been reported are *Arthrobacter, Variovorax, Azospirillum, Alcaligenes, Enterobacter, Bradyrhizobium, Burkholderia, Serratia, Azotobacter, Klebsiella, Mesorhizobium, Rhodococcus, Streptomyces, Flavobacterium, Bacillus,* and *Pseudomonas*, among others (Katiyar et al. 2016; Odoh 2017; Singh 2018).

PGPR exhibit a special role by preventing plant infestation with the disease, increasing nutrient absorption, promoting root and shoot formation, improving seed germination, and making the plant more tolerant to environmental stress (Odoh 2017; Widnyana 2018). The application of microbial inoculants or microbial base substances on seeds, plant surfaces, or soil to colonize the rhizosphere or the interior part of the plant is called bio-fertilization. Research has demonstrated that inoculating crop plants with certain strains of PGPR at an early stage of development can be an effective strategy to stimulate crop growth and can also improve crop tolerance for biotic and abiotic stresses (Backer et al. 2018; Bhat et al. 2019; Odoh 2017).

Another technology that has been considered promising for commercial organic agriculture is the co-inoculation. Recently, Zeffa et al. (2020) realized a quantification of the effects of co-inoculation of *Bradyrhizobium* and PGPR on the soybean crop using a meta-analysis approach. They found that the co-inoculation technology resulted in a significant increase in nodule number (11.40%), nodule biomass (6.47%), root biomass (12.84%), and shoot biomass (6.53%) and in general, *Azospirillum*, *Bacillus*, and *Pseudomonas* were more effective than *Serratia*.

The action of the PGPR can occur extracellularly (ePGPR), in the rhizosphere, or the spaces between cells of the root cortex, or intracellularly (iPGPR), inside root cells, generally in specialized nodular structures (Jha and Saraf 2015). The PGPR can exert effects on plants through direct and indirect mechanisms. The direct mechanism entails either providing the plant with a compound that is synthesized by the bacterium, for example, phytohormones, or facilitating the uptake of certain nutrients from the environment. The indirect mechanisms occur when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms by producing antagonistic substances or by inducing resistance to pathogens (Singh 2018; Tariq et al. 2017). Direct and indirect mechanisms of action in PGPR are summarized in Fig. 22.2.

22.3.1 PGPR as Biofertilizers

Plant-growth-promoting rhizobacteria (PGPR) can participate in soil fertilization through biological nitrogen fixation of atmospheric nitrogen and biosolubilization of insoluble nutrients such as phosphorus, potassium, and some micronutrients.

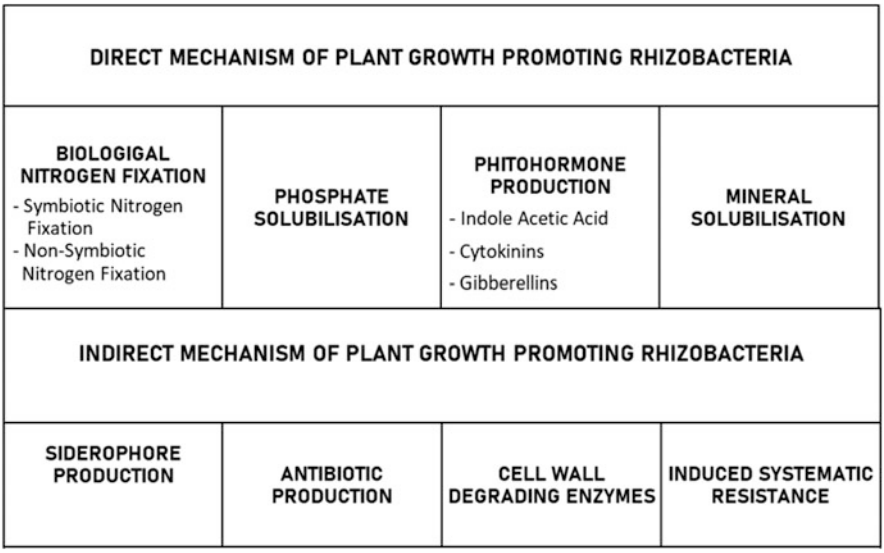

Fig. 22.2 Direct and indirect mechanisms of plant-growth-promoting rhizobacteria

Nitrogen occurs in the atmosphere as N2, a form that is not useable by plants. Biological nitrogen fixation is the process by which molecular nitrogen can be fixed by some organism prokaryotes; hence, it is important in improving the fertility and productivity of low-N soils (Singh 2018). The selection and proliferation of bacteria depend on exudates from roots and the ability of the microorganism to utilize organic matter as the source of nutrition. They are categorized into two major groups; (1) symbiotic rhizobacteria that invade the interior of the cell and provoke the formation of nodules and (2) free-living rhizobacteria that exist outside the plant cells (Odoh 2017).

In symbiotic nitrogen fixation, bacteria such as *Azorhizobium*, *Allorhizobium*, *Rhizobium, Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* fix nitrogen only in association with certain plants (Bhat et al. 2019; Odoh 2017). Symbiotic nitrogen fixation by *Rhizobium* has been extensively studied in leguminous plants; however, only certain legume crops are benefited from this symbiosis (Katiyar et al. 2016). In the case of non-leguminous plants, a symbiotic relationship with *Frankia* (a nitrogen-fixing Actinomycete) has been reported, leading to the formation of root nodules in trees and shrubs, assigning an important ecological role to this symbiosis (Cissoko et al. 2018).

Microorganisms living freely show the non-symbiotic nitrogen fixation. The genera identified in this group include, e.g., *Azotobacter*, *Azospirillum*, *Clostridium*, *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Corynebacterium*, *Derxia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas*, and *Xanthobacter* (Bhat et al. 2019). *Azospirillum*–plant association enhanced development and yield of different

plants, among them grasses, mainly by an increase in water and mineral uptake and, to a lesser extent, biological N2 fixation (Katiyar et al. 2016). N-fixing bacterial strains *Pseudomonas putida* RC06, *Paenibacillus polymyxa* RC05, and RC14, *Bacillus* strains, and *Azospirillum brasiliense* sp. 246 have great potential, and as formulations, they are used as biofertilizers for better yield and the quality of barley (Cakmakci et al. 2007; Canbolat et al. 2006).

Phosphorus is the other nutrient that limits the growth of crops due to the form in which it is mainly present in the soil, which is insoluble phosphate; thus it is difficult for plants to absorb it. Therefore, microbial solubilization of inorganic phosphate compounds is of great economic importance in crop nutrition. Certain PGPR are capable of solubilizing phosphate through various mechanisms, and it has resulted in the lesser requirement of phosphate along with the enhanced quality of yield, efficient use of fertilizer, lesser pollution, and more eco-friendly agriculture (Bechtaoui et al. 2019; Singh 2018). One of the common mechanisms of phosphate solubilization by phosphate solubilizing bacteria (PSB) strains involves secretion of organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms. Also, some PSB produce phosphatase-like phytase that hydrolyzes organic forms of the phosphate compound efficiently (Katiyar et al. 2016; Zaidi et al. 2009). Among the PSB genera are *Arthrobacter, Pseudomonas, Alcaligenes, Bacillus, Burkholderia, Serratia, Enterobacter, Acinetobacter, Azospirillum, Azotobacter, Flavobacterium, Rhizobium,* and *Erwinia* (Zaidi et al. 2009).

The ability of PGPR to solubilize potassium rock by producing and secreting organic acids has also been reported. Potassium-solubilizing PGPR, such as *Acidithiobacillus* sp., *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Ferrooxidans* sp., *Pseudomonas* sp., *Burkholderia* sp., and *Paenibacillus* sp., have been reported to release potassium in accessible form from potassium-bearing minerals in soil (Kumar et al. 2018).

Iron is one of the essential elements for the growth and development of a plant particularly toward respiration, nitrogen fixation, and photosynthesis. Despite the enormous amount in which it is present at the surface of the earth, it is very rarely available for the plant because it is present in the form Fe3+, which is insoluble and hence unavailable for plant uptake (Lal 2018). Certain PGPR contribute to improving the availability of iron for the plant by releasing chelating compounds called siderophores that attract iron to the rhizosphere and reversibly bind to it. Once inside the cell, the iron is released and is then available to support microbial growth. Siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit the growth of other microorganisms with the release of their antibiotic molecule, and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron siderophore complex (Bhat et al. 2019). Indirectly, rhizobacteria help plant growth by releasing biocontrol agents for protecting plants against phytopathogens (Jha and Saraf 2015). Bacteria usually belonging to *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Rhodococcus* genus are known for the production of siderophores. The most researched microorganisms for the production of siderophores are *Pseudomonas*

aeruginosa and *P. fluorescens,* which produce pyoverdine and pyochelin kinds of siderophores (Singh 2018).

22.3.2 Phytohormone Production by PGPR

One of the most widely documented beneficial effects of PGPR is in the production of phytohormones (indole acetic acid, gibberellins, cytokinins, and ethylene), needed for growth promotion and adaptation in a stressed environment, being an efficient tool for agriculture (Odoh 2017). The major hormones produced are indole acetic acid (IAA), which plays an important role in rhizobacteria–plant interactions. It has been reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Spaepen and Vanderleyden 2011). The IAA synthesized by PGPR promotes root hair development and root proliferation, which in turn improves the absorption capacity of nutrients from the soil. Another significant result of inoculation with auxin-producing bacteria is the formation of adventitious roots, which derive from the stem by induction of stem tissues to redifferentiate as root tissue (Jha and Saraf 2015).

Microbes that induce production of phytohormones play a vital role in shoot and root invigoration such as *Rhizobium leguminosarum*, *Pantoea agglomerans*, *Rhodospirillum rubrum*, *Pseudomonas fluorescens*, *P. aeruginosa*, *P. putida*, *Bacillus subtilis*, *Azotobacter chroococcum*, *Enterobacter asburiae*, *Paenibacillus polymyxa*, *Stenotrophomonas maltophilia*, *Mesorhizobium ciceri*, and *Klebsiella oxytoca*, which are regarded as PGPR (Bhat et al. 2019). The improvement of plant growth by some rhizobacteria (PGPR) producing gibberellins was reported by Kang et al. (2009). Research has shown that inoculating seedlings with cytokinin-producing strains of *Bacillus subtilis* confers the plant's resistance against environmental stress (Odoh 2017).

PGPR capable of inducing exogenous production of ethylene via degradation of the endogenous product using enzyme include *Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia*, and *Rhizobium* (Odoh 2017). Pierik et al. (2006) suggested that at a low concentration of ethylene mediated by PGPR, the plant yield, growth performance, and germination properties *of Arabidopsis thaliana* get accelerated.

22.4 Biological Nitrogen Fixation and Its Importance for Grain Crops

All organisms require nitrogen (N) to grow and incorporate into organic molecules like DNA. There are different ways to N availability; one is the mineralization of organic matter of crops that responds to N availability in the soil and favors a better root development of plants and indirectly by releasing cationic micronutrients with acidification of the root zone. Another way by nitrogen is biological fixation (BNF) due to the natural consumption of the large N reservoir in the gaseous atmosphere that transformed N2 into NH_3 through two processes. BNF is mediated by microorganisms that are distributed in several ecological and physiological groups and belong to several taxa, which include aerobic heterotrophic, facultative and obligatory anaerobic bacteria, chemical-autotrophic and photo-autotrophic bacteria, associative bacteria (*Azospirillum* sp., *Herbaspirillum* sp.), and endophytic bacteria. The natural bacteria of the soils (telluric), Gram-negative, generically called rhizobia, capable of forming nodular structures, are located, generally, in the roots of legumes and intermediate the process of biological N_2 fixation (Giller 2001). BNF is performed by a large functional group of microorganisms, which are prokaryotes (eubacteria and archaebacteria) in association or symbiosis with plants (Giller 2001).

Briefly, BNF is a set of oxide-reduction chain reactions for the enzymatic breakdown of the atmospheric N_2 triple bond, transforming it into forms readily assimilated by plants.

$$N \equiv N + 8H^{+} + 8^{e-} \rightarrow 2NH_3 + H_2$$

Oxidation-reduction reactions aiming at transforming N_2 to the combined inorganic form (NH_3) are mediated by prokaryotic microorganisms that acquired, during its evolution, the ability to synthesize the enzymatic set composed of two proteins, Mo-Fe protein and dinitrogenase reductase (Fe-protein).

The symbiotic efficiency association of nitrogen-fixing bacteria, called *rhizobia*, with plants, depends on the interaction between chemical and physical factors in the soil. The supply of N to plants via BNF presents a series of agronomic, economic, and environmental advantages to the use of N fertilizers. The crop plant response to inoculation with rhizobia depends, featuring in the interaction between the rhizobia (micro-symbiont) and the legume (macro-symbiont), on a series of edaphic-climatic factors that affect the BNF efficiency, in addition to factors such as photosynthesis, light, temperature and humidity that will also alter the effectiveness of the symbiosis between plant bacteria, being essential for the growth of the plant.

BNF represents a clean N source for the agroecosystem; however, for high yields of some crops, e.g., common bean (*Phaseolus vulgaris*), after seed inoculation, lower doses of N fertilizers applied as topdressing in the initial stages are still necessary. In tropical and subtropical agriculture, the interactions of the physical, chemical, and biological factors are strong and all these could affect the nodulation

and the efficiency of the symbiosis as well as the crop management in the rhizobia-leguminous symbiosis.

The symbiotic association between rhizobia and legumes, like any biological interaction, is altered by changes in the soil environment and by several biotic factors, for example, microbial antagonism, diseases, pests, and it also depends on several factors inherent to the host plant and the bacteria inoculated or present in the soil.

The benefits from the interaction between leguminous plants and N_2-fixing bacteria depend on many factors, including the bacteria number inoculated on the seeds after sowing, the water, and the temperature in the soil. Inadequate soil management, crop rotation exclusively with grasses, reduced bradyrhizobia diversity in soybean (Ferreira et al. (2000), and soil acidity decreased the rhizobia diversity in common bean sites (Andrade et al. 2002).

When alterations occur in the soil-plant system, the diversity of microorganisms seems to be the first to demonstrate how tillage and its predecessor crops could affect bacterial N-fixers' occurrence and diversity in the soil (Andrade et al. 2003). The positive effects of no-tillage, especially under tropical conditions, could be attributed to several factors, such as lower soil temperature, higher soil moisture content, preservation of soil aggregates, and higher carbon content, favoring soil biomass and many classes of microorganisms, including rhizobia, which results in higher nodulation.

The liming of acidic soils is a fundamental practice not only for the use of nutrients by plants and this agricultural practice, this correction of acidity, is also essential for the success of BNF, because of its direct effect on the revision of active acidity (H+) and in neutralizing the exchangeable acidity (Al^{3+}) of the soil. Soil acidity is a severe problem to rhizobia survival due to the low pH and the toxicity of aluminum, iron, manganese, and deficiencies of some other micronutrients. In acidic and low-fertility soils, after liming to correct associative acidity, the population of naturalized rhizobia nodulating common bean increased in number and diversity, and consequently, the possibility of more efficient strains also increased (Andrade et al. 2002) (Fig. 22.3).

There was a significant relationship between grain yields, production of common bean, and rhizobia diversity, measured by the number of groups formed by cluster analysis based on the polymorphism of the intergenic region (ITS) of the 16-23S rRNA, according to limestone doses in acidic soil (Fig. 22.3a, b)

Co-inoculation studies have been encouraged with the use of Azospirillum with *Bradyrhizobium* bacteria, in a current approach aimed at agricultural, economic, social, and environmental sustainability (Hungria et al. 2013b; Hungria et al. 2015a, b) besides contributing to the main limitations of BNF, obtained through the traditional inoculation of soybean only with bacteria of the genus *Bradyrhizobium* and for common bean. The inoculation with *Rhizobium tropici* alone or combined with *Azospirillum brasilense* enhanced root nodulation and grain yield of the common beans when the crop was grown during the dry season in the Brazilian savanna region. This suggests that the positive response of inoculation with *Rhizobium* alone or with *Azospirillum brasilense* on the grain yield is

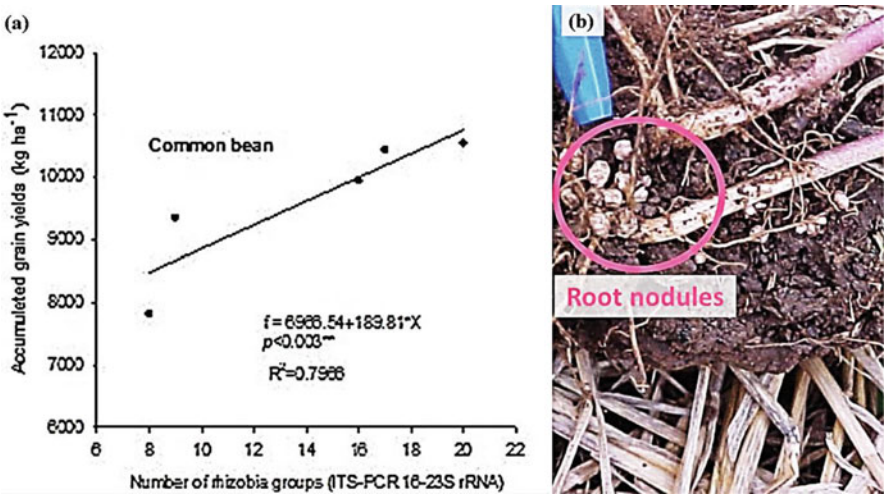

Fig. 22.3 (**a**) Relationship between rhizobia diversity and five years of accumulated grain yields of the common bean, in an acidic soil with different liming doses' graphical regression based on data from Andrade et al. (2002) and Parra and collaborators (1996) (**b**) Root nodules effective in the common bean. Photo Diva S Andrade

dependent on the environmental conditions during the growing season, especially the lower rainfall rate by stimulating root nodulation (Steiner et al. 2019).

22.4.1 Bacterial Inoculant Products and Efficiency

Inoculation can be defined as the technique of placing viable cells from the microsymbiont, the specific and efficient rhizobia strain, in sufficient quantity and the closest to the seed to provide conditions to form the symbiosis. Inoculation represents a way of supplying N continuously and according to the requirements of the plant, without risk of loss of nitrate (NO_3) via leaching, which is highly polluting.

In Brazil, at the Agronomic Research Institute of The State of Rio Grande do Sul (IPAGRO) group, in 1949, professor João Rui Jardim Freire started a program of rhizobia strain collection and the small-scale production of inoculants in the state of Sao Paulo (Freire 1982). This collection has about a 1000 species, being recognized worldwide for the deposit and maintenance of nitrogen-fixing bacteria.

In Brazil, the inoculants produced, imported, and marketed in the national territory must be registered with the competent agency of the Ministry of Agriculture, Livestock and Supply because the inoculation of the crop seeds must be done with rhizobia inoculant produced according to the norms of guarantees, registration, packaging, and labeling and containing nitrogen-fixing microorganisms authorized and recommended for the crop according to Brazilian legislation (MAPA—Ministério da Agricultura PeA 2011). For soybean cultivated under tropical

conditions, independent of whether in soils with or without previous history, it was recommended that the minimum inoculum rate could be 1.2×10^6 cells seed^{-1} (Hungria et al. 2017). In Brazil, inoculants used for soybean in the year 2018–2919 had excellent quality, with the number of cells above guarantee (de Souza et al. 2019). Soybean is the most inoculant-consuming crop worldwide, carrying bacteria belonging to the genus *Bradyrhizobium* sp.. In Brazil, of a total of 70 million doses of inoculant commercialized per year, approximately 90% is for soybean crops (Santos et al. 2019).

When grain leguminous crops such as soybeans, groundnuts, and common beans are inoculated, most of the nodules are concentrated in the crown region of the roots, and generally, this quantity of nodules are correlated positively with the total nodulation; therefore, the procedure for assessing nodulation can be simplified and restricted to an area of 7–10 cm of roots from the cotyledon (Cardoso et al. 2009). The efficiency of the inoculation can be evaluated as follows: (a) Number and size of nodules: indicative of sufficiency is the presence of 10–15 nodules, with 2–4 mm in diameter each, in the region of the root crown; or more than 20 nodules per plant; (b) distribution of nodules: the presence of nodules in secondary roots is indicative of nodulation by native population; (c) internal staining: pink or reddish color of the nodule is indicative of an active nodule in fixing N as well as striated and rough surface nodules; (d) aspect of the plants: the leaves must present an intense green color, indicating an adequate supply of N; plants with insufficient development and chlorotic are indicative of N deficiency.

22.4.2 Crop Responses to Microbial Inoculation

The contribution of biological nitrogen fixation to grain production depends on factors such as soil, climate, crop management, planting system, host plant, and rhizobia or *Bradyrhizobium* strain. Considering the interdependence of the chemical, physical, and biological factors that contribute and/or alter the N_2 rates fixed by the rhizobia-leguminous symbiosis, the goal of a productive and sustainable agricultural system in terms of N is the search for the balance of these factors. The increase in the amount of N_2 fixed biologically by symbiosis with native and inoculated strains represents a rational alternative for the supply of nitrogen to crops and the balance of this nutrient in agro-systems, without risk of environmental pollution. BNF needs to be treated as a source of N of fundamental importance, and that can result in increases in productivity associated with quality and sustainability.

The use of bacterial inoculants such as *Rhizobium, Bradyrhizobium*, and *Azospirillum* for production has been providing a consistent response for high levels of yield and cost/benefit as for soybean (Amaral and Lucas 2019; Döbereiner 1997) for common bean (Soares et al. 2016) and soybean and common bean inoculation in northern Tanzania (Ndakidemi et al. 2006). According to Santos et al. (2019) using *Bradyrhizobium* sp. inoculant for soybean cropping without any N-fertilizer has generated an annual economy that is estimated at about 20 billion dollars. Based on

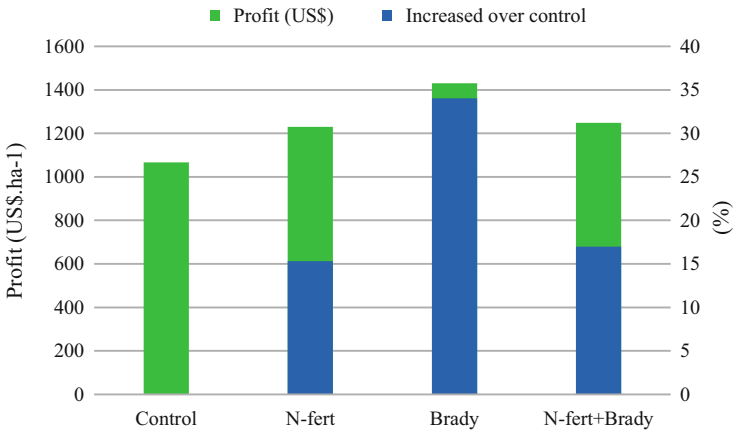

Fig. 22.4 Estimate increase of profit for soybean inoculated with *Bradyrhizobium* recommended strains. Adapted from Amaral and Lucas (2019)

the Ndakidemi et al. (2006) data for financial *profit*, and modified from Amaral and Lucas (2019), the use of *Bradyrhizobium* sp. inoculation for soybeans corresponded to a profit of US $ 363 per hectare and 34% concerning the control and US $ 199 per hectare and 18.7% concerning N-fertilizer (Fig. 22.4).

Although there are some arguments, such as time, handling, and adverse environmental conditions that are used to avoid using rhizobia/bradyrhizobia, there is no doubt about the economy of inputs for N-fertilizer, which would already compensate the farmer for carrying out bacterial inoculation carefully.

22.5 Cyanobacteria Based in Inoculants for Plants in the Crop Production

Cyanobacteria (blue-green algae) are Gram-negative photosynthetic oxygen prokaryotes with a long evolutionary history and are probably the most primitive photosynthetic organisms found on earth and in the polar and alpine regions, where they are major primary producers in the food chain, carbon and participate in the nitrogen cycle (Larkum 2020; Margesin et al. 2008).

Cyanobacteria are species that vary in unicellular or colonial and filamentous forms and morphologically resemble some fossils that occurred 1–3 billion years ago (Gupta et al. 2013; Stewart 1971). They have two types of specialized cells called heterocysts and akinetes. Heterocysts protect the enzyme nitrogenase from oxygen, catalyzing the conversion of N_2 to ammonia, making N available and developing from vegetative cells. Akinetes are cells resistant to unfavorable environmental conditions (Grover et al. 2020). Their function can be compared to that of bacterial spores, and their protoplasm accumulates a large amount of nutrient reserves

(Fleming and Haselkorn 1973). Their reproduction is asexual and occurs either through cell division, colony fractionation, the formation of hormogonies, or akinetes (Barsanti and Gualtieri 2014).

Cyanobacteria have attracted interest in the production of sustainable energy. As they are aquatic photoautotrophic organisms, they can be produced on non-arable land and do not compete for land for food production. Their abundant genetic resources offer a means of conducting metabolic pathways for the synthesis of a valuable biological basis for various products (Rosgaard et al. 2012). They can be used as food, feed, fuel, fertilizer, dyes, in production of various metabolites, including vitamins, enzymes, medications, and pharmacological probes, increasing their use day by day. The soil is the habitat of some species of terrestrial cyanobacteria that are beneficial for soil fertility, fixing atmospheric nitrogen (N), helping to structure the soil, maintaining humidity, and preventing erosion (Shariatmadari et al. 2013).

In agriculture, there is potential for the use of its biomass as a soil conditioner and/or biofertilizer. Also, there is a little-studied effect on plant growth, which can occur due to substances that stimulate this growth or even BNF. Exploring these potentials opens up possibilities for studies related to the inoculation of cyanobacteria capable of providing nitrogen for crops of economic interest, such as plants of the Poaceae families (grasses), such as wheat *Triticum sativum* L. (EL-Shinnawil et al. 2016) maize *Zea mays* L. (Zambrano Gavilanes et al. 2019), rice *Oryza sativa* L. (Jing et al. 2019), sorghum *Sorghum bicolor* L. Moench (Ramond et al. 2013), and Fabaceae (legumes), such as peanuts *Arachis hypogaea* L. (Dai et al. 2019), common beans *Phaseolus vulgaris* L. (Pichardo and Pflugmacher 2011), and soybean *Glycine max* L. (Jadhav and Talekar 2020), or even the inoculation of them with other N-fixing bacteria, to increase the supply of this nutrient to plants (Andrade and Colozzi Filho 2014) as the co-inoculation of *Anabaena cylindrica* with *Azospirillum brasilense* that increases the grain yield of maize hybrids (Gavilanes et al. 2020), the co-inoculation of rhizobium, azospirillum, and cyanobacteria in increasing common bean production (Horácio et al. 2020).

The taxonomy of cyanobacteria is developed in botany because they are morphologically visible organisms, similar in appearance to algae, and have traditionally been identified as such. Therefore, its nomenclature was covered by the provisions of the botanical code of nomenclature, currently the International Code of Nomenclature for algae, fungi, and plants, ICN, (including previous versions, known as the International Code of Botanical Nomenclature, ICBN) (Oren and Ventura 2017).

In view of the morphological diversity of cyanobacteria, an obvious issue is the extent to which this is accompanied by physiological, biochemical, and molecular diversity. Most experimental studies have focused on a limited number of genera and species, and relatively few studies have deliberately established to investigate diversity at these other organizational levels. While most physiologists and biochemists perceive this problem in theory, in practice, they often appear in the

literature that does not take into account the less-studied organisms (Whitton 1992; Whitton 1992).

The genera *Nostoc* and *Anabaena* have been widely studied due to several biotechnological uses. *Nostoc* is a genus of cyanobacteria of the order Nostocales and family Nostocaceae, which can form macroscopic or microscopic colonies and is common in terrestrial and aquatic habitats. Much of Nostoc's success in terrestrial habitats is related to its ability to remain desiccated for months or years and to recover metabolic activity within hours to days after rehydration fully. *Nostoc* can also withstand repeated freeze-thaw cycles and is, therefore, an essential component of extreme terrestrial habitats in the Arctic and Antarctic. The ability to fix atmospheric N can provide an advantage in nitrogen-poor environments (Dodds et al. 1995).

Nostoc muscorum is a free-living microorganism with filamentous, Gram-negative cells (Allison et al. 1937). Differentiation of 5–10% of the cells is observed when the filaments are transferred from a container of medium containing NH_4Cl or Ca $(NO_3)_2$ to a medium free of N. Differentiated cells, called heterocysts, can be observed at intervals regular across the length of the filament 24 hours after the transfer (Fleming and Haselkorn 1973).

The ideal environment for *N. muscorum* is a pH in the range of 7.0–8.5, with a lower pH limit of 5.7. It grows best when the light intensity is lower than direct sunlight, but it can continue to grow and fix nitrogen in the presence of glucose and the absence of sunlight (Allison et al. 1937).

Anabaena is a genus of cyanobacteria of the order Nostocales and family Nostocaceae, photoautotrophic of asexual reproduction, commonly found in freshwater, forming heterocysts and performing oxygenic photosynthesis. *Anabaena* grows in long filaments of vegetative cells (Starr et al. 2013). Some cyanobacteria of the *Anabaena* genus produce pigments of commercial interest, biofertilizers, and polysaccharides (Loreto et al. 2003).

Anabaena cylindrica, in addition to contributing to BNF, is active in the solubility of iron by producing siderophores (Itou et al. 2001). Another very important use is for biofuels such as hydrogen production (Benemann and Weare 1974) and biomass for biodiesel (Patel et al. 2017).

Cell differentiation in Anabaena cylindrica is accompanied by characteristic changes in the composition of pigments from heterocysts and spores (Fay 1969).

22.5.1 Cultivation of Cyanobacteria

For a culture of microorganisms to be successful, several environmental and operational factors affect biology and habitat and must be taken into account. These factors also affect the biomass productivity of cyanobacteria as well as their composition. The most important factors are nutrients, pH, light, cell density, temperature, and contamination by other microorganisms (Markou and Georgakakis 2011).

Cyanobacteria have a wide variety of forms due to the morphological, physiological, and biochemical adaptations acquired throughout their evolutionary history. The ability to grow in the most different media is one of the hallmarks of cyanobacteria. However, freshwater environments are the most favorable for their growth. Most species show better growth in waters with pH values in the range 6–9, temperature between 15 and 30 °C, weak and moderate winds, and a high concentration of nutrients. Its vital processes require only water, carbon dioxide, inorganic substances, and light, obtaining energy, mainly through photosynthesis (Barbosa 2009).

Cyanobacteria have three types of metabolism, regarding the use of carbon for the synthesis of biomass: Mandatory photoautotrophic: grow only in the presence of light in an inorganic medium and are unable to assimilate organic C (Huang et al. 2010).

Optional chemoheterotrophic: live in an environment without light in a medium containing a source of organic C (maltose, glucose, sucrose, lactose, glycerol, and starch) or phototrophically in light. Optional photoheterotrophic: use organic compounds as a source of C in light but not in the dark or phototrophically in light (Moon et al. 2013; Zhang et al. 2011).

Cyanobacteria require minimal concentrations of nutrients (N, P, S, K, Fe, etc.), which constitute the different synthetic culture media specific to their development, the most used being shown in Table 22.1.

22.5.2 Biological Nitrogen Fixation in Cyanobacteria

Cyanobacteria are the most abundant photosynthetic organisms on earth, playing essential roles in the renewal of atmospheric oxygen and the production of biologically available nitrogen, both in free life and in symbiosis (Muro-Pastor et al. 2017).

Cyanobacteria with symbiotic competence are found mainly in the genus *Nostoc*, which is common in terrestrial environments. However, some other heterocyclic cyanobacteria (symbiotic cyanobacteria), such as *Calothrix* and *Scytonema* (in lichens and cycads) and *Richelia* (in diatoms), also form a symbiosis (Bergman et al. 2007).

Cyanobacteria have a broad ecological distribution that varies from deserts to all types of water reservoirs, suggesting sophisticated adaptation mechanisms. Filamentous are complex prokaryotes that exhibit a variety of cell differentiation processes, including the formation of spore-like cells, mobile hormogonia, or nitrogen-fixing heterocysts. Heterocytic cyanobacteria are filamentous photosynthetic organisms that grow as strands of undifferentiated photosynthetic cells (called vegetative) or, due to nitrogen deficiency, as filaments containing vegetative cells and heterocysts, the latter specialized in fixing atmospheric nitrogen (Muro-Pastor et al. 2017).

N_2 fixation is performed exclusively by prokaryotes (Bacteria or Archaea) in an anoxic environment or in intracellular micro-toxic conditions that are appropriate for the expression and function of the N_2 fixation enzyme, nitrogenase. This enzyme is

Table 22.1 Synthetic culture media BG-11, Zarrouk's medium, and high salt medium, for growth and maintenance of cyanobacteria

	(Stanier et al. 1971)	(Zarrouk 1966)	(Sueoka 1960)
Reagents	BG-11	Zarrouk's medium	High salt medium
K_2HPO_4 (g L^{-1})	0.04	0.50	1.44
$MgSO_4$ $7H_2O$ (g L^{-1})	0.075	0.20	0.02
$CaCl_2$ $2H_2O$ (g L^{-1})	0.036	0.40	0.01
$C_6H_8O_7$ (g L^{-1})	0.006	–	–
$C_6H_{5\ +\ 4}FexNyO_7$ (g L^{-1})	0.006	–	–
EDTA (g L^{-1})	0.001	0.08	–
Na_2CO_3 (g L^{-1})	0.02	–	–
Trace metal mix (mL^{-1})	1.00	–	–
$NaHCO_3$ (g L^{-1})	–	16.8	–
$NaNO_3$ (g L^{-1})	1.5	2.50	–
K_2SO_4 (g L^{-1})	–	1.00	–
NaCl (g L^{-1})	–	1.00	–
$FeSO_4$ $7H_2O$ (g L^{-1})	–	0.01	–
Solution A5 mL	–	1.00	–
Solution B6 mL	–	1.00	–
KH_2PO_4 (g L^{-1})	–	–	0.72
NH_4Cl (g L^{-1})	–	–	0.50

Solution A5 (g L^{-1}): 2.86 × 10^{-3} H_3BO_3; 1.81 × 10^{-3} $MnCl_2$ $4H_2O$; 0.22 $ZnSO_4$ $7H_2O$; 0.39 Na_2MoO_4 $2H_2O$; 0.079 $CuSO_4$ $5H_2O$. Solution B6 (mg L^{-1}): 22.86 NH_4VO_3; 192 KCr $(SO_4)_2$ $12H_2O$; 44.8 $NiSO_4$ $6H_2O$; 17.94 Na_2WO_4 $2H_2O$; 61.1 $TiOSO_2$ $8H_2O$; 43.98 $Co(NO_3)_2$ $6H_2O$

encoded by the nifHDK operon and is extremely sensitive to oxygen. In a photosynthetic cyanobacterial filament, the heterocyst provides a micro-toxic site for N_2 fixation. Therefore, in these organisms, the compartmentalization of oxygenic photosynthesis and the fixation of N_2 in different types of cells is the solution to the problem of incompatibility between the two processes (Flores and Herrero 2010).

22.5.3 Use of Cyanobacteria as an Inoculant in Several Cultures

Cyanobacteria can fix atmospheric nitrogen and transform it into forms available for cultivation, which has increased interest in its use as an inoculant (Zambrano Gavilanes et al. 2019) and can be applied in different cultures.

Nostoc commune, *Anabaena iyengarii*, *Nostoc linckia*, and *Nostoc sp*. in proportions (45, 25, 25, and 5% respectively) were used as biofertilizer in rice culture (Pereira et al. 2009), allowing a reduction of up to 50% in the use of synthetic nitrogen fertilizer (50 kg N ha^{-1}), resulting in the same grain yield (7.4 t ha^{-1}) and quality in relation to the fertilized control. The use of biofertilizers based on local strains of cyanobacteria is promising to increase the efficiency of nitrogen use in rice.

Inoculation with the cyanobacterium *Calothrix elenkinii* under cowpea resulted in growth and may help in the general improvement of the N status of plants and soil (Dey et al. 2017). Ahmed et al. (2010) studied inoculation with cyanobacteria *Chroococcidiopsis sp*., *Leptolyngbya sp*., and *Phormidium sp*. extracted from a rice field, in seedlings 10 days old of *Triticum aestivum*, *Vigna radiata,* and *Pisum sativum* incubated for 1 week, have shown to have positive effects on the biological fixation of N. Such cyanobacteria can be applied in the field as soil inoculants in combination with fertilizers. Nitrogen-fixing cyanobacteria have also been reported in sugarcane cultivation in India (Thajuddin and Subramanian 2010).

Jäger et al. (2010) made the first report on the effect of microalgae and cyanobacteria on anthers in maize. Their research was on the activity of cytokinin and auxin, improving the androgenic response and they were able to reduce the amount of synthetic auxin of 2.4 dichlorophenoxy-acetic acid (2,4-D) needed or to replace it completely.

Research conducted in South Africa was carried out to evaluate the effects of inoculation of a *Nostoc* cyanobacterial strain on soil structure and fertility and maize growth. The *Nostoc* suspension was applied uniformly to the soil in pots at a rate of 6 g (dry weight) per square meter right after the germination of the maize. Inoculation with *Nostoc* increased the N content in the soil from 17% to 40%. The carbon content has also increased significantly. Inoculation with Nostoc increased the dry matter production of maize by 40% and 49%. The corresponding increases in N content in the maize tissue were between 14% and 23% (Maqubela et al. 2009).

Three species of red seaweed *Laurencia obtusa*, *Corallina elongata*, and *Jania rubens* were studied for their effect as a biofertilizer on the growth of maize plants. *Laurencia obtusa* + *Jania rubens* was the best treatment to increase the growth of maize plants during the first stages of the crop, with the best growth of the aerial part of the plants increasing by 48.21% after 60 days of planting (Sainaz and Ragaa 2013).

In Egypt, research with the cyanobacteria *Nostoc muscorum* and *N. rivulare* indicated that *Nostoc* nitrogenase activity was significantly increased compared to the root system of uninoculated maize (Sholkamy et al. 2012).

In order to evaluate the influence of cyanobacteria inoculation from the IPR collection of IAPAR on the development of maize plants, an experiment was conducted at the Instituto Agronômico Paraná, carried out in a greenhouse, with sterile soil for 35 days. The maize seeds used were from the cultivar IPR 164, soaked in a suspension of each of the cyanobacteria, which were grown in the BG-11 medium, using seeds embedded in the culture medium as control. Plant height, root length, stem diameter, dry shoot weight, dry root weight, and nitrogen content of the shoot were evaluated. There was an increase in the dry mass of the root, promoted by the inoculation of the cyanobacterium *IPR7029* in relation to the control and those inoculated with *IPRSyn7062*. Although cyanobacteria species show the ability to perform BNF in vitro, the significant effect of inoculation on the N concentration of the aerial part was not observed. In the other evaluations, with

the exception of dry root mass in natural soil, there were no significant differences between treatments (Andrade and Colozzi Filho 2014).

Inoculation of cyanobacteria in maize results in higher plant growth, improving nitrogen absorption (Maqubela et al. 2009). (Prasanna et al. 2016) found significant differences in plant height, higher than the control when inoculated with cyanobacteria. In the evaluation of the maize grain yield, however, they did not find significant differences in relation to the control treatment, with a yield between 6.45 and 6.88 g per plant.

In a research were tested four species of cyanobacteria *Nostoc muscorum*, *Anabaena sp*., *Anabaena cylindrica,* and *Calothrix brevissima* (Zambrano Gavilanes et al. 2019) as an inoculant in maize, determined that the most effective inoculation treatments were when *Nostoc muscorum* and *Anabaena cylindrica* were used. The results show the stimulating effect of cyanobacteria to promote growth in maize.

(Gavilanes et al. 2020) tested the effects of inoculation and co-inoculation of *Anabaena cylindrica* with *Azospirillum brasilense* on the development and productive performance of four maize hybrids. Co-inoculation favored the development and yield of maize hybrids, with an increase between 9.37% and 23.1%, compared to the uninoculated control. Another co-inoculation has been investigated among rhizobia, azospirilla, and cyanobacteria in the production of common beans, which has caused an increase in nodulation, plant growth, and production (Horácio et al. 2020), demonstrating that cyanobacteria together with growth-promoting bacteria can live together and add benefits to cultivars.

22.6 Soil Fertility Attributes Related to Symbiosis Efficiency

The main abiotic factors linked to soil fertility and fertilization that affect BNF are soil acidity (low pH), aluminum toxicity, low soil fertility, and availability of mineral N (Howieson and Ballard 2004; Hungria and Vargas 2000). These limitations can act both in bacteria and the host, affecting symbiosis.

According to Câmara (2014), an efficient BNF occurs if the soil has a balanced chemical fertility, which makes nutrients available to plants. According to Marschner (2011), almost all nutrients have specific functions that are directly or indirectly linked to BNF, as shown in Table 22.2.

However, in the literature, in addition to pH and toxicity caused by excess Al, the presence of mineral N, the deficiencies of P, S, Co, and Mo are the ones that most affect BNF.

Table 22.2 Mineral nutrients, functions, and consequences of their deficiencies for BNF

Nutrient	Functions	Effects of the element's deficiency
Phosphorus	Directly related to ATP production and consumption	Nodulation and BNF reduction, leading to N deficiency
Potassium	Activator of various enzymes in photosynthesis and respiration	Reduction of nodule dry mass with reduction in BNF
Calcium	Root growth and performance in messenger proteins of chemical signals	Nodulation reduction due to reduction in root surface
Magnesium	Constituent of chlorophyll, it is directly related	N deficiency induced by BNF reduction
Sulfur	Constituent of secondary metabolites that act on soybean nodulation factors (Nod)	Delay and reduction in root nodulation
Boron	Cell division	Decrease in nodule sizes.
Copper	Not yet understood	Reduction in BNF
Iron	Nitrogenase iron-protein constituent	Delayed onset, decreased nodulation and reduced BNF
Molybdenum	Constituent of nitrogenase molybdate-iron-protein	Ineffective nodulation and N deficiency
Nickel	Plant enzyme constituent (urease) and required for H_2 reprocessing (hydrogenase)	Delay in nodulation and reduction of BNF
Zinc	Operates in the synthesis of leghemoglobin	Reduction of the number and size of nodules
Cobalt (beneficial element)	Component of cobalamin (B12), precursor of leghemoglobin	Delayed onset and decreased nodulation

22.6.1 Acidic pH and Excess of Al

Soil acidity is measured by the concentration of H+ ions in the soil solution. In acidic soils, aluminum is one of the most important factors limiting production. When the soil pH is reduced (pH <5.5), aluminum and low-solubility minerals that contain aluminum are dissolved and toxic levels of this element are released into the soil solution (Silva et al. 2002).

In addition to Al toxicity, other factors are associated with soil acidity, especially in tropical soils, such as Mn toxicity, low Cation Exchange Capacity (CEC), and availability of nutrients.

In addition to the hosts having development difficulties in acidic soils, microsymbionts are also affected, sometimes to a greater degree. Rhizobia generally have their survival and multiplication hampered in conditions of soil acidity, which reduces nodulation and BNF (Albareda et al. 2009; España et al. 2006; Graham 1992). The acidity of the soil makes it difficult to exchange signals between rhizobia and plant roots (Rufini et al. 2014).

Souza et al. (2010), evaluating the soybean culture, found that the pH of the soil influenced the number of nodules and found that the initial pH changed the production of nodules, with a higher number in higher soil pH (pH 6.3).

22.6.2 Mineral N

The presence of mineral N negatively affects BNF. Brady and Weil (2009) claim that legumes invest energy in BNF only when N levels are low in the soil.

Nitrogen fertilization reduced the nodulation process and the symbiotic nitrogen fixation in soybean crops (Novo et al. 1999). Zuffo et al. (2019) found that nitrogen fertilization in soybean culture inhibited nodule production, volume, and mass. Yagi et al. (2015) also found that nitrogen fertilization caused a significant reduction in the amount of nodules produced by bean cultivars inoculated with *Rhizobium freirei*.

Giller and Wilson (1991) affirm that the N dose necessary to cause suppression of nodulation varies with the legume species and that its addition did not alter the number of nodules per plant, but its mass.

Santos et al. (2014) also found that nitrogen fertilization reduced the production of nodules, with a more evident effect in reducing the mass of nodules in the crown region and secondary nodulation.

22.6.3 Phosphorus

As functions of P, we can mention its participation in energy production processes for the plant's metabolic activity. Thus, it participates in the breathing, synthesis, and transport of carbohydrates and active absorption of nutrients. It is a structural component of nucleic acids, nucleotides, and phospholipids in the cell membrane.

Under P deficiency, plants show reduced growth and root and shoot development. The stems become shorter and thinner. The plants produce few flowers and pods per plant (Fig. 22.5).

P deficiency can directly affect BNF, influencing nodule formation, growth, and activity. According to Al-Niemi et al. (1997) for nitrogenase activity, high demand for ATP is required, which depends on the availability of P. P deficiency altered the nodulation of three forage legumes (Krolow et al. 2004). In soybeans, increasing the doses of P also increased the mass of the rhizobial nodules (Soares 2013).

Fig. 22.5 In the two photos, the plants on the left were grown without application of P and those on the right, with application of this element (100 kg/ha of P_2O_5). (**a**) Bean crop in bloom. (**b**) Plants uprooted on the day of harvest. Photos: Luiz Antônio Zanão Júnior

22.6.4 Sulfur

It is a nutrient component of enzymes and vitamins in plants, a necessary part for the formation of chlorophyll, even though it is not a constituent of it and promotes nodulation for nitrogen fixation (Sfredo and Lantmann 2007).

22.6.5 Molybdenum and Cobalt

Mo is one of the nutrients required in the least amount by plants. It plays an important role in the use and metabolism of N. It is a component of the enzymes involved in oxi-reduction reactions: nitrate reductase and nitrogenase. The first is responsible for the reduction of nitrate (NO_3-) to nitrite (NO_2-) and the second, for the reduction of atmospheric N (N_2) to ammonium (NH_4+). Nitrogenase, therefore, is important for nodulating legumes. They are electron transfer enzymes and they are transferred directly from Mo. Nitrogenase disrupts the triple bond of N_2 atoms for biological N fixation (BNF) and is produced by diazotrophic bacteria. Plants that receive nitrogen fertilization in nitric form and that depend exclusively on BNF, therefore, present a higher requirement in Mo.

Cobalt is not considered a nutrient for plants and is classified as a beneficial element, especially for legumes. In some conditions, the application of Co in the seeds has provided an increase in the production and quality of seeds in legumes such as peas, soybeans, beans, and peanuts. It is an essential element for bacteria of the *Rhizobium* genus.

It is important in the synthesis of cobalamin (vitamin B12), which interferes with the synthesis of the leghemoglobin protein in the nodules of legumes infected by these bacteria, which is important for BNF (Mengel and Kirkby 2001). In these cases, Co deficiency inhibits the synthesis of leghemoglobin and therefore, the biological fixation of N.

In Brazil, due to the importance of these two elements in the soybean BNF, Co and Mo are recommended in the treatment of seeds together with inoculation with *Bradyrhizobium* and the grain yield generally responds positively to this practice (Sfredo and Oliveira 2010).

22.7 Effect of Crop and Soil Management on Microbial Inoculant Efficiency

Microorganisms play a fundamental role in the balance and sustainability of terrestrial ecosystems, as they directly and indirectly influence the chemical, physical, and biological attributes of the soil. Several studies have demonstrated the influence of these microorganisms and their exudates in the region in the rhizosphere on soil aggregation and other dynamic processes that occur in the soil.

However, changes in the physical environment of the soil can influence the activity and efficiency of these microorganisms in the soil microbiome as well as their interactions with plants. The suitability of a soil as a means for plant development depends not only on the presence and quantity of nutrients, but also on how air and water are found and moved in it, as well as on the mechanical properties of the soil and its thermal regiment (Hillel 1998). The author points out that for the proper development of plants, soil must have porosity, size, and convenient distribution of solid particles in order to ensure the optimal conditions for the retention and the movement of water and air. Therefore, the physical properties of the soil determine many key processes for terrestrial ecosystems and, therefore, the agronomic potential of soils.

Soil physical environment factors such soil water content, aeration, temperature, and mechanical impedance affected nodulation and biological N_2 fixation (Siczek and Lipiec 2011). Also, the soil structure which refers to distribution of solid particles in relation to pores space plays a key role in determining the biogeochemical ambience of the rhizosphere, due to most important components of soil air CO_2 and O_2 (Hinsinger et al. 2009).

Studies of Siczek and Lipiec (2011) and Siczek et al. (2015) examined the effects of soil compaction levels (three and five tractor passes compared to no-compaction) exerted by a tractor URSUS®, model C-360 with 3.4 Mg of total load and covering of soil surface with straw chopped into pieces approximately 3–5 cm long and applied on the soil surface at a rate of 0.5 kg m^{-2} in a Haplic Luvisol with 7 dag kg^{-1} of clay at 0–20 cm soil layer and showed that nitrogenase activity was the most sensitive indicator to levels of soil compaction.

One of the most important conclusions was that mulch affected nodulation and nitrogenase activity to a higher extent than compaction and the inverse was true with respect to seed and protein yields (Siczek and Lipiec 2011). Another important conclusion was that the sensitivity of soybean growth to soil deformation and mulching was related to developmental stage and weather conditions (Siczek et al. 2015). Also, these authors showed that most yield components of soybean increased in the soil without compaction compared to moderate compaction (three passes of a tractor), which was attributed to enhanced root water and nutrient uptake rates.

At 0–10 cm depth, soil bulk density increased from 1.29 Mg m^{-3} in the plot without soil compaction to 1.58 Mg m^{-3} in the plot with severe soil compaction with five passes of the tractor (Siczek and Lipiec 2011), which represents 22.4% of increment rate of soil bulk density. The increase of soil compaction levels increased the contribution of nodules with diameters of 0.41–0.61 cm and decreased the contributions of nodules smaller than 0.20 cm and 0.21–4.40 cm.

Soil organic carbon content and composition affect both soil structure and adsorption properties and therefore soil bulk density, water retention, and hydraulic conductivity may be affected by soil organic carbon.

In order to obtain knowledge of the soil physical environment for the microorganisms, it is essential to determine the relationship between solids particles and pore system and aeration status.

Soil physical degradation processes like compaction and shearing change the relations between soil particles and pore space and committing the water and air movement through the soil. Root growth and soil microbial communities are severely affected by transmission of water and air (Frey et al. 2009). Microbial processes in soil are known to be heavily dependent on the physical pore structure that influences microbial habitats (Marshall 2000). Alterations in soil structure caused by mechanical stress lead to modifications in soil pore system and compromise the availability of soil water to plants, tortuosity, and soil functions (Siczek et al. 2015).

In an experiment developed in a greenhouse of the Agronomic Institute of the State of Paraná—IAPAR in Londrina county, Brazil, PVC columns with dimensions of 50 cm of height by 14 cm of diameter cultivated with the common bean cultivar IPR Eldorado on a clayey in a Dystropheric Red Latosol with 51 dag kg^{-1} of clay for assessment of the effects of degree of soil compaction (soil bulk density in the pot divided by maximum soil bulk density obtained in the laboratory from compaction curve determined in Proctor essay) in the subsurface soil layer at 10–25 cm, the increases of soil compaction from 60% (soil bulk density equal to 1.0 Mg m^{-3}) to 90% (soil bulk density equal to 1.5 Mg m^{-3}) on nutrients uptake and accumulation in the root system (Table 22.3). The soil layer of 10–25 cm depth was chosen for compaction due to a presence of no-tillage or plough pan layers in the field soils in the State of Paraná.

Based on the results from our study, it was possible to define 78% as a degree of compaction close to ideal to shoot and root growth of common bean (Fig. 22.6) however, when it was studied, a higher degree of soil compaction 90% (Bd = 1.50 Mg m^{-3}) root and shoot growth was committed and this result was probably due to

Table 22.3 Nutrient contents in the root system of two leguminous crops submitted to soil compaction degrees

Nutrients	Mean[a]	SEM	CV	MIN	MAX
Nitrogen, g kg^{-1}	23.19	0.99	18.22	17.88	30.65
Phosphorus, g kg^{-1}	2.28	0.34	1.42	0.82	5.77
Potassium, g kg^{-1}	1.78	0.22	51.45	0.20	3.30
Calcium, g kg^{-1}	21.48	1.14	4.84	14.94	32.51
Magnesium, g kg^{-1}	1.65	0.06	15.65	1.23	2.14
Copper, mg kg^{-1}	25.28	4.02	67.53	8.84	77.47
Zinc, mg kg^{-1}	27.05	5.09	79.85	7.45	80.90
Boron, mg kg^{-1}	10.82	1.87	73.34	0.17	26.38
Manganese, mg kg^{-1}	45.79	1.72	15.98	34.47	61.35

[a]Averages from 18 samples; *SEM* standard error of the mean, *MAX* maximum value, *MIN* minimum value, *CV* coefficient of variation

Fig. 22.6 PVC columns with common bean cultivar IPR Eldorado cultivated in a clayey Dystropherric Red Latosol (51 dag kg^{-1} clay) submitted to degrees of soil compaction in a subsurface layer of 10–25 cm depth. From left to right: Degree of compaction = 60% (Soil bulk density = 1.0 Mg m^{-3}); 78% (Soil bulk density = 1.3 Mg m^{-3}) and 90% (Soil bulk density = 1.5 Mg m^{-3})

mechanical impedance to root growth, aeration deficiencies to gas diffusion, lower nodulation and N_2 fixation, nitrogenase activity decreased as soil compaction level increased as a highlighted for Siczek and Lipiec (2011). Our results are in agreement with those obtained by Siczek et al. (2015) , which showed that moderate compaction could increase root water and nutrient uptake rates.

These results demonstrated that soil compaction degrees and mechanical impedance to root growth are the factors that govern the ability of bean plant growth and development when the other factors such as water and nutrients are not restrictive.

The soil physical environment affects plants and associated microorganisms through mechanical impedance, soil air deficiencies, nutrient uptake and accumulation, nodulation, biological fixation, and other biogeochemical processes. Also, the soil structure and mechanical resistance determine the accessibility and availability of water to the roots and associated microorganism.

22.8 Biocontrol Activity

Due to the constant increase in food supply requirement, the environmental friendly microbe biocontrol agents (MBCAs) play an important role as an alternative to reduce the impacts of chemicals and pesticides, which, despite their importance, are not sustainable, leading to negative effects on non-target beneficial microorganisms, and present contamination risks for humans, animals, and the environment (Gilden et al. 2010; Kim et al. 2017; Stanley and Preetha 2016). In the past decades, plant growth-promoting microbes (PGPM) have been also described as effective in controlling diseases and pests (Beneduzi et al. 2012; Hyakumachi and Kubota 2003). Herein, we discuss the most recent data about the reliability of plant-growth-promoting microbes as biocontrol agents against pathogens and pests.

22.8.1 Biocontrol Mechanisms

The main action mechanisms involved in suppressing plant biotic stresses are mentioned above.

22.8.1.1 Production of Antibiotics and Other Bioactive Compounds

MBCAs can enhance the plant host resistance against harmful agents by the production of bioactive compounds. Antibiotics like amphisin, DAPG, hydrogen cyanide, oomycin A, phenazine, polymyxin, and others, cell-wall degrading enzymes such as beta-glucanases, chitinases, and proteases, toxins (Ruiu 2020) and VOCs (volatile organic compounds) like hydrogen cyanide (HCN) (Farbo et al. 2018; Tewari and Sharma 2020) are well-known antagonists to detrimental organisms. These compounds act directly, suppressing the invader organism. Tewari and Sharma (2020) observed a good biocontrol potential of the *Bradyrhizobium* IC-4059 strain against *Fusarium udum*, a damaging pathogen of pigeon pea, reporting a reduction of 52.6% of mycelial growth inhibition in vitro. The authors also observed the presence of antagonistic compounds in the supernatant of the same strain.

22.8.1.2 Hyperparasitism (or Mycoparasitism)

The hyperparasitism consists in a bacterial or fungal in the rhizosphere which parasites a fungal disease agent (Jacob and Sudini 2016), where the beneficial microorganism feeds up of the hyphal tips and/ or reproductive structures. Hyperparasitism also suppresses the harmful organism (a fungal pathogen in most of the cases) in a direct manner. Sharif et al. (2003) suggests that the pathogen fungal growth is inhibited through the covering of its hyphal tips with subsequent degradation by antibiotics and enzymes.

22.8.1.3 Induced Systemic Resistance (ISR)

Beneficial microbes are characterized by the capacity of induced host resistance to multiple pathogens. This kind of resistance is triggered by its presence by specific recognition receptors (MAMPs) (Pieterse et al. 1996). The induced systemic resistance (ISR) occurrence is facilitated by the synthesis of the phytohormones jasmonic acid and ethylene (Verma et al. 2019). Pangesti et al. (2016) demonstrated that it can be achieved even in non-exposed tissues. These authors observed that the rhizobacterium *Pseudomonas simiae* WCS417r negatively affected the performance of the caterpillar *Mamestra brassicae* by triggering ISR. Sharma et al. (2019) observed that the rhizobacteria *Klebsiella* spp. strain MBE02 activates ISR-related genes in peanut, involved in jasmonic acid, ethylene, and pathogen-defense signaling. They also observed a growth reduction of fungal pathogens in the presence of the MBE02 strain. Jayapala et al. (2019) observed that a *Bacillus* spp. triggered ISR against *Colletotrichum* species, the causal agents of anthracnose in chili. Jiang et al. (2020) showed that the *Bacillus cereus* strain AR156 induced systemic resistance against *Pseudomonas syringae* pv. *Tomato* DC3000 through the activation of CNLs, an intracellular immune receptor.

22.8.1.4 Antagonism by Competition

Competition for infection sites and organic nutrients can also contribute to the antagonism of MBCAs against pathogens and pests (Raymaekers et al. 2020; Schouteden et al. 2015). Due to the fact that the microbes' growth and reproduction, of both pathogenic and beneficial, depends of the nutrient uptake from its living host (Olanrewaju et al. 2017), coupled to the fact that MBCAs are more competent in nutrient uptake than phytopathogens (Verma et al. 2019), competition is indeed a limiting factor to the pathogen binding.

22.8.1.5 Siderophores

Rhizobacteria can prevent or reduce the pathogen's proliferation indirectly through the production of siderophores. These low-molecular-weight organic compounds are able to chelate insoluble iron from the environment (Kraemer et al. 2015), making it available to the plant (Jacob and Sudini 2016; Olanrewaju et al. 2017). As a consequence, its availability to the harmful organisms is reduced as much as its growth capacity (Lugtenberg and Kamilova 2009; Shen et al. 2013). Sasirekha and Srividya (2016) evidenced a significant reduction in the anthracnose causing *Rhizoctonia solani* growth due to the presence of a *Pseudomonas aeruginosa* FP6 strain, suggesting a siderophore-mediated antagonism. Arya et al. (2018) also suggest a biocontrol of a *Fusarium* Wilt in Tomato (75–100%) to the siderophore production by two *Pseudomonas fluorescens* strains.

Several papers have extensively discussed the role of PGPM as biocontrol agents against economically important pests and pathogens (Dukare et al. 2019; Köhl et al. 2019; Mhatre et al. 2019; Ruiu 2020; Verma et al. 2019). A wide range of targets was proved to be affected by these beneficial microorganisms, like bacterial and fungal diseases (Suárez-Moreno et al. 2019; Tewari and Sharma 2020), as much as the parasitism by nematodes (Viljoen et al. 2019) and insects (Pangesti et al. 2016).

22.8.2 Current Perspectives in Biocontrol Activity

Within the diversity of mechanisms involved in the biocontrol, microorganisms that present more than one of these characteristics can increase the reach spectrum and have attracted major interest. Patel et al. (2019) in a study of the rhizosphere of sugarcane crops investigated the prospect of bacterial plant-growth promoters with biocontrol activity on sugarcane' red rot pathogen *Colletotrichum falcatum*, and identified 26 different isolates with inhibitory effect on the causal agent of the red rot disease. Twenty-three of the 26 strains showed over 50% of inhibition against *C. falcatum*. Cell wall-degrading and siderophores-producing strains were also represented. Tewari et al. (2020) characterized a *Bradyrhizobium* strain IC-4059 as having multiple traits of interest for biocontrol, as siderophore production, HCN production, and enzyme secretion. Myo et al. (2019) observed that a *Bacillus velezensis* NKG-2 strain is able to secrete fungal cell-wall-degrading enzymes, VOCs, and siderophores, which makes it capable of reducing the disease severity of *Fusarium oxysporum* in tomato. Despite the highly efficient MBCAs as individual strains, formulations containing more than one microorganism and/or other bioactive compounds are targets for commercial products. Tewari et al. (2020) enhanced the pigeon pea productivity under field conditions using a combination of *Bradyrhizobium*, cell free culture supernatant, and exopolysaccharides. Liu et al. (2016) evaluated the inoculation of a bacteria mixture and observed a reduction of lesions incidence caused by black rot disease in cabbage.

Since the increase in the availability of genome, transcriptome, and proteome data, several studies have been describing the role of specific proteins in the interaction between pathogens/pests and their hosts. As a consequence, biotechnological approaches emerged as an alternative for further application.

Genetically engineered microorganisms constitute a promising strategy. It can be made by the enhancing of a natural capacity of MBCAs through the expression increase of a naturally produced protein or by the insertion of a protein from an organism to another more suitable and adapted (Jishma et al. 2019). For example, the production of antibiotics, enzymes, and other metabolics can improve the resistance against diseases and pests. Peng et al. (2019) engineered a *Bacillus velezensis* strain to produce high amounts of the VOC acetoin and obtained as result a strong ISR. Jing et al. (2020) improved the antifungal activity of a *Pseudomonas protegens* strain against *Rhizoctonia solani* due to the enhanced production of DAPG.

Bacteria-mediated RNAi also presents potential for future application (Goodfellow et al. 2019). This mechanism consists in a post-transcriptional gene silence induced by double-stranded RNAs through the degradation of targeted mRNA, in a very specific manner (Fire et al. 1998). Goodfellow et al. (2019) suggest the dsRNA delivery through living bacteria expressing these molecules. Then, the harmful organism can uptake the dsRNA during infection, resulting in reduction of the target expression. Although bacteria are not suitable to RNAi due to the absence of the necessary machinery, its potential has been successfully demonstrated by many authors. Several eukaryotic organisms, such as virus (Lu et al. 2005), fungi (Koch et al. 2016), nematodes (Yadav et al. 2006), and insects (Fishilevich et al. 2016) had been proved to be significantly affected by dsRNA. These molecules also have potential to be incorporated in biocontrol formulations.

22.9 Crop Responses to Microbial Inoculation

Microbial inoculants are recognized worldwide and their beneficial effects in economically important crops have been broadly discussed (Ahmad et al. 2018; Havugimana et al. 2016; Patra and Singh 2019; Santos et al. 2019). We summarized below the data about the microbial inoculation responses in some important crops (Table 22.4).

Significant yield increases are reported so far. Economically important crops as soybean, maize, wheat, and common bean are positively affected by microbial inoculation (Table 22.4). For soybean, it is reported increases over 190% due to inoculation with *Bradyrhizobium japonicum* (Tairo and Ndakidemi 2013). Beneficial effects were also observed due to the co-inoculation with *A. brasilense* strains (Hungria et al. 2013a; Hungria et al. 2015a, b). Increase in wheat and maize production of around 31 and 47.8%, respectively, was observed, especially in response to inoculation with *A. brasilense* strains (Hungria et al. 2010; Martins et al. 2018). Inoculation with *Rhizobium tropici* significantly contributes to a yield increase of common bean, of almost 70%. Co-inoculation of *Rhizobium* spp. with

Table 22.4 Yield increase range by PGPM in economically important crops

Crop	Microorganism	Yield increase range (%)	Reference
Soybean	*Bradyrhizobium japonicum*	1.67–192	(Leggett et al. 2017; Solomon et al. 2012; Tairo and Ndakidemi 2013)
	Bradyrhizobium spp.	10.69–46.9	(Afzal et al. 2010; Hungria et al. 2015a, b)
	Bradyrhizobium spp. + *Azospirillum brasilense*	17.12	(Hungria et al. 2015a, b)
	B. japonicum + *A. brasilense*	14.1	(Hungria et al. 2013a)
Maize	*A. brasilense*	7.4–47.8	(Di Salvo et al. 2018; Galindo et al. 2019; Hungria et al. 2010; Lana et al. 2012; Martins et al. 2018)
	A. brasilense + *Anabaena cylindrica*	23.1	(Gavilanes et al. 2020)
	Azospirillum spp. + *Azotobacter* spp.	2.6	(Naserirad et al. 2011)
Wheat	*A. brasilense*	11.9–31	(Hungria et al. 2010; Ozturk et al. 2003; Salantur et al. 2006)
	Ralstonia spp.	18.6	(Salantur et al. 2006)
Common bean	*Rhizobium tropici*	8.3–66.36	(de Souza and de Brito Ferreira 2017; Hungria et al. 2013a; Mercante et al. 2017; Nogueira et al. 2017)
	Rhizobium spp.	4.87–48	(Argaw and Akuma 2015; Morad et al. 2013; Samago et al. 2018)
	Rhizobium spp. + *Pseudomonas fluorescens*	98	(Yadegari and Rahmani 2010)
	R. tropici + *A. brasilense*	53.7	(de Souza and de Brito Ferreira 2017)
	R. leguminosarum bv. phaseoli	6.78	(Elkoca et al. 2010)
	Bacillus subtilis	12.1	(Elkoca et al. 2010)
	R. tropici + *R. freirei* + *A. brasilense*	62	(Horácio et al. 2020)
	R. tropici + *R. freirei* + *A. brasilense* + *A. cylindrica*	84	(Horácio et al. 2020)

Pseudomonas fluorescens resulted in a yield increase of almost 100% (Yadegari and Rahmani 2010).

Inoculation has been proved to have beneficial effects on plant growth under abiotic stresses, such as salinity (Chen et al. 2016; El-Esawi et al. 2018; Fukami et al. 2018; Hamdia et al. 2004; Khan et al. 2019; Latef et al. 2020; Li and Jiang 2017; Nadeem et al. 2007; Rojas-Tapias et al. 2012), drought (Bano et al. 2013; Basal and Szabó 2019; Creus et al. 2004; Ghorchiani et al. 2018; Naveed et al. 2014; Silva et al.

2019), extreme pH (Singh and Reddy 2011), arsenic (Armendariz et al. 2019), and heat (Khan et al. 2020).

Within the several possible approaches that can be applied to the increase of the productivity in a sustainable manner, microbial inoculants are for sure a likely alternative to chemical fertilizers.

22.10 Conclusion

The potential use of microorganism inoculant is now seriously considered as a means to reduce the amount of chemicals required for crop production, to minimize pollution and soil infertility, and above all, reduce grower's costs. Plant-growth-promoting microbes such as Rhizobacteria, AMF, and cyanobacteria are for sure a huge part of sustainable agriculture development, since they play a significant role in the improvement of agricultural practices and yield, thereby reducing the application of chemical fertilizers and pesticides. Although the presented data demonstrated the remarkable abilities of these microorganisms, there is still much to be done on both explorations as well as the implementation of PGPM. Single microorganism inoculants as well as formulations have been proved to significantly increase crop production at a very low cost when compared to chemical fertilizers. Exploration that involves the understanding of the mechanism at same time as implementation needs to take care of a great deal of optimization on field application.

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Chapter 23
Microalgae: Cultivation, Biotechnological, Environmental, and Agricultural Applications

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Contents

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Abstract This chapter presents general aspects regarding microalgae biology and growth under ex situ conditions. Emphasis is given on some aspects of microalgae responses to major environmental and nutritional factors, for example, temperature, light, nutrients, and pH. Then, management of photobioreactor systems where microalgae are grown to achieve the objectives of producing high biomass and bioactive compounds for biotechnological applications is addressed. The feasibility of producing multiproducts has led to more efficient production pathways and use of materials and energy. Most of the studies about microalgae are addressed in an interrelated way with environment and agricultural applications.

Keywords Biostimulants · Bioenergy · Chlorophyll · Lipid · Microalgae supply chain · Wastewater

23.1 Introduction

Microalgae are a noteworthy photosynthetic microbial group, which grow in a wide range of aquatic environment such as freshwater, seawater, and also wastewater; they also occur in soil and plant rhizosphere microhabitat. These microorganisms present higher photosynthetic efficiency, faster growth rates, and higher yields per unit of area than terrestrial plants. From the point of view of the environmental and biotechnological applications, microalgae have an essential role due to their versatile metabolism as they have broad potential for the production of biomass under different conditions.

Microalgae have been used as food, bioingredients, and by-products that are biologically active compounds, such as polyunsaturated fatty acids (PUFAs), carotenoids, phycobiliproteins, sterols, vitamins, and polysaccharides. These byproducts have proved to have many important biological functions, making them biomaterials and bioactive products of increasing importance for a wide range of applications, from industry to agricultural activities.

Most biotechnologically relevant microalgae are the green algae (Chlorophyceae), for example, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina*, and the Cyanobacteria *Spirulina maxima* (*Arthrospira*), which are already widely commercialized and used mainly as food supplements for humans, as animal feed additives (Gouveia et al. 2008; Nethravathy et al. 2019), as nutraceuticals compounds (Matos 2017), as feed in aquaculture industry (Hemaiswarya et al. 2011), for cosmetic industry, and as bioenergy feedstock production (Converti et al. 2009; Yin et al. 2019).

Microalgae can be used for bioremediation of wastewater (Tonhato Junior et al. 2019; Zhu et al. 2019), reducing the concentration of polluting compounds that alter the quality of natural resources. These microalgae biomass and by-products can be

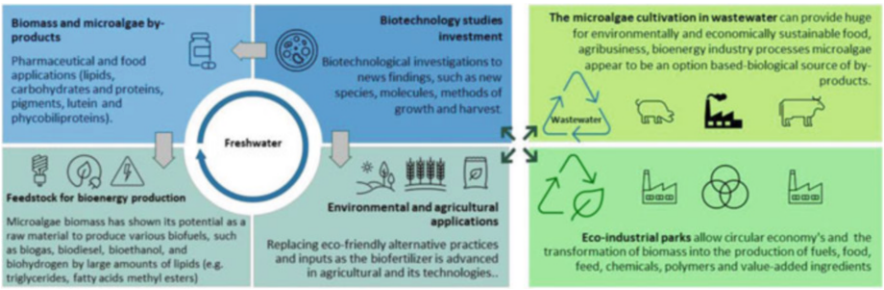

Fig. 23.1 An overview of microalgae biomass production in freshwater and wastewater

obtained from wastewater or renewable resources, reducing the environmental impact of anthropic activities (Fig. 23.1).

In this chapter, the following points are presented: (1) general aspects of microalgae biology and the nutritional and environmental factors affecting their growth; (2) production systems using photobioreactors and procedures of biomass harvesting; (3) applications of microalgae biomass as a feedstock for bioenergy and for other biotechnological purposes; and (4) microalgae cultivation in wastewater is addressed, since they reduce polluting compounds that alter the quality of natural resources and to the environmental function aiming to generate biomass that can be used for several purposes, mainly as biofertilizers in agriculture.

23.2 General Aspects of Microalgae

Microalgae are a diverse group of microscopic photosynthetic microorganisms (i.e., the prokaryotic cyanobacteria and the eukaryotic microalgae) that inhabit continental freshwater and seawater, and they are microhabitats of air, soil, and rhizosphere environments. As primary producers, they are important in food chain in these environments, and some of them are with symbiotic capacity. In applied phycology, the term microalgae refers to any microorganisms (prokaryotic or eukaryotic) with chlorophyll *a* and a thallus not differentiated into roots, stem, and leaves. The akinetes and heterocyst are the differential cellular structure that, respectively, are vegetative in chlorophyte and cyanophyte cells that accumulate oil, pigments, and other reserve substance, and also resistance spores (Fig. 23.2a, b).

These photosynthetic microorganisms include species from different phyla such as Cyanophyta (blue green algae, cyanoprokaryotes, and cyanobacteria), Chlorophyta (green algae), Rhodophyta (red algae), Cryptophyta, Haptophyta, Pyrrophyta, Streptophyta, and Heterokontophyta. In general, microalgae have different types of cell organization, for example, unicellular, colonial, and filamentous, and the main structure is thallus called "stalk," being able to be unicellular or multicellular, colonial, filamentous, or siphonaceous, and some species have centrioles, one or two flagella (Richmond 2004).

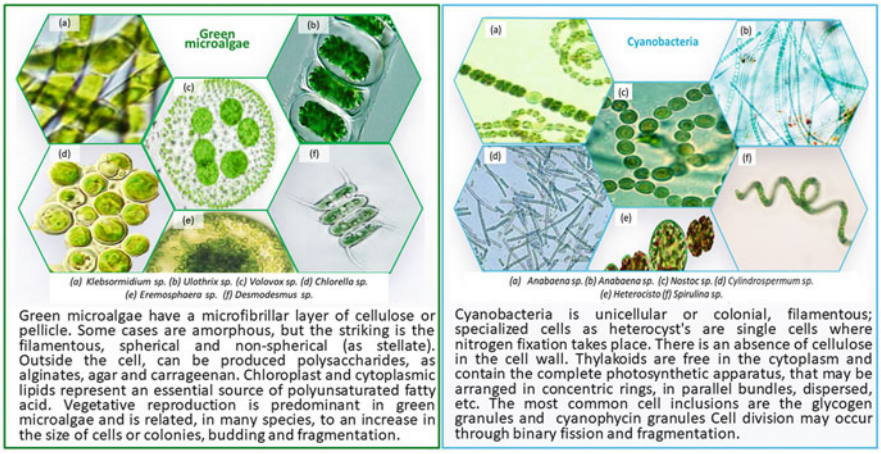

Fig. 23.2 Diagram showing general aspects of cell morphology of (**a**) green microalgae and (**b**) cyanobacteria

The origins of applied phycology most probably date back to the establishment of a culture of *Chlorella* by Beijerinck in 1890. According to Richmond (2004), several species to genus *Chlorella* take up the first place in the commercial use of these microorganisms alike others genera belonging to Chlorophyta, green microalgae, which have great morphological variability (Fig. 23.2a).

Cyanobacteria were the first organisms to evolve oxygenic photosynthesis, and in addition, as prokaryotes, some species are N_2 fixing. In many cyanobacteria, single heterocyst develops at intervals of approximately 10–15 vegetative cells forming a one-dimensional pattern. Most filamentous cyanobacteria species present cells differentiated from vegetative cells called heterocyst, which has ability to fix atmospheric nitrogen even under N-limiting medium (Fig. 23.2b). Heterocyst development is repressed in the presence of a rich source of combined nitrogen, such as ammonium or nitrate (Adams 2000).

Biological nitrogen fixation (BNF) relies on CO_2 fixation as a source of carbon skeletons and reduced organic compounds as observed in the freshwater filamentous cyanobacterium *Anabaena oscillarioides* (Paerl and Bland 1982).

Microalgae displays a significant ecological plasticity by the ability to adapt to changing extreme environmental conditions such as temperature, light, pH, salinity, and moisture, which describes their worldwide distribution. They have different pathways to fix atmospheric carbon dioxide and to efficiently utilize the nutrients to convert it into biomass (Alwathnani and Johansen 2011; Sharma and Sharma 2017).

Microalgae belong to the fastest-growing photosynthetic organisms since their cell doubling time can be as little as a few hours. They are the highly efficient biological approach for converting CO_2 and nutrients to biomass (Sigamani et al. 2016).

23.3 Microalgal Growth

Like other microbial groups, microalgae growing is altered by the nutrients, pH, and temperature, and when under phototrophic conditions, they also depend on the light that is an essential factor for this particular group. These microorganisms are still capable of tolerating fluctuations in humidity, lighting, salinity, and nutrients (Tiwari et al. 2019), but for ex situ intensive growth, microalga depends on the balance of nutrients in the culture media according to needs for cell multiplication and by-product production and on the adjustment of light intensity and photoperiod cycles.

The lipid content of microalgae is strongly influenced by the variation of nutrients and temperature in the cultivation medium; under stress conditions, it results in high lipid accumulation but as also in low biomass productivity, overall lipid productivity is consequently lower. An increase in temperature from 20 to 25 °C resulted in significant increase in the lipid content of *Nannochloropsis oculata* (from 7.90% to 14.92%), while an increase in temperature from 25 to 30 °C brought about a decrease of the lipid content of *Chlorella vulgaris* (from 14.71% to 5.90%) (Converti et al. 2009).

23.3.1 Factors Affecting Microalgal Growth

An economical process of microalgae mass culture for oil production depends on both high biomass productivity and high lipid content in cells, which can increase or decrease under advantageous or disadvantageous nutrient such for N content in the cultivation medium. It should also be considered that variation in temperature seems to influence lipid content, and this effect depends on the microalgae species.

Several microalgae species have metabolic capacity to produce large amounts of lipids as a storage product using an inorganic carbon source and light energy, which makes microalgal biomass an attractive resource for biodiesel production and other biotechnological applications.

Microalgae biomass can be obtained through heterotrophic, autotrophic, or mixotrophic metabolism and growth conditions, but they are preferably derived from photosynthesis, and some species of Chlorophyceae, Volvocales, show an average biochemical composition: 30–50% proteins, 20–40% carbohydrate, and 8–15% of lipids under favorable environmental conditions, but under unfavorable conditions up to 80% of fatty acids, 80% of hydrocarbons, and 40% of glycerol on dry weight (Richmond 2004).

Among these environmental factors, we can point out that the light, temperature, nutrient status, and salinity not only affect photosynthesis and productivity of cell biomass but also change the cellular metabolic activity resulting broad biotechnological implications, as an example, lutein content in microalgae adapts according to pH, temperature, salinity, nitrogen availability, and mainly the specific growth rate

of the cultured strain (Guedes et al. 2011) and light intensity (Coulombier et al. 2020).

As a photosynthetic microorganism, the photoperiod is an essential factor, especially in the photoautotrophic; both natural light (sunlight) and artificial light (lamps) are key areas of microorganism life development. Maintaining an adequate level of light throughout the life of cell culture or using it efficiently is a significant factor (Richmond 2004). At the high light intensity on microalgae, cells can cause photoinhibition, decreasing photosynthetic efficiency and biomass production (Borowitzka 2018; Richmond 2004); still, in the superficial part, they have a high luminosity incidence.

In the open cultivate, the sun is the light supplier for cultivation, and at night, additional artificial light can be provided to obtain better efficiency in the use of nutrients within the shortest time, besides. Light and chemical energies are vital to increasing biomass photoautotrophic and mixotrophic cultivation systems (Sipaúba-Tavares et al. 2019; Sirisansaneeyakul et al. 2011).

Photobioreactors illuminated with mixed light-emitting diode (LED) wavelength have been showing more efficient performance for microalgae growing (Che et al. 2019), mostly when the aims are to removal of pollutants such as carbonaceous organic matter, nitrogen, phosphorus, and other compounds from wastewater.

Microalgae do not distinguish between natural and artificial light, for instance, LED-illuminated photobioreactors with microalgae are a promising technology for wastewater treatment applications (Silva et al. 2020), but they are susceptible to high light intensities, as well as changes in the light/dark cycle.

Photoautotrophic microalgae productivity is limited mainly by the irregular light supply of which generates a low efficiency of energy conversion inside the cultures. Some enzymes as a photoenzyme acting on lipids show that light-driven catalysis is not restricted to the processes of light capture and use or to the repair of UV damages in DNA. Some microalgal enzymes involved in metabolic or signaling pathways are regulated by light, for instance, in *Chlorella variabilis*, a photoenzyme called fatty acid photodecarboxylase converts fatty acids to hydrocarbons (n-alkanes or n-alkenes) in response to blue light (Sorigué et al. 2017). Light intensity has a strong influence on production and activity of compounds with antioxidant capacity of microalgae as shown by 12 microalgae species that were cultivated at two light intensities (Coulombier et al. 2020).

Lipid accumulation and carbohydrate degradation of *Chlamydomonas* sp. were deferred under the light/dark when compared to the continuous light photoautotrophic cultivation condition, for instance, phosphoenolpyruvate accumulates and glycerol 3-phosphate decreases under the light/dark condition, suggesting that it was the imbalance of the metabolites which seems to be the cause of delay in the accumulation of lipids (Kato et al. 2019). Also, in this study, the metabolic dynamic profile showed higher levels of lipid/carbohydrate anabolism (as the production of 3-phosphoglycerate and acetyl-CoA) of CO_2 and the highest biomass yield in the light/dark, indicating a more significant fixation of CO_2 than in the light condition.

Temperature is a relevant factor for biomass and by-product production, independent if cultivation is in an open or closed system, although microalgae can easily

withstand a wide range of temperatures; variation in their ideal temperature might result in total yield loss. Strain selection can be important for optimizing productivity, and some of the major characteristics that need to be considered are range of temperature tolerance, resistance to photoinhibition, harvest ability (trichome size), and quality (composition) of the biomass produced (Borowitzka 2018).

In a study that examined the hypothesis whether temperature alters biomass and metabolite production of microalgae according to species and even strain, Maroubo et al. (2018) concluded from long-term data collection that it is possible to choose a strain suitable for growing in each season depending on the temperature of a given region. The genetic of species has an ideal temperature range for cultivation and full crop development, depending on the location where the species was isolated. According to Metsoviti et al. (2019), both temperature and light intensity influence the growth rate, as well as the biomass production of five species *Chlorella vulgaris*, *Botryococcus braunii*, *Chlamydomonas reinhardtii*, *Euglena gracilis*, and *Nannochloropsis oculate*.

In the cultivation of microalgae, the process of homogenization and aeration is essential for a greater distribution of gases and nutrients, and with that, there is increase in the productivity of biomass and some compounds of interest. Aeration, injection of gases, into the culture medium allows movement and greater maintenance of the cells in suspension, improving the light efficiency and exchange of gases, preventing thermal stratification, aiding in the homogeneous distribution of nutrients, preventing the accumulation of organic matter at the bottom of the bioreactor, and reducing photooxidation, factors that influence the biomass productivity (Uggetti et al. 2018; Yin et al. 2019).

In order to provide microalgae cell growth ex situ, there is a need to properly balance the supply of essential nutrients, generally in liquid media in which must meet all the nutritional needs for cell synthesis and for the production of wanted metabolites of biotechnological interest. As an essential approach to reach higher microalgal biomass production, it is necessary to study the nutrient requirements to meet the specific needs of each species. The nutritional elements of more significant quantitative proportion are carbon (C), nitrogen (N), phosphorus (P), magnesium (Mg), potassium (K), and calcium (Ca). Already manganese (Mn) and cobalt (Co) favor their vital activities to a lesser extent, for example, the ratio of C:N:P in the cells of microalgae is 100:16:1 (Geider and La Roche 2002).

The hydrogen potential (pH) control can be done using buffer compounds. This factor is one of the most relevant and related to the solubility of CO_2 and availability of other chemical elements in the culture media, and, consequently, to optimal cell metabolism (Richmond 2004).

By controlling the pH for cultivation of *Nannochloropsis gaditana* at an optimum value, ranging from 8 to 9, exhibited higher CO_2 conversions in biomass, which suggests reduction in the cost of the microalgae production process using tubular photobioreactors under outdoor conditions (Moraes et al. 2020).

Microalgae as photoautotrophic microorganisms using light and CO_2 are responsible for large amount of global photosynthesis and CO_2 fixation, but these microorganisms also can grow under heterotrophic conditions by using organic

compounds such as energy and C source, or also in mixotrophic metabolism, where light, CO_2, and organic substrate are simultaneously used (Sabia et al. 2015; Silva et al. 2016; Sipaúba-Tavares et al. 2019).

Overall, in microalgae culture for CO_2 sequestration, increasing CO_2 bubbling depth and keeping higher carbon concentration and higher pH can improve CO_2 absorption ratio, which will optimize the biofixation of CO_2 by microalgae furthermore (Yin et al. 2019).

Using untreated urban wastewater dominated by *Scenedesmus* sp. from the treatment plant, the addition of CO_2 resulted in an increase in biomass ranging from 66% to 100% (Uggetti et al. 2018), and for *Scenedesmus obliquus* in the culture medium, the injection of 10% CO_2 increased the biomass production, suggesting that microalgae grown at high CO_2 levels that are equivalent to those of power plant emissions can be nutritious and sustainable animal feed (Molitor et al. 2019).

Chlorella sp. cells growing in buffered medium showed that the stress of pH induces a shift in lipid metabolism from membrane lipid syntheses to storage, as in the alkaline pH had a greater accumulation of triglycerides with a decrease in the membrane lipids classes, glycolipid, and polar lipid, regardless of the content of N or carbon (Guckert and Cooksey 1990). These authors highlighted the use of suitable buffer in the growth media to avoid misinterpretation of results when studying changes of pH on biochemical and biomass production of microalgae. For instance, a great potential for the production of astaxanthin in *Haematococcus lacustris* was detected when there was the application of pH shock, which suggests some advantages, such as low cost, rapid induction, and wide applicability (Han et al. 2020).

Due to the cost of buffers when using scale-up production systems, other techniques are used to control the pH, such as pumping natural or CO_2-enriched air, contributing to stimulate growth and microalgae biomass production (Moraes et al. 2020). Also as pointed out by Galès et al. (2020) using polycultures of microalgae cultivated in outdoor raceways at high rate algal ponds, when by using control at pH 8.0, it was observed that higher biomass productivities and CO_2 use efficiencies were reducing carbon losses to the atmosphere and overall process costs. A key point for CO_2 fixation improvement in intensive cultures is control of pH that can eliminate contaminants and unwanted organisms since they are not tolerant to pH below 6.0 or 9.0, and this is a variable that can help and be useful on a large scale. At pH 8.0, using wastewater from dairy was considered as an optimum value for chemical oxygen demand (COD) removal by *C. vulgaris* (Valizadeh and Davarpanah 2020).

The key to obtain high biomass productivity and to reduce costs is recycling autotrophic and mixotrophic growth media of the microalgae, which provides a more sustainable impact on water resources; however, the presence of free fatty acids and metabolites from the oxidation of unsaturated fatty acids has inhibitory effects on microalgae cells, reducing the production of biomass (Sabia et al. 2015).

Photosynthetic carbon fixation in microalgae cell suspension can been measured in solutions with special electrodes in which is the partial pressure of carbon dioxide (pCO_2) (Richmond 2004). Increasing CO_2 bubbling depth and keeping higher carbon concentration and higher pH when growing *Scenedesmus* sp., *Porphyridium* sp., and *Dunaliella salina*, an increase in CO_2 absorption ratio was detected (Yin

et al. 2019); overall, there is an interaction between environmental and nutritional factors when growing microalgae, which will optimize the biofixation of CO_2.

23.3.2 Microalgal Bioreactor Systems and Biomass Harvest

There are many systems to cultivate microalgae, including raceway pond and photobioreactors. There are a variety of open cultivation systems and different designs about the size, material, type of construction, agitation, and inclination. One more widely used photobioreactors is made up of transparent plastic bags supported by a metal structure, which can be supported or hung in this structure (Patent BR1020140215670) (Silva et al. 2014), which seems to be suitable to grow microalgae for inoculum for scale-up system cultivation as shown in Fig. 23.3a.

The open systems called as raceway pond are extensive circular ponds with the presence of a rotating arm, the lake in agitated track with rotating paddles and inclined systems forming a thin layer of cascade culture medium mirror, which has lower construction and operating costs when compared to closed photobioreactor systems. For this reason, most microalgal producers are still using this system of cultivation despite the concerns regarding the difficulties to keep an extensive system contamination-free by microorganisms and by other animals.

In general, most of the open raceways run at a depth of 20–40 cm, as the light energy must cover the entire cell mass and also allowing the aeration system and homogenization of the medium (Fig. 23.3b).

Most open systems are homogenized, especially in large-scale production, promoting the rapid circulation of microalgae cells from the dark to the light zone of the bioreactor, for example, rotating blades, as the cultivation must be mixed by moving the cell from the bottom of the tank to the top to avoid a decline in productivity because light and aeration are essential to microalgae growth (Richmond 2004).

Photobioreactors are classified as the closed tower, plate, tubes, bags, or tank reactors. There are different shapes of closed photobioreactor systems in the form of plates that are built from glass or acrylic to metal structures that have a thin layer of plastic bags or honeycomb panels with internal partitions. Constructed of glass, transparent Teflon tubes, or transparent PVC tubes, they are organized in parallel lines or helically wound around central support. The tower-shaped system consists of vertical cylinders, usually constructed of acrylic or fiberglass.

For these photobioreactors, the culture medium circulation has been carried out of air injection by a compressor and the temperature control is done by heat exchangers or evaporative cooling by spraying water on the reactor surface. Large tubular photobioreactors had the tubes floating in a large pool for temperature control; microalgae advantages cultivation system in "closed" photobioreactors which eliminates contamination. Among the techniques for sterilizing photobioreactors are the use of water vapor and the use of chemical compounds (Richmond 2004). Outdoor pilot-scale tubular photobioreactors have been used or located inside a greenhouse which controls temperature and light (Moraes et al. 2020).

Fig. 23.3 (**a**) Transparent tubular photobioreactor for the cultivation of microalgae aiming inoculum production and (**b**) open tank

Overall, microalgae grow in systems and environments that are aqueous with nutrients and aeration with O_2 or CO_2 in the appropriate proportions for each species; therefore, one of the important aspects of large-scale cultivation is the harvest that is the concentration of cells when the biomass should be removed. From the point of view of microalgae farming, harvest comprises the separation of the solid–liquid phases of the cultures, the solid part being composed of cells and the liquid part being composed of water and the other compounds, including nutrients.

For food applications, the harvested and concentrated algal biomass is to be further utilized, a product with a water content of less than 10% is required. Moisture affects the spoilage of the dried algal product by supporting the growth of bacteria, mold, and fungi (Becker 2013). By physical, chemical, or biological stages, the main stage can be performed by centrifugation, filtration, flotation, sedimentation by gravity, flocculation, and coagulation. Harvesting, which consists of separating the microalgae cells from the liquid part of the culture medium and drying process, represents a significant proportion of the production cost due to the general low concentration of biomass. Overall, the cost for harvesting and drying processes has been reported as ranging from 20% to 30% of the total value of the production cost, which presents great challenges for the commercial use of microalgae, mainly for the production of biofuels.

To select harvesting method, whether filtration, centrifugation, flocculation, or sedimentation, some factors must be considered, such as cell morphology, for example, the shape (spherical cells, in chains or filaments), the size (usually between 2 and 30μm), specific weight, area of charge surface (typically negative), and in which system and how microalgae are growing; and the cost and efficiency of the process will depend on the final application of biomass (Richmond 2004).

The recovery for harvesting biomass is crucial in the cost–benefit of producing microalgae, since the cells are relatively small varying between 2 and 30 μm in

diameter, with few exceptions (e.g., *Arthrospira*), and with very low concentration of 0.1–5 g L^{-1} of dry biomass. There is no single harvesting method recognized as the best or the most suitable for all microalgae species; flocculation is a more convenient harvesting method, such as centrifugation and filtration, as allowing the treatment of large volumes of microalgae culture.

Filtration is a technique that allows to accurately determine the equivalent volume of the culture with high efficiency in separating the biomass from the culture medium. The filtration method is the operation in which a solid is separated from a liquid employing a porous medium, which retains the solid fraction and allows the liquid fraction to pass (Richmond 2004). The filter medium can be composed of paper, fabric, or other porous solid, such as ceramic or a thin layer of sand. However, in large-scale cultivation, the separation of large quantities of microalgae is only viable if the species has large cells or filamentous structure since small cells cause the rapid clogging of any significant volume filtration system.

Centrifugation is a practical and straightforward method of harvesting cells and can be performed without adding chemicals, preserving the original characteristics of the biomass. However, on a commercial scale, sometimes this is not feasible due to the high energy expenditure for operating the system, the difficulty in processing large volumes of cultivation, and the need for high investments in the acquisition of large equipment.

To address this challenge, flocculation has been identified as a low cost and promising technique. To reduce harvesting costs, some flocculation methods are being explored, including auto-flocculation with titanium dioxide (TiO_2) plus intense pulsed light as reported for *Tribonema* sp. and *Synechocystis* sp. cultivated together in swine wastewater (Cheng et al. 2020) by bio-flocculation with bacteria and filamentous fungi for *Chlorella pyrenoidosa* (Jiang et al. 2020). There is no single harvesting method recognized as the best or the most suitable for all microalgae species; flocculation is a more convenient harvesting method, such as centrifugation and filtration, as allowing the treatment of large volumes of microalgae culture. A wide variety of chemicals are studied as a flocculating agent for microalgae as it was emphasized in a review on flocculation methodologies (Li et al. 2020). Flocculation consists of removing the cells' ability to remain in suspension or stimulating aggregation to form flakes that can settle or float (Fig. 23.4).

By using nonstarch-based cationic polymer as flocculant for harvesting *Chlorella* sp., *Micractinium* sp., and *Scenedesmus* sp., the obtained efficiency ranged from 96% to 97% at an optimized dosage (Kumar et al. 2019b). Also, there are other studies comparing different bio-based organic polymers flocculants and also doses, for example, for *N. oculata*, the biomass harvesting efficiency of flocculation when using cationic cellulose nanocrystals was 90% and when using chitosan was >95% (Verfaillie et al. 2020), and the values range from 85% to 95% according to doses of two cationic polymers (Vu et al. 2020), and there is the coagulation–flocculation by alkaline pH induction (Ajala and Alexander 2020).

The advantages of flocculation methods using organic polymers are related to the low cost of operation and high efficiency (>90%) because chemicals are not used to

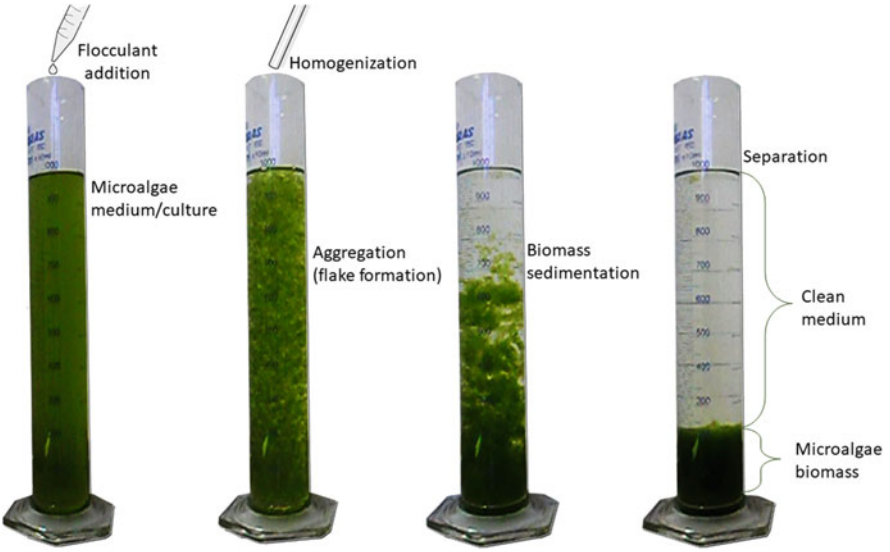

Fig. 23.4 Illustrations on flocculation of microalgal cells and sedimentation process using flocculants. Modified from Silva et al. (2014)

concentrate the biomass, which avoids the use of anions based on chlorine or sulfate (Mubarak et al. 2019).

In a study of coagulation/flocculation treatment of brewery wastewater using flocculant based of vegetable tannin showed to be efficient (Tonhato Junior et al. 2019). These authors point out that the flocculation adds the cost 0.335 per kg of dry biomass recovered and cost of flocculant per volume of treated effluent of U of US 0.13 m^{-3}. It achieved approximately 98% efficiency in nutrient removal from a municipal wastewater, when *Chlorella vulgaris* was cultivated for 30 days in a laboratory scale hybrid process by combining an aerobic membrane bioreactor with a membrane microalgal reactor and using flocculation cationic polyacrylamide polymers to harvest (Nguyen et al. 2020).

23.4 Microalgal Biomass and By-Products: Pharmaceuticals and Food Applications

One of the major current challenges for the planet is to provide enough food for its population. As predictions of the world population will have increased by another 2 billion by 2050, current estimations have indicated that sufficient water and arable land are not available to support such demand (Smithers 2016). Microalgae (including cyanobacteria) are promising organisms for sustainable products for use as

feedstocks for food, feed, fine chemicals, biofuels, and agro-industrial. They can synthesize a broad range of products with high-value market price such as polysaccharide, single-cell protein (SCP), carotenoids and phycobilin pigments, and long-chain polyunsaturated fatty acids. These products are commercialized in the food industry as dietary supplements and functional foods, in the pharmaceutical and chemical industries as cosmeceuticals and flavorings, and in the therapeutic field as nutraceutical compounds (Matos 2017).

The addition of microalgal biomass to food products is an interesting tool for providing nutritional supplementation with biologically active compounds (e.g., antioxidants, PUFA-ω3) besides coloring purposes. Accordingly, the selection of microalgae species with balanced nutritional profiles is fundamental for successful novel food development. A detailed physicochemical characterization of the microalgae is an essential stage that will allow determining which algae are best suited for different applications and purposes (Batista et al. 2013).

Some eukaryotic microalgae species produce a hug diversity of compounds that are widely studied for their bioactivities in the fields of cosmetics and nutrition especially to prevent overweight, including two molecular families, omega-3 long-chain polyunsaturated fatty acids (PUFAs) and carotenoids that comprise two major subfamilies, carotenes and xanthophylls (Delbrut et al. 2018; Sathasivam and Ki 2018).

Microalgae by-products are significant source of fine chemicals, such as natural pigments, carotenoids, vitamins, proteins, fatty acids, sterols, among other biologically active compounds, presenting potential benefits for human and animal health (Gouveia et al. 2008; Soares et al. 2019) and polysaccharides (Vishwakarma and Sirisha 2020).

23.4.1 Enzymes, Polysaccharides, and Proteins

Enzymes are essential components of biological reactions and play important roles in the scaling and optimization of many industrial processes. Due to the growing commercial demand for new and more efficient enzymes to help further optimize these processes, many studies are now focusing their attention on more renewable and environmentally sustainable sources for the production of these enzymes. Microalgae are very promising from this perspective since they can be cultivated in photobioreactors, allowing the production of high biomass levels in a cost-efficient manner. This is reflected in the increased number of publications in this area, especially in the use of microalgae as a source of novel enzymes (Vingiani et al. 2019).

Enzymes for healthcare applications can include L-asparaginase. Paul (1982) first purified the L-asparaginase in *Chlamydomonas* spp. with limited anticancer activity and tested it in an in vivo anti-lymphoma assay. Ebrahiminezhad et al. (2014) screened 40 microalgal isolates via activity assays and reported that *C. vulgaris* was a potential feedstock for L-asparaginase production. There are other microalgal

enzymes involved in the synthesis of bioactive compounds; some studies have focused on polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPSs). PKSs produce polyketides, while NRPSs produce nonribosomal peptides. Polyketides and nonribosomal peptides have been reported to have antipredator, allelopathic, anticancer, and antifungal activities (Kohli et al. 2016). PKS can be multidomain enzymes (Type I PKS), large enzyme complexes (Type II), or homodimeric complexes (Type III). Genes potentially encoding these first two types of PKSs have been identified in several microalgae (e.g., *Amphidinium carterae*, *Gambierdiscus* spp., *Karenia brevis* in cyanobacteria, for instance, *Anabaena* sp. PCC 7120, *Nostoc punctiforme*, *Gloeobacter violaceus*, *Crocosphaera watsonii*, and *Anabaena variabilis*) (Jenke-Kodama et al. 2005), also in *Azadinium spinosum* (Meyer et al. 2015), in *Gambierdiscus excentricus* and *Gambierdiscus polynesiensis* (Kohli et al. 2017), and in *Amphidinium carterae* (Lauritano et al. 2017).

Enzymes for bioremediation can be as follows: (a) enzymes directly used for the degradation of toxicant compounds to less or nontoxic versions (e.g., the hexavalent chromium is converted to the less toxic trivalent chromium due to the activity of chromium reductase); and (b) enzymes involved in cellular stress response mechanisms such as peroxidases (Px), superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). SOD, Px, and CAT typically function in helping detoxify the cell from oxygen reactive species, while GR replenishes bioavailable glutathione, catalyzing the reduction of glutathione disulfide (GSSG) to the sulfhydryl form (GSH) (Vingiani et al. 2019).

Reactive oxygen species (ROSs) are generated in aerobic organism as result of respiration and substrate oxidation. Environmental stresses such as intense light, heavy metals, herbicides, UV radiation, high salt concentrations, and extreme temperatures stimulate ROS production. Consequently, microalgae possess antioxidant defense mechanisms that combat ROS cell damage. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione reductase, catalase, and peroxidase (Mallick and Mohn 2000). Superoxide dismutase (EC 1.15.1.1) is a metalloenzyme that converts superoxide radicals (O_2) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The applications of SOD include therapeutic and prophylactic applications in humans, in the preservation of biological materials (organs for transplantation and sperm), in the preservation of perishable materials such as foodstuffs and vaccination agents, and as an antigenic agent for the serodiagnosis of pathogens (Bafana et al. 2011). By comparing SOD activities in three cyanobacteria, Gunes et al. (2015) found that the maximum specific activities in *Synechococcus nidulans*, *Arthrospira platensis*, and *Pseudanabaena* sp. were 50.4, 30.0, and 18.4 U mg^{-1} of protein, respectively. Because SOD is a promising and potent antioxidant enzyme, future studies should evaluate SOD synthesis in microalgae.

The presence of the enzymes in microalgae has important biological properties. In a study in silico, it was described in different microalgal classes that the enzymes, such as monogalactosyldiacylglycerols and sulfoquinovosyldiacylglycerols, maintaining in conserved domains, could be effectively involved in the synthesis

of compounds with recognized anticancer and immune-modulatory activities (Riccio et al. 2020). Other compounds with antioxidant activity are also produced by microalgae, for example, *Pediastrum boryanum* that showed ability to produce free phenolic compounds with potential antioxidant activity in vitro (Corrêa da Silva et al. 2020).

Polysaccharides are widely used in the food industry primarily as gelling and/or thickening agents. Beta-1,3-glucan, a natural soluble fiber active as immune-stimulator, antioxidant, and reducer of blood cholesterol, has to be mentioned, which is accessible from the cultivation of *Chlorella* strains (Spolaore et al. 2006). In addition to the therapeutic use, this carbohydrate can be implemented in food and beverage manufacturing, mainly as fat substitute for texturizing. It is possible to add beta-1,3-glucan to novel food products such as functional beverage, functional bread, ready-to-serve soups, functional snack foods and a variety of sauces, creamers, bakery products, and additional food products (Ahmad et al. 2012). It has to be emphasized that beta-1,3-glucan displays a considerably higher market value if compared with other algal carbohydrates that are of importance for technical applications, such as the gelling or thickening compound agar (produced by macroalgae belonging to the Rhodophyta group), alginates, cellulose, or carrageenan that is used as emulsifier and stabilizer in various food products. Carrageenan, also known as food-additive E407, can similarly be implemented for pharmaceutical applications (Koller et al. 2014).

Comprehensive analyses and nutritional studies have shown that microalgae proteins are of high quality and comparable to conventional vegetable proteins. The protein content of microalgae can be in the range between 6% and 71% depending on the species (Becker 2007; Nethravathy et al. 2019). The content of standard amino acids in almost all microalgae is favorable compared to that of the WHO/FAO reference and other food proteins such as soy and egg (Richmond 2004). Microalgae can synthesize high protein content, for example, *Spirulina platensis* (60–65%) and *C. vulgaris* (51–58%) of dry matter, and this outstanding capacity has been one of the main reasons to consider these organism as a source of proteins (Henrikson 2010).

Since protein is one of the most valuable algal components, four important parameters of protein quality are used to determine the appropriate nutritive value of algal protein, that is, protein efficiency ratio (PER), biological value (BV), digestibility coefficient (DC) or true digestibility, and net protein utilization (NPU). The nutritive value of the alga-protein depends on the type of postharvesting process, and most of the microalgae have relatively thick cell wall, which makes improperly treated algal biomass indigestible for humans (Becker 2013).

23.4.2 Chlorophylls, Carotenoids, Lutein, and Phycobiliproteins

There is a growing interest in the field of biotechnology for obtaining nonvegetable sources of dyes. The use of natural pigment production by biotechnology involving microalgae has advantages such as continuous cultivation and rapid multiplication of these microorganisms, which can guarantee such productivity for the process. A large number of pigments associated with light occurrence are found in microalgae. Expect chlorophyll as primary photosynthetic compound, the important ones are carotenoids and phycobiliproteins. Similar to what occurs in other organisms, each class of microalgae has its own combination of natural pigments and, consequently, different coloring. Carotenoids extracted from microalgae have various applications in market: β-carotene from *Dunaliella* as vitamin supplement in health foods; lutein, zeaxanthin, and canthaxanthin for pharmaceutical uses and chicken skin coloration; and astaxanthin for aquaculture to provide natural red color for some fishes like salmon, extracted from *Haematococcus*. The phycobiliproteins like phycocyanin and phycoerythrin which are unique in algae are already in use as food and cosmetic applications (Pulz and Gross 2004).

According to Borowitzka (2013), it is possible to increase the synthesis of these bioactive compounds through the manipulation of cultivation conditions, usually due to some environmental stress. New microalgal bio-products from microalgae are being produced, and they are being developed for the scale-up production of health foods from *Chlorella* and *Arthrospira* (*Spirulina*), valuable fine chemicals such as β-carotene from *Dunaliella salina*, astaxanthin from *Haematococcus pluvialis*, and long-chain polyunsaturated fatty acids from *Crypthecodinium cohnii* and *Schizochytrium* (Borowitzka 2018).

Chlorophylls stand out among the most well-known pigments being responsible for capturing sunlight and producing oxygen and sugar through photosynthesis. Chlorophyll is registered and approved as a colorant additive (E140) and is mostly used in the food pigmentation and dietary supplement industries. Famous “chefs de cuisine” use chlorophyll to provide a green coloring to foodstuffs and beverages, such as pasta, pesto, and absinthe (Koller et al. 2014).

In general, most chlorophylls available on the market are in the form of derivative sodium copper chlorophyllin, which makes these structural changes favorable to convert fat-soluble chlorophyll into a water-soluble compound, and this derivative molecule (chlorophyllin) has shown antimutagenic effects to various polycyclic procarcinogens such as aflatoxin-B1, polycyclic aromatic hydrocarbons, and some heterocyclic amines, demonstrating potential chemopreventive agent (Coates et al. 2013).

Carotenoids are primarily a major class of fat-soluble pigments and antioxidants, and the intake of some carotenoids is associated with lowered risk of diseases through their involvement in cell signaling pathways (Stahl and Sies 2005). In the case of β-carotene, due to its antioxidant activity and the nutritional value as pro-vitamin A (Grune et al. 2010), it has been widely applied in food products and

cosmetics (Edge et al. 1997). Several microalgal species can accumulate a high concentration of β-carotene, astaxanthin, or canthaxanthin, for example, which have a wide application as natural dyes and antioxidants (Pulz and Gross 2004).

Carotenoids display so-called secondary light harvesting pigments, supporting the "primary pigment" chlorophyll in capturing light energy. They also act as antioxidants that inactivate reactive oxygen species (ROS) formed by exposure to excessive solar radiation. However, in a review, Gong and Bassi (2016) argued that a better understanding of suitable and economically feasible biotechnological strategies for carotenoids from microalgae is needed.

The industrial interest in these natural pigments can be explained by the ability attributed to them to prevent degenerative diseases: combating free radicals and functioning as anticancer agents and stimulators of the immune system (Orosa et al. 2000; Pangestuti and Kim 2011). Compared to synthetic dyes, they are more resistant to the presence of ascorbic acid, to heat, and to freezing processes, and they are efficient even when applied to food in small quantities (Skulberg 2004). The strict regulation for the application of synthetic dyes in the food industry stimulates research aimed at the productive development and the use of microalgal carotenoids as a food additive (Del Campo et al. 2000).

Microalgal species like *Chlorella zofingiensis*, *Spirulina platensis*, and *Caulerpa taxifolia* are known to synthesize β-carotene at an average yield of 0.1% and 2% of their dry biomass weight (Rammuni et al. 2019). However, the halophilic green biflagellate *Dunaliella salina*, which accumulates up to 13% of β-carotene on its dry biomass, is the predominant source for commercial production of natural β-carotene (Rammuni et al. 2019). In fact, the first high-value product commercially produced from microalgae was β-carotene from *D. salina*. In contrast to synthetic β-carotene, which is limited to its all-trans isomer, natural β-carotene consists of a mixture of cis-trans isomers (9-cis-β-carotene isomer) which shows higher bioavailability, thus considered as a superior product (Raja et al. 2007). Natural β-carotene finds application as a food colorant to enhance appearance and consumer acceptability of products like margarine, cheese, fruit juices, baked goods, dairy products, canned foods, and others (Begum et al. 2016). Global market for carotenoids such as overall is a reality in some countries, but still it is being a potential field in demand in most of them.

Lutein (b,ε-carotene-3,3′-diol) is a naturally occurring pigment belonging to the xanthophyll division of carotenoids. The role of this compound in human health and in particular visual function (lutein is accumulated in the macula) is well established from epidemiological, clinical, and interventional studies (Abdel-Aal et al. 2013). Astaxanthin and β-carotene have been well recognized in prevention and treatment of various diseases. Also, there is an evidence that lutein may have biological effects that include anti-inflammatory and antioxidant properties and play a role in cognitive function (Johnson 2014).

Studies on astaxanthin esters, *cis* and *trans* forms of carotenoids, lutein, and fucoxanthin in vitro and in vivo models are essential for the development in biotechnological applications. *Dunaliella*, *Muriellopsis*, *Scenedesmus*, and *Chlorella* accumulate high lutein content, which varies between 3.4 and 7.6 mg g^{-1}

dry weight of biomass (Fernández-Sevilla et al. 2010). Ambati et al. (2019) discussed some studies that reported the major carotenoid pigments from microalgae with commercial values, such as astaxanthin and astaxanthin esters in *H. pluvialis*, *Chlorococcum* spp., and *Chlorella* spp.; β-carotene in *D. salina*, *S. platensis*, and *Scenedesmus* spp.; lutein in *B. braunii*; canthaxanthin in *Nannochloropsis* spp.; and fucoxanthin from diatoms. These carotenoids have high demand in the global market for health food applications. In the European Union, plant origin lutein is allowed as a food and feed additive and finds applications as a color enhancer of poultry products. In 2015, the global market of lutein was estimated at 135 million US$, with a predicted annual growth rate of 5.3% until 2024 (Hu et al. 2018).

Most of the light energy used by any photosynthetic organism is absorbed by a collection of accessory pigments, since chlorophyll absorbs light energy only in a limited region of the solar spectrum. Phycobiliproteins are a hydrophilic family of pigments of a protein nature, which is soluble in water and functions as accessory pigments of the photosynthetic apparatus in cyanobacteria and in various groups of eukaryotic algae. More specifically, they have antennas of light-collecting pigments and have chromophores called bilins. Phycobiliproteins are classified by the three main pigments or chromophores depending on the color and the absorbance properties: phycoerythrin (red), phycocyanin (bright blue), and allophycocyanin (green-blue) (Matos 2017).

Natural pigments, among their various functions in the food, pharmaceutical, and biochemical areas, have antioxidant activity. Microalgae are photoautotrophic organisms that are exposed to high rates of oxygen and radical stress and, consequently, have developed several efficient protection systems against reactive oxygen species and free radicals. The content and type of antioxidant compounds depend on the microalgae species and their growing conditions (Pulz and Gross 2004).

In addition to carotenoids and other bioactive compounds, microalgae lipids have gained attention not only due to their potential applications in many areas but also as great source of essential polyunsaturated fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In addition to the interest in the production of biofuels, long chains of omega-3 polyunsaturated fatty acids (PUFAs) are valuable lipids produced from microalgae, which cannot be synthesized by higher plants or animals, and they are widely used as nutritional supplements (Matos 2017), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have attention due to their bioactivities. The production of DHA from microalgae has already been commercially exploited (Vingiani et al. 2019), contributing for the feasibility of the microalgae supply chain (Andrade et al. 2020b).

As for the health benefits of EPA and docosahexaenoic acid DHA, both compounds have been well recognized for the prevention of cardiovascular diseases by alleviating adipose tissue inflammation and insulin resistance (Kalupahana et al. 2011). Also, EPA- and DHA-derived lipids such as oxylipins have played an extremely important role in the resolution of inflammation. For instance, PUFAs produced in microalgae can relieve inflammatory bowel disease (IBD) symptoms when consumed in diet (de los Reyes et al. 2014).

23.5 Feedstock for Bioenergy Production

In the past few decades, the use of fossil fuels has significantly increased greenhouse gas emissions. These problems have aroused interest in the search for cleaner energy production to help environmental conservation, with biofuels being a great alternative in solving these problems (Gavilanes et al. 2017). The most important advantages of biofuels lie in the fact that their physical properties and combustion characteristics are very similar to those of fossil fuels and, therefore, could be used without any significant modification of the existing infrastructure for storage, transport, and combustion. Also, all forms of renewable energy have the exceptional merit of being sustainable, potentially CO_2 neutral, and of low or zero air pollution (Peng et al. 2020).

First-generation biofuels are derived from edible raw materials such as wheat, palm, corn, soybeans, sugar cane, rapeseed, oilseeds, beets, and corn. In contrast, second-generation biofuels use dedicated lignocellulosic materials and waste, such as raw materials: jatropha and grass. One of the main disadvantages of first- and second-generation biofuels is that the cultivation of these food or nonfood crops for the production of biofuels will compete for limited arable land, which should be used to grow crops for food production. Microalgae biofuels, known as third-generation biofuels, are treated as a technically viable alternative energy solution that overcomes the main disadvantages related to the first and second generations (Noraini et al. 2014).

Compared to first- and second-generation biofuels, microalgae biofuels offer many more advantages in addition to lipid yields, such as high growth rate, high-efficiency CO_2 mitigation, do not compete with land-based food crops, less water demand than terrestrial crops, tolerance to wastewater during cultivation, use of low-quality land and water, integration with point sources of carbon dioxide, such as coal plants, and more economical agriculture (Bennion et al. 2015; Bharadwaj et al. 2020; Noraini et al. 2014).

Microalgae biomass has shown its potential as a raw material for the production of various biofuels, such as biogas, biodiesel, bioethanol, and biohydrogen. At the current stage of biofuel development, it is still too early to indicate which would be the most beneficial route for the production of biofuels from algae biomass. However, anaerobic digestion appears to be the least complex of all; besides, it can play an important source of energy combined with other biofuel production. In this context, the specific characteristic of the strain of the selected microalgae is essential (González-Fernández et al. 2012).

23.5.1 Biogas, Biodiesel, Biohydrogen, and Bioethanol

Biogas

The generation of biogas is a biochemical process with cooperative action by multiple microorganisms, involving several mandatory or optional anaerobic microorganisms. Microorganisms play a decisive role in the efficiency of biogas production (Deng et al. 2020). The composition of biogas can vary according to the type of raw material, and the operating conditions of the digester contain from 50% to 75% CH_4 and 25% to 50% CO_2, together with other components, such as water vapor (H_2O), hydrogen sulfide (H_2S), and ammonia (NH_3) (Andrade et al. 2020a)

In comparison to the production of biodiesel, relatively few studies have been published on the anaerobic digestion of microalgae; although it was already studied in the 1960s, the hype of microalgae in recent years has revived the research on methane production. It is essential to mention that anaerobic digestion is a more direct process of energy production, as it does not need an intense concentration of cells, drying, and oil extraction that increases production costs (Ayala-Parra et al. 2017), making more financially feasible its use in biogas generation.

The methane yield of anaerobic digestion of microalgae can be achieved in several stages, including cultivation, harvesting, pretreatment, and, finally, some pretreatment techniques for microalgae before biodigestion. However, due to the wide variation in the composition of several microalgae species, the potential of methane also varies significantly between species (Table 23.1), which must be considered before selecting any strain as a methane producer. In addition, several other factors, mainly process parameters, significantly affect the throughput and efficiency of the overall process (Chu and Phang 2019).

Factors that must be taken into account in the process of anaerobic digestion are the pH and temperature of the substrate, which play a crucial role in the production of methane gas. Alkaline conditions are more suitable for the generation of biogas by microalgae since alkaline conditions can increase the solubility of the biogas CO_2 remaining in the form of dissolved carbonate generating a highly pure biogas (Chu and Phang 2019).

From different species of microalgae found in Table 23.1, it is shown that the methane gas content varied from 40% to 78.6%, with the majority of the research being carried out with a batch-type bioreactor with retention varying between 2.2 and 64 days.

Microalgae, in addition to the isolated effect for the generation of biogas, may have an effect accompanied by other microorganisms such as endophytic bacteria. In one study, it was demonstrated that the cocultivation of the microalgae *Chlorella vulgaris* with endophytic bacteria resulted in higher removal of nutrients and CO_2 than the monoculture of microalgae, besides efficiency in removing the chemical oxygen demand showing essential implications for improving wastewater purification and biogas (Xu et al. 2020).

Another alternative is the cocultivation of microalgae with fungi, with excellent results in the generation of methane gas and the treatment of wastewater (Muradov

Table 23.1 Microalgae species used in anaerobic biodigestion of biomass

Microalgae species	Study domain/emphasis	Reactor type	Temp.	HRT	CH_4 %	References
Chlamydomonas reinhardtii and *Chlorella vulgaris*	Enhanced methane production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* by hydrolytic enzymes addition	Batch	35	22	72–75	Mahdy et al. (2014)
Chlamydomonas reinhardtii, *Chlorella kessleri*, *Dunaliella salina*, *Euglena gracilis*, and *Scenedesmus obliquus*	Microalgae as substrates for fermentative biogas production in a combined biorefinery concept	Batch	38	32	66–67	Mussgnug et al. (2010)
Chlamydomonas reinhardtii and *Pseudokirchneriella subcapitata*	Sunlight to biogas energy conversion system	Batch, semicontinuous	34–41	2.5	40–65	De Schamphelaire and Verstraete (2009)
Scenedesmus sp. and *Chlorella* sp.	The superiority of co-digestion as a step toward maximizing methane production from microalgae	Batch	35	40	–	Zhen et al. (2016)
Chlorella sorokiniana	Anaerobic digestion of residual algal biomass	Batch	30	42	–	Ayala-Parra et al. (2017)
Chlorella vulgaris	Anaerobic digestion of *Chlorella vulgaris* for energy production	Batch	28–31	64	67.8–75.3	Sánchez and Travieso (1993)
Dunaliella tertiolecta	Methane production	Batch	37	49		Lakaniemi et al. (2011)
Nannochloropsis sp., *Nanofrustulum* sp., and *Phaeodactylum tricornutum*	Efficient anaerobic digestion of whole microalgae biomass and lipid-	Batch	35	30	64.81–69.56	Zhao et al. (2014)

(continued)

Table 23.1 (continued)

Microalgae species	Study domain/emphasis	Reactor type	Temp.	HRT	CH_4 %	References
	extracted microalgae residues for methane energy production					
Nannochloropsis salina (lipid-extracted biomass)	Thermal pretreatment on anaerobic digestion of *biomass*	Batch	40	49	–	Schwede et al. (2013)
Scenedesmus obliquus	Anaerobic digestibility of *Scenedesmus obliquus* under mesophilic and thermophilic conditions	Hybrid flowthrough anaerobic reactor	54	2.2–22.3	77.1–78.6	Zamalloa et al. (2012)
Tetraselmis	Biomethanation of *Tetraselmis*	Completely stirred tank reactor	35	14	72–74	Di et al. (2007)
Isochrysis galbana	Biogas production from dry and wet lipid extraction	Batch	38	30	–	Sánchez-Bayo et al. (2020)

et al. 2015). Cocultivation between *Chlorella vulgaris* and the fungus *Ganoderma lucidum* resulted in 64.92% CH_4 and 35.08% CO_2, with the removal of the chemical oxygen demand, total nitrogen, and total phosphorus of 86% (Wang et al. 2017).

The integration of the microalgae cultivation process to remove CO_2 from biogas and treat wastewater is a promising strategy for the economic viability of producing microalgae-based biofuels (Srinuanpan et al. 2020). The cocultivation of microalgae with other microorganisms and their applications have great potential in the generation of biogas and wastewater treatment to reduce contamination.

Biodiesel

All biodiesels have the same renewable and primary origin; they are produced from the photosynthetic conversion of solar energy into chemical energy, which makes them isolated from the initial photosynthesis. According to the American Society of Testing and Materials (ASTM), the term biodiesel is attributed to monoalkyl esters of long-chain fatty acids resulting from edible oils, nonedible oils, and used oils, produced from the process of transesterification of triglycerides using methanol and catalyst (Singh et al. 2020a) thus producing biodiesel and glycerin (soap) (Chua et al. 2020).

Microalgae have been identified as the most promising third-generation raw material with great potential for the production of biodiesel since its cultivation requires less cultivated land compared to conventional oilseeds and the high growth rate of microalgae (Goh et al. 2019; Yin et al. 2020). Its lipid content varies according to the different species of algae and growth periods, generally between 20% and 50% of the biomass, and reaches 70% under certain culture conditions. Lipid productivity instead of lipid content is generally accepted as an indicator for assessing the performance of microalgae in oil production. Lipid content is the concentration of lipids in the microalgae cells, regardless of biomass production, and lipid productivity depends on the production of biomass and refers to the accumulation of lipids in the cells in the total biomass produced (Xue et al. 2020). Under normal cultivation conditions, it can reach from 1.9% to 54% by weight of the lipid content, and in species under conditions of lack of nitrogen, it reaches between 18.42% and 64% (Goh et al. 2019).

The lipid content of microalgae biomass can vary between 2% and 41% of dry weight (Gouveia et al. 2008). It represents a very diverse group of compounds that have essential biological functions, such as the formation of structural components of cell membranes, serving as a source of energy and storage, and participation in metabolic pathways.

Microalgae cells are known to accumulate large amounts of lipids, with triglycerides (TAGs) which can be converted into fatty acids methyl esters (FAME) that can be used as feedstock for biodiesel production (Soares et al. 2019) and polyunsaturated fatty acids (PUFA) being the most studied from a biotechnological application (Bellou et al. 2014; Khan et al. 2018). Algae lipids are composed of polar and nonpolar lipids. Polar lipids are produced in the growth phase and are enriched in the chloroplast and cell membrane system (Guckert and Cooksey 1990).

The most frequently studied enzyme involved in lipid synthesis is acyl-CoA diacylglycerol acyltransferase (DGAT), which is involved in the final reaction of the TAG biosynthetic pathway (Merchant et al. 2012; Xu et al. 2018). Three independent groups of enzymes, referred to as acyl-CoA diacylglycerol acyltransferases types 1, 2, and 3 (DGATs 1-2-3), take part in the acyl-CoA-dependent formation of TAGs, which has been analyzed in different microalgae, for instance, in *Ostreococcus tauri* (Wagner et al. 2010), *Chlorella ellipsoidea* (Guo et al. 2017), and *Nannochloropsis oceanica* (Wei et al. 2017).

Different isoforms of DGAT2 (NoDGAT2A, 2C, 2D) have successively been identified in *N. oceanica*, and different combinations of either overexpression or under expression have been analyzed. These combinations gave different fatty acid production profiles, with some optimized for nutritional applications and others for biofuel purposes (Xin et al. 2017). In *Chlorella variabilis* lipid metabolism, an enzyme named fatty acid photodecarboxylase was identified, which belongs to a microalgae-specific clade of the glucose–methanol–choline oxidoreductase family and which catalyzes the decarboxylation of free fatty acids to hydrocarbons (n-alkanes or n-alkenes) important for biofuel production (Sorigué et al. 2017).

Microalgal biomass pretreatment is essential for further processing, which depends on microalgae cell structure and composition and energy demands during the process. According to de Carvalho et al. (2020), high-pressure homogenization and acid hydrolysis remain economically competitive, and that those could be upgraded to increase the viability. These authors argued that operations reliable at a small scale, such as sonication and lyophilization, may prove impractical or too expensive on an industrial scale. In contrast, uncommon steps, such as freeze-thawing and pulsed electric fields, can end up having a positive energy balance.

The high content of free fatty acids in the lipids of microalgae biomass is an important topic, which must be addressed when considering the production of biodiesel from microalgae biomass (Krohn et al. 2011). The oils extracted from the microalgae biomass are characterized by having a high content of free fatty acids that can reach up to 85% of the total lipids, depending on the cultivated microalgae strain and the cultivation conditions (Chen et al. 2012; Krohn et al. 2011).

Lipids extracted from microalgae biomass, which have a high content of free fatty acids, are unsuitable for the synthesis of biodiesel when transesterified with primary catalysts since the high content of free fatty acids decreases the catalytic activity due to saponification. An alternative to overcome this limitation is the use of two steps in the crude oil treatment process, which involves the esterification reaction of microalgae lipids with a high content of free fatty acids with methanol to convert free fatty acids into ester fatty acid methyl and then followed by transesterification (Dong et al. 2013). When the level of free fatty acids in oils is higher than 5%, saponification will inhibit the separation of methyl and glycerol esters, which causes the formation of an emulsion during washing with water; therefore, it is necessary to convert free fatty acids into methyl esters (Huang et al. 2010).

The two-step proposal for catalytic conversion was also proposed by Chen et al. (2012) because it had a high potential for the production of biodiesel from microalgae oil rich in free fatty acid. After optimized esterification–

transesterification procedures, the conversion rate of triacylglycerols and free fatty acids to methyl esters reached 100%.

Regarding the extraction of lipids and proteins from wet microalgal biomass in a 3G biorefinery by comparing supercritical fluid extraction (SFE) and low-pressure solvent extraction (LPSE), it was showed that supercritical fluid extraction for wet microalgae processing is not economically attractive, as it increases the total investment by 71% (Albarelli et al. 2018).

For microalgal biomass, the extraction of the crude hexane lipid fraction, using mechanical stirred associated with ultrasound technique, allowed greater extraction of the crude hexane lipid fraction (Gomes et al. 2019). These authors argued that the ester profile with relatively elevated concentration of polyunsaturated fatty acids (C18:3) is unfeasible in their application for biodiesel production.

After obtaining the biomass of microalgae depending on the type of microalgae, it is necessary to carry out the cell rupture process, which is a pretreatment for the extraction, to facilitate the extraction of the metabolites of interest, in the case of biodiesel production, lipids (Fig. 23.5). It is not a mandatory step, and the decision for its use depends on the extraction method to be used. According to Mata et al. (2010), rupture and extraction can occur in two ways:

- By mechanical action: through the high-pressure homogenizer, ball mill, ultrasound, autoclaves or freeze-drying, microwave or
- By nonmechanical action: by freezing, using organic solvents, osmotic shock, or reactions of acids, bases, or enzymes, for example.

These pretreatment steps use energy intensively and, therefore, can only be carried out by increasing the efficiency of lipid extraction from microalgae. Most cell disruption pretreatments require water, and therefore must be performed before the drying process.

Once the pretreatment is carried out, oil extraction follows. Peralta-Ruiz et al. (2013) mentioned that there are several methods of oil extraction used in microalgae; these methods can be divided into as follows:

- Methods assisted by mechanical interruption using homogenizing cells, ball mills, pressing systems, among others. These methods are not suitable for the extraction of oil on a laboratory scale, as they present high biomass losses and low selectivity for lipids.
- Enzyme-assisted extraction methods, in which the microalgae cell wall is degraded by enzymes that allow the release of lipids. However, enzyme activity is affected by several variables, including concentration and ratio of system compounds, acid profile greases, microalgae composition, temperature, among others. These issues make it challenging to maintain this route at this time for large-scale biomass processing.

Other methods are also used for the extraction of microalgae oil. One of the most used method is the extraction with an organic solvent. Currently, hexane and ethanol have been widely used in the extraction of edible oil, but chloroform, methane, benzene, and other organic solvents are toxic and therefore are not applicable.

Fig. 23.5 Examples of methodologies for lipid extraction. Source: Modified from Kumar et al. (2015)

According to the similarity compatibility principle, nonpolar solvents dissolve and destroy nonpolar lipids in the cell membrane of microalgae to extract the oil. Because organic solvents are toxic, volatile, and difficult to recycle, some green solvents are also used, such as bio-based solvents, ionic liquids, convertible solvents, supercritical fluids, subcritical water, and pressurized solvents (Xue et al. 2020).

A widely used technology is supercritical fluid extraction (SFE), which allows the preservation of the natural qualities of bioactive compounds, reducing the environmental impact and minimizing energy costs at the same time (da Silva et al. 2016). Also, SFE allows us to prevent the presence of traces of solvent in the final extracts, with the possibility on a large scale of recovery of CO_2 in a closed circuit with an economic advantage with the use of other solvents (Molino et al. 2020).

Supercritical fluid technology is an analytical process in which the extraction and separation of organic compounds from a matrix can be carried out effectively. A pure substance is in a supercritical state when it is above its critical temperature and pressure (Akalın et al. 2017). Carbon dioxide (CO_2) and water are the most used supercritical fluids, which can potentially be used in the production of biofuels; supercritical CO_2 has several advantages, especially for the extraction of low polarity chemicals, such as biomass lipids (Li et al. 2019).

Transesterification is a multistep reaction, including three reversible steps in series: triglycerides are converted to diglycerides, then diglycerides are converted to monoglycerides, and monoglycerides are converted to esters (biodiesel) and glycerol (by-product). The transesterification reaction is where the radicals R1, R2, R3 represent long-chain hydrocarbons, known as fatty acids (Mata et al. 2010). For the conventional transesterification reaction, oil or fat and short-chain alcohol (the alcohols commonly used are methanol, ethanol, propanol, butanol, and amyl alcohol, but methanol is applied more widely due to its physical advantages and low cost) (Huang et al. 2010) are used as reagents in the presence of a catalyst (usually NaOH). Although the theoretical molar ratio of alcohol:oil is 3:1, the molar ratio of 6:1 is generally used to complete the reaction accurately. The ratio between the mass input of raw material and the mass production of biodiesel is about 1:1, which means that, theoretically, 1 kg of oil results in about 1 kg of biodiesel (Mata et al. 2010).

An alternative for obtaining a higher biodiesel content is the use of heterogeneous catalysts in addition to the use of ultrasound and microwave techniques and supercritical alcohols that generally improve biodiesel production (Goh et al. 2019).

In research carried out by Levine et al. (2010), wet *Chlorella vulgaris* biomass was directly processed, eliminating the use of organic solvents during lipid extraction, recovering nutrients and glycerol. They developed a catalyst-free technique for the production of biodiesel. First, wet biomass (about 80% humidity) reacted in subcritical water to hydrolyze intracellular lipids. In another step, solids rich in moist, fatty acids underwent supercritical transesterification in situ with ethanol to produce biodiesel in the form of ethyl esters of fatty acids. They examined hydrolysis at 250 °C for 15–60 min; the solids recovered by filtration contained 77–90% of the lipid initially present in algae biomass, mainly in the form of fatty acids. They determined that the higher time and temperature and higher ethanol load tended to

increase the gross yields of biodiesel and fatty acid ethyl esters, which ranged about 56–100% and 34–66%, respectively, based on lipids in hydrolysis solids.

Another study with *Chlorella* sp. (Chauhan et al. 2020) reported the development of an efficient method of direct conversion to biodiesel via supercritical transesterification of methanol. The method involved the evaluation and optimization of the neutral lipid content and water content of the biomass as two critical attributes of the biomass quality to maximize the yield of fatty acid methyl esters (FAME). They obtained the highest FAME yield of 96.9% reaching an ideal value of lipid content, the water content of the biomass and methanol load of 52% (w/w), 5.75 mL g^{-1} and 115 mL g^{-1}, respectively. In general, the use of microalgae as raw material for biodiesel is technically viable, but not economically viable.

Biohydrogen

Biohydrogen is a natural and transitory by-product of several biochemical reactions of microbial origin; generation of H_2 gas either by biological machinery or by thermochemical treatment of biomass can be defined as "biohydrogen." The thermochemically produced H_2 is also being called bio-hydrogen due to the use of biomass as a substrate/raw material. On the contrary, several biological routes are available for the production of bio-hydrogen belonging to anaerobic/fermentation, photobiological, enzymatic, and electrogenic mechanisms (Mohan and Pandey 2013).

Biohydrogen can be generated by various biological forms and classified into two main categories (Aslam et al. 2018): light-dependent and dark fermentation processes. The primary light-independent process is dark fermentation, while light-dependent processes include photofermentation and photolysis. All bio-hydrogen production pathways depend on nitrogenase or hydrogenase for the evolution of hydrogen. These technologies derive energy directly from light energy or indirectly through the consumption of photosynthetically derived carbon compounds.

Some species of microalgae have potential indirect biophotolysis, especially *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Nannochloropsis* sp., *Scenedesmus obliquus*, *Cosmarium* sp., *Thalassiosira weissflogii*, among others (Eroglu and Melis 2016). Their hydrogen can be obtained by different methods as follows:

– Chu and Phang (2019) reported that in direct biophotolysis, the photosynthetic apparatus chlorophyll and other pigments of eukaryotic green microalgae can retain light and energy from the sun. In addition, it is being improved with water separation to produce a low potential reducer or ferredoxin, which can moderate a hydrogenase or nitrogenase enzyme directly without temporary CO_2 fixation. The hydrogen ions generated are transformed into hydrogen gas in the medium with electrons donated by reduced ferredoxin in the presence of the enzyme hydrogenase. Naturally, direct bio-photolysis is a desirable method due to the use of solar energy to alter an easily obtained substrate, water, oxygen, and hydrogen, but, in practice, it is restricted by other problems such as enzyme hydrogenase

activity affected by O_2, because it is related during the process of direct bio-photolysis and, therefore, inhibits the H_2 yield.

- In indirect bio-photolysis, the sensitivity problems of the hydrogen evolution process are potentially circumvented by the temporal and spatial separation of the evolution of oxygen and the evolution of hydrogen. Indirect bio-photolysis processes involve the separation of H_2 and O_2 evolution reactions in separate stages, coupled through CO_2 fixation/evolution; in this case, cyanobacteria have the unique characteristics of using CO_2 in the air as a source of carbon and energy solar as a source of energy. The cells absorb CO_2 first to produce cellular substances, which are later used in the production of hydrogen (Pareek et al. 2020).
- In dark fermentation, hydrogen is produced in the absence of sunlight, oxygen, and water. Fermentative microorganisms convert complex organic matter into a mixture of alcohol and organic acid, together with the production of hydrogen. Various carbon-rich waste resources can be processed by dark fermentation, producing hydrogen, and other significant by-products like volatile fatty acids, acetic acid, and butyric acid (Ren et al. 2019).
- Photo-fermentation is a fermentative conversion of organic substrates into hydrogen and carbon dioxide by using sunlight as an energy source. Using the light as a power source, organic acid substrates are oxidized using the tricarboxylic acid cycle, producing electrons, protons, and carbon dioxide. Its advantages are the removal of environmental pollutants, the use of industrial residues, and the use of organic acids produced from dark fermentation. The disadvantages are the need to limit the nitrogen condition and the pretreatment of the industrial effluent, as it can be toxic (Sharma and Arya 2017).
- When the process is carried out in two stages (integration of dark and photo-fermentation), during the first stage of dark fermentation, the substrate containing carbohydrates is converted into organic acids, CO_2, and hydrogen by mesophilic and thermophilic bacteria. In the second stage, dark fermentation residues containing organic acids, such as acetic and lactic bacteria, used in photosynthetic photofermentation or without purple sulfur, are transformed for later production of hydrogen (Mohan and Pandey 2013).
- Biocatalysis electrolysis is a technology that is related to the microbial fuel cell and overcomes thermodynamic barrier utilizing a small electrical energy input, making the process independent of the reactor's surface area. Biocatalyzed electrolysis achieves this by using electrochemically active microorganisms, which convert dissolved organic material into bicarbonate, protons, and electrons. By direct contact with the electrode surface or aided by redox mediators (excreted), these microorganisms release the electrons produced to the electrode surface, in order to generate current. When coupling this biological anode to a proton reducing cathode through a power source, direct conversion of dissolved organic material into hydrogen is carried out. The complete process takes place in an electrochemical cell in which the oxidation of dissolved organic material and the reduction of protons are separated into two chambers. The separation between these chambers is established through a cation exchange membrane (e.g.,

Nafion). Externally, the anode and cathode are connected to the power supply using an electrical circuit. As the power supply conducts electrons released from the anode to the cathode, an equal number of protons permeates through the membrane. At the cathode, protons and electrons combine to form pure hydrogen gas (Eroglu and Melis 2016).

Bioethanol

Ethanol is the most widely used liquid biofuel. It is an alcohol and is fermented from sugars, starches, or cellulosic biomass. Most commercial ethanol production is from sugarcane or beet, as starches and cellulosic biomass generally require expensive pretreatment. Usually, it is used as a source of renewable fuel as well as in the manufacture of cosmetics, pharmaceuticals, and also in the production of alcoholic beverages (Demirbaş 2005).

The production of bioethanol involves different stages of the process, including pretreatment of biomass, hydrolysis, fermentation, and product recovery. Pretreatment of biomass is one of the most important and expensive stages of the process. The pretreatment step is necessary to reduce the crystallinity of the biomass and increase the surface area to improve the digestibility of the substrate (Harun et al. 2011; Sarkar et al. 2012).

Certain microalgae species can produce high levels of carbohydrates instead of lipids as reserve polymers. These species are ideal candidates for the production of bioethanol since the microalgae carbohydrates can be extracted to produce fermentable sugars (Mussatto et al. 2010), among which stands out *Laminaria japonica*, *Sargassum fulvellum*, *Hizikia fusiformis* (Lee et al. 2009), *Chlorococum* sp. (Harun et al. 2010), *Gelidium corneum* (Yoon et al. 2012), *Schizocytrium* sp. (Kim et al. 2012), *Scenedesmus obliquus* (Miranda et al. 2012), *Chlorella vulgaris* (Ho et al. 2013), *Chlorella* sp. (Ngamsirisomsakul et al. 2019), *Chlorella sorokiniana* (Tatel and Madrazo 2020), *Scenedesmus acuminatus* (Chandra et al. 2020), *Chlamydomonas* sp. (Kim et al. 2020), *Ulva intestinalis*, *Amphiroa compressa* (Osman et al. 2020) among other species.

Some microalgae have great potential for the generation of different biofuels. Different technologies are ready for their development on a large scale, but there are still several obstacles that need to be overcome, such as the high costs of cultivation, harvesting, and processing, which consequently causes the price of microalgae biofuels higher than fossil fuels. Anaerobic biodigestion for the generation of biogas seems to be most financially viable due to its less complex processing compared with other biofuels.

23.6 Environmental and Agricultural Applications

The microalgae have the capability to remove more than 90% of nutrients and some extend of toxic chemicals and heavy metals from the industrial effluent, and it can be further increased by using growth stimulators or by developing growth. In addition,

from the perspective of improvement of soil environments, microalgae, mainly cyanobacteria, are thought-out as a potential producer of exopolysaccharide and biomass production in large scale, aiming dispersion of inoculum in the field as efficient, eco-friendly method (Tiwari et al. 2019). Evidence reveals a higher amount of lipids, proteins, and pigments in biomass of these microorganisms plus to recycling water, generating applications in bioenergy (Zhu et al. 2019) in agriculture as biofertilizers (Castro et al. 2020).

23.6.1 Environmental Bioremediation Using Microalgae

For environmentally and economically sustainable food, agribusiness, and bioenergy industry processes, microalgae appears to be an option-based biological source of by-products. The microalgae cultivation can provide recovery of recyclable nutrients from secondary sources, which has an enormous role to global sustainable demands. The wastewaters have different origins, and most of them are rich in energy and nutrient sources that can be recovered and reused in a circular bioeconomy viewpoint (Nagarajan et al. 2020). Concomitantly, photosynthetic microorganisms when integrated with existing facilities to intensive cultivation that can be in different agro-industrial types of wastewater provide biomass production, environmental bioremediation, and reasonable return.

Both, cyanobacteria and microalgae in a mixotrophic or heterotrophic system, can utilize carbon, N, P, and other nutrients from different types of wastewater. Using industrial effluents through cultivation of microalgae is an alternative to synthetic media and viable to the increase of its biomass generated, with effects on both in the investments in the agriculture and in the reuse of wastewater from agro-industries by remediation and/or energy production recovery (Umamaheswari and Shanthakumar 2016).

23.6.2 Agro-Industrial Wastewater Treatments

Besides phototrophic growing using CO_2 as energy source, microalgae can use other carbon sources to increase biomass production in heterotrophic or mixotrophic cultivation systems, including organic carbon. The high availability of wastewater or effluents with high organic content, especially those derived from agro-industries, and the ability of microalgae to thrive in unsuitable waters benefit the generation of biomass for the production of biofuels. For instance, effluents from the brewing industry that generates a large amount of wastewater (Tonhato Junior et al. 2019) and unsterilized dairy-derived liquid digestate can be used for simultaneous biofuel feedstock production and contaminant removal (Zhu et al. 2019).

Studies have highlighted the significant potential and economic value of combining biorefinery treatment to recover wastewater with a high organic load. For

sugarcane, Sydney et al. (2019) proposed an efficient process of growing microalgae and cyanobacteria for reusing vinasse from the bioethanol industries of bioethanol production. For food and agro-industrial activities, Vu et al. (2020) projected a hybrid system to collect energy, nutrients and microalgal biomass from highly organic and nutritious wastewater, which comprises an anaerobic membrane bioreactor to produce biogas and a microalgal membrane reactor.

Wastewater is a resource for the recovery of clean water, energy, and nutrients (Kehrein et al. 2020). Table 23.2 shows the wastewater types, microalgal genus or species used, the cultivation system, and the main contributions or relevant findings that were found in each study mentioned. The reviews were chosen to present how the microalgae cultivation in wastewater or agro-industrial waste reduced environmental impacts and to produce biomass as raw material for bioenergy and also as biofertilizers for agriculture.

In these works, chlorophytes and cyanobacteria were evaluated using growth media that included dairy residues; animal residues as pig-slurry, poultry, cattle, and fish; processing of cassava, maize, potatoes, coffee, sugar cane, grapes, palm oil, and soybean; animal feed production and yeast production; and brewery, tannery, and sewage (Table 23.2).

An option to bioremediation of swine/piggery wastewater is fermentation for biogas and after microalgae cultivation, mainly with green microalgae that are strong candidate for biomass production by using piggery wastewater. Addressing the treatment of swine/pig wastewater, some studies were highlighted, mainly those that evaluated the participation of microalgae-based processes in the removal of phosphorus, nitrogen, and organic matter, avoiding soil and surface water contamination. *C. vulgaris* and *Scenedesmus dimorphus* have the ability to remove P and N from pig slurry and dairy residues (González et al. 1997), while *C. zofingiensis* was able to adapt and grow well outdoors using pig slurry sterilized, which can expand the potential biomass production for biodiesel with a cost–benefit advantage (Zhu et al. 2013).

Anaerobic digestion and subsequent microalgal cultivation with the digestate under a circular economy concept might help improve the economic feasibility of in-farm biogas plants with net positive values (Nagarajan et al. 2020). Cultivation of *C. sorokiniana* on thin stillage digestate that was pretreated with struvite was able to remove chemical oxygen demand, ammonia–nitrogen, and total phosphorus with biomass production containing high protein, starch, and lipid contents (Sayedin et al. 2020), which is a value-added product for application as fertilizer. There are some biological processes (e.g., aerobic processes and anaerobic digestion) that are beneficial in nitrogen removal, but they are relatively expensive. To replace these processes, an option to remove organic carbon and nutrients from a wastewater purification perspective, a diluted soybean wastewater as a cultivation medium for *Chlorella* sp. promoted removal rate of 50–65% chemical oxygen demand (COD), 70–80% NH_3–N, and 95–100% total phosphorus (Song et al. 2019).

When growing in heterotrophic medium from untreated dairy wastewater or dairy farm effluent, green microalgae have been identified as a good candidate for biomass production for bioenergy and simultaneously for nutrients recycling, for instance,

Table 23.2 Example of agro-industrial waste and microalgae species used in cultivation system as nutrient removal and biomass production

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
Dairy residues and pig-slurry	*Chlorella vulgaris* *Scenedesmus dimorphus*	Cylindrical and triangular bioreactors of glass	*C. vulgaris* and *S. dimorphus* have been shown to reduce pollutant elements in effluents in different ways *S. dimorphus* was more efficient in removing ammonia, at the end of the cultivation cycle, both removed the same amount of P from the residue *C. vulgaris*, the triangular bioreactor, was adequate to remove ammonia and cylindrical for phosphorus	González et al. (1997)
Pig-slurry	*Chlorella zofingiensis*	Photobioreactors bubble column	The combination of *C. zofingiensis* cultivation in swine wastewater can improve the expansion of algae biodiesel production and improve the cost–benefit ratio. Wastewater can replace the use of fertilizers The expansion will depend on the policy of governments to reduce carbon emissions, in addition to future research, through investments and subsidies	Zhu et al. (2013)
Municipal and piggery slurry	*C. sorokiniana*	UASB reactor + flat panel photobioreactors	UASB's high efficiency in removing organic matter (>90%) and biomass production (1 g L^{-1}), with average removal of dissolved inorganic carbon, phosphate,	Leite et al. (2019)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			and ammonia around 46–56%, 40–60%, and 100%, respectively	
Swine/piggery wastewater	*C. zofingiensis*, *Synechocystis* sp., *Tribonema* sp., and *Botryococcus braunii*	Glass bubbling bottles	Cultivation wastewater from anaerobic digestion of swine wastewater which was pretreated by sterilizing swine. pH 7.0. Microalgae grown in the pretreated wastewater were higher than that in the nontreated wastewater, but the protein content was lower	Cheng et al. (2020)
Processing of potato, fish, coffee, animal feed production, and yeast production	Consortium of *Phormidium* sp., and green microalgae *Oocystis* sp. and *Microspora* sp.	Glass bottles	The biodegradable total organic carbon was the limiting component during wastewater treatment in most of the evaluated agro-industrial effluents and dilutions The results highlight the need for an external carbon source (CO_2) supply, pH control strategies, and the dilution of the high ammonia concentrations	Posadas et al. (2014)
Meat-processing industry	*Scenedesmus* sp.	Photobioreactor	In a meat-processing industry after flotation treatment (PE) and after an activated sludge unit (SE) The dominance of the genus *Scenedesmus* (mixotrophic) in all	Tango et al. (2018)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			the operations showed the ability of that genus to survive in extreme environments	
Vinasse	*Scenedesmus* sp.	Air-lift photobioreactors	Light intensity and percentage of vinasse (up to 40%) influenced the amount of biomass to be produced by *Scenedesmus* sp.	Ramirez et al. (2014)
Vinasse	*Chlorella vulgaris*	Tubular reactors	Reduction in the concentration of most metabolites in the first days of microalgae growth in the dark under continuous air flow, due to the transition from autotrophic to heterotrophic metabolism	Quintero-Dallos et al. (2019)
Sugarcane	*Chlorella vulgaris*		The treatment of conventional filtration and bio-digested of sugarcane (vinasse) resulted in cleaner residues that supported the *C. vulgaris* growth put on 32× more cell density and higher final biomass	Candido and Lombardi (2017)
Soybean	*Chlorella vulgaris*	Conical bottles	The ammonia escape rate could be reduced to 15.8% and the carbon conversion capacity and efficiency of the hybrid process was around 44.3 mg/L/day and 60.8% with efficient purification of soy effluents Associated with	Song et al. (2019)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			nitrogen and carbon biotransformation, 78.8 mg/L/day of microalgae biomass could be grown to produce value-added ingredients to enhance the techno-economic viability of the hybrid microalgae absorption process	
Tofu whey wastewater	*C. pyrenoidosa*	Filtrated and sterilized	Growing at heterotrophic and mixotrophic conditions using 100% Tofu whey wastewater The biomass productivity was improved when using TWW, which resulted in higher lipid and protein productivity	Wang et al. (2018)
Grape processing	*Auxenochlorella protothecoides* and *Chlorella sorokiniana*	Photobioreactors	Two microalgae analyzed removed >90% nitrogen, >50% phosphate, and 100% acetic acid in the residual water of the winery Organic carbon did not play a limiting role in the growth of microalgae Bacteria and algae provided benefits to synergistic growth, contributing to wastewater treatments	Higgins et al. (2018)
Palm oil mill effluent	*Tetraselmis suecica*	Bottles	Cocultivation of microalgae with oil palm empty fruit bunch and palm oil mill effluent	Ahmad et al. (2014)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			presented the highest specific biogas production and biomethane yield was achieved with microalgae for anaerobic biomethane production	
Olive mill wastewater	A mix of *Spirulina platensis*, *Nostoc muscorum,* and *Anabaena oryzae*	–	Cyanobacteria biomass from the growth on olive mill wastewater was s applied as biofertilizers for celery in sandy soil	Rashad et al. (2019)
Fish farming	*Spirulina platensis*	Two boxes for raising fish and a swimming pool (pilot scale)	Two boxes for raising fish and a swimming pool (pilot scale). Two boxes for fish cultivation and a swimming pool (pilot scale) Carry out was successful to the consortium *S. platensis* with other organisms in polyculture systems, or integrated agriculture, with or without water recirculation	Nogueira et al. (2018)
Manure wastewaters (poultry, pig, and cattle), brewery, dairy residues, and sewage	*Scenedesmus oblíquus*	Bubble photobioreactors and flat plate	The biomass productivity achieved using the different wastewater was higher than the synthetic medium, except for birds, with a higher volume obtained in brewery wastewater The reduction of environmental impacts, in addition to indicating strategies for the future of	

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			bioenergy production and circular economy	
Brewery effluent	*Chlorella* sp.	Flask	The SL Tanfloc tannin proved to be efficient in flocculating the brewery effluent, allowing the reuse of water and recovered biomass containing nutrients The sludge generated and accumulated in the brewery's effluent treatment process can be applied as a biofertilizer, after predrying, since it has considerable amounts of nitrogen and phosphate in its composition	Tonhato Junior et al. (2019)
Tannery (animal leather processing)	*Arthrospira* (Spirulina)	Open lane ponds	The odor emission was reduced with the cultivation of microalgae compared to the other lagoons. Thus, with treatment based on microalgae, the disposal of wastewater from tanneries can be compatible with the environmental and social acceptability. For the local community, the odor has decreased substantially	Dunn and Rose (2013)
Sewage and tannery	*C. vulgaris* and *Pseudochlorella pringsheimii*	Conical flask	Both species are apparent to treat tannery effluent in three dilutions, with the substantial removal of polluting	Saranya and Shanthakumar (2019)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			compounds, like NH_3, PO_4, and heavy metal chromium *P. pringsheimii* has higher lipid accumulation potential than *C. vulgaris* irrespective of the saline stress	
Dairy residues	*Chlorella* sp.	Suspended solid supports and polyethene foam	By using polyethene foam, it allows the cultivation of *Chlorella* sp. easily with large accumulated biomass, and for a relatively long period	Johnson and Wen (2010)
Dairy residues	*Neochloris oleoabundans*	Horizontal photobioreactors	Increase lipids in its biomass, and this is useful in biodiesel. In the same way, it demonstrates the potential capacity of larger photobioreactors and low cost for biomass production Microalgae monocultures have increased the possibility to reuse wastewater and produce high-quality biodiesel during wastewater treatment	Levine et al. (2011)
Dairy residues	*Chlorella pyrenoidosa*	Conical glass balloon	*C. pyrenoidosa* can remove up to 85% phosphorus and 80% nitrogen, and excellent lipid (oil) conversion	Kothari et al. (2012)
Dairy residues	*Chlorella* sp. and *Scenedesmus* sp.	Bottles in vitro and in situ	Microalgae grew in different dairy effluents, and *Chlorella* became best in high	Labbé et al. (2017)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			organic content and high ammonium loads, like the effluents from the cattle yard. *Scenedesmus* grew best with a high chemical and detergent load, like the waters of the milking parlor The potential use of dairy effluents does not produce microalgae for purposes and treatment and improves the finances of small- and medium-sized dairy farms	
Dairy residues	*Chlorella sorokiniana* (DS6)	Conical vials	Green unicellular microalgae *C. sorokiniana* isolated from the holding tanks of farm wastewater treatment plant using multistep screening and acclimation procedures was found high-lipid producing facultative heterotrophic microalgae strain capable of growing on dairy farm effluent (DFE) for biodiesel feedstock and wastewater treatment	Hena et al. (2015)
Dairy residues	*Chlorella vulgaris*	Micro-photo bioreactor	The maximum percent of chemical oxygen (COD) removal efficiency was 42.57% after 52 h, and the optimum conditions of COD were equal to flow rate =	Valizadeh and Davarpanah (2020)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			0.0125 Cm^3 min^{-1}, length of photo micro-bioreactor = 16 m, temperature 30 °C, and at initial pH 8.00	
Dairy residues	*C. vulgaris*	Discontinuous photobioreactors	To cultivate *C. vulgaris* at 25% was ideal for the complete removal of ammonium and phosphorus in addition to achieving high lipid yield that favors the production of biofuels. Bacteria do not influence microalgae growth but decreased microbial diversity. These findings contribute to a mix of bacteria in the cultivation of large-scale microalgae	Zhu et al. (2019)
Dairy residues	*Ascochloris* sp.	Column and flat plate photobioreactor	The cultivation of microalgae in photobioreactors outdoors generates yields and bioremediation of wastewater from untreated dairy products was used to produce biomass, lipids, other value-added by-products with a reduced organic compound. There is 100% use of raw effluent from untreated dairy products for the production of biomass, lipids, other value-added by-products, and	Kumar et al. (2019a, b)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
			clean, odorless water for recycling and reuse	
Dairy wastewater	*Ascochloris* sp.	Ponds	Production of 504-ton biomass per year at $0.482/kg with ~240,000 m^3 of treated clean water and high-volume V-shape ponds was one of the cost-effective and area-efficient microalgal cultivation systems for mass production	Kumar et al. (2020)
Cassava	*Chlorella pyrenoidosa*	Tubular photobioreactor	*C. pyrenoidosa* significantly reduces the organic amount of the residue, without altering the production of ethanol, and this residue can be reused up to four times	Yang et al. (2008)
Cassava	*Acutodesmus obliquus*	Open tank	*A. obliquus* cultivated biomass improved by the addition of cassava than the control, and the quantity of lipids and carbohydrates increased by 96.8% bioethanol and 98.7% biodiesel	Selvan et al. (2019)
Cassava	*Spirulina platensis*	Microbial fuel cell and microalgae-assisted cathode	A combination of two biocatalysts with indigenous microorganisms from wastewater at the anode and microalgae grown in situ at the cathode was able to reduce 67% of the initial organic quantity, to generate renewable bioelectricity and produce microalgae biomass	Hadiyanto et al. (2019)

(continued)

Table 23.2 (continued)

Agro-industrial waste types	Microalgae Genus/species	System of cultivation	Highlight applications	Ref.
Tapioca wastewater	*Scenedesmus* sp.	100 mL filtered in 250 mL flask	*Scenedesmus* sp. which was cultured in 50% of tapioca wastewater gives highest lipid production	Romaidi et al. (2018)
Maize	*Chlorella vulgaris*	Erlenmeyer and balloon bottles	The cultivation of *C. vulgaris* in vinasse removed 84–86% of total carbon with substrate degradation of 76–79% and high biomass production New phytoremediation strategy to treat effluents generated from the corn industry to ethanol and simultaneous production of value-added coproducts	Beigbeder et al. (2019)
Corn/maize stillage digestate	*C. sorokiniana*; *Scenedesmus obliquus*; *C. saccharophila*	Glass bottle	Pretreatment (centrifugation, chemical add) *C. sorokiniana* removed chemical oxygen demand, ammonia-N, and total P and produced biomass with high content of protein (37.8 ± 3.4%), starch (17.8 ± 0.8%), and lipid (8.9 ± 0.3%) Potential to integrate into an existing corn ethanol plant to reduce the corn consumption, increase the protein content of the dried distiller's grain and corn-oil yield	Sayedin et al. (2020)

Chlorella sp. (Johnson and Wen 2010), *C. pyrenoidosa* (Kothari et al. 2012), *C. sorokiniana* (Hena et al. 2015), and *C. vulgaris* (Valizadeh and Davarpanah 2020).

Levine et al. (2011) showed that using anaerobically digested dairy manure wastewater for growth Neochloris oleoabundans approximately 90–95% of the initial nitrate and ammonium was assimilated and yielded 10–30% fatty acid methyl esters of dry biomass after 6 d. These authors concluded that this microalgae species is an excellent green microalga for combined biodiesel feedstock production.

The selection of species and strains with the best performance to cultivation aiming for chemical removal and bioenergy feedstock production must be thoughtful, due to the high complexity in terms of nutritional composition and adaptability of the microorganisms, including isolation and screening for adaptation in media similar to wastewater (Chu 2017; Moreno Osorio et al. 2020).

The chemical, microbiological composition, and organic load of the wastewater also interfere with the growth of microalgae. Consequently, not only residues must be carefully characterized but also the growth of the species/strain must be evaluated in screening to better choose the wastewater cleaning technology based on microalgae cultivation. In a study using polluted effluents from the dairy industry for biomass production and phytoremediation, it was observed that *Chlorella* sp. grew better in effluents from the cattle yard with high organic and ammonium contents, while *Scenedesmus* sp. presented better growth in the milking parlous effluents with higher inorganic compounds and detergent cargo (Labbé et al. 2017). Biomass produced by *Ascochloris* sp., a strain isolated from dairy industrial effluent, exhibited a relevant lipid increase, showing potential for bioenergy production and for simultaneous bioremediation of raw dairy wastewater (Kumar et al. 2019a).

The feasibility of *C. vulgaris* cultivation in unsterilized dairy-derived liquid digestate diluted to 25% was observed by Zhu et al. (2019), who also estimated that for each ton of biomass produced, approximately 102 tons of wastewater can be treated with removal of N and P, allowing coproduction of bioenergy feedstock and chemical removal. In this study, also it was observed that bacteria do not influence microalgae growth but microbial diversity was decreased, inferring that the presence of bacteria does not affect the cultivation of large-scale microalgae using wastewater.

The use of effluents to generate biomass from microalgae is an approach that benefits both bioremediation and the production of biofuels. Regarding wastewater from leather industries, an effort has been made to treat them using phycoremediation, aiming to produce biomass for bioenergy, as it was showed with the cultivation of *C. vulgaris* and *Pseudochlorella pringsheimii* for treating the tannery effluent in dilutions <30%, which resulted in significant removal of polluting compounds like NH_3, PO_4, and heavy metal chromium (Saranya and Shanthakumar 2019).

Considering that wastewater is used to replace freshwater in the microalgal cultivation, according to Chu (2017), it is possible to reduce by 94%, which is indeed a strategic approach to use agro-industrial wastewater to enhance biomass productivity for biofuel production because such combine system also reduces the pollutants in the effluents before discharge.

Cyanobacteria species thrive well in wastewater from different effluents, for example, *Arthrospira* sp. (*Spirulina*) growing in tannery effluent and *Spirulina platensis* lessened odor characteristic of perfume activity (Dunn and Rose 2013) in pisciculture wastewater. There was a reduction of 19.8% in ammonia, 100% in nitrite, 98.7% in nitrate, and 94.8% in phosphate content, with nutrient levels within the standards those required by Brazilian environmental standards to release (Nogueira et al. 2018).

High organic and nutritional amount in the wastewater and effluents, especially from agro-industries, is synergistic to the ability of microalgae to thrive in such wastewater, and for example, biomass production for biofuels and conservation of natural resources (Zhu et al. 2019). For industrial and versatile scale, microalgae are aimed at different purposes, especially for the ability to convert the contents in the growth medium to a high content of lipids and carbohydrates and for being considered promising as a raw material in the production of biofuels (Ramirez et al. 2014; Zhu et al. 2019).

Effluents may contain growth inhibitors such as high concentrations of toxic compounds and high turbidity that reduces the availability of light that induce anaerobic conditions and hinder treatments of aiming biological degradation of organic matter (Panchangam and Janakiraman 2015). Additionally, photosynthetic microorganisms (e.g., photoautotrophic microalgae) when in medium with high turbidity that reduces the availability of light tend to have their growth limited. To overcome these limitations, some strategies can be considered: the choice of effluents to be treated and the adaptation or selection of microalgae species or strains to be used. Overall, biodegradable total organic carbon was the main factor limiting to remove nutrients and other compounds in effluents from the processing potato, fish, coffee, animal feed production, and yeast production; when growing a microalgae consortium (*Phormidium* sp., *Oocystis* sp., and *Microspora* sp.), also, it was observed that the initial C/N/P ratio of these wastewaters was correlated with its biodegradability (Posadas et al. 2014).

By using vinasse from ethanol industry, the amount of biomass produced by *Scenedesmus* sp. is altered with light intensity and percentage of vinasse added in the medium culture, showing that this effluent can be used as a nutrient source for microalgae production (Ramirez et al. 2014). Growing microalgae in a thin stillage effluent (vinasse) generated by a starch-based (maize) ethanol production industry, it was found that *C. vulgaris* were able to degrade both organic and inorganic compounds during mixotrophic growth producing biomass at a rate of 0.9 g^{-1} L^{-1} day^{-1} (Beigbeder et al. 2019). In addition, biomass had a high protein and carbohydrate contents, and natural photosynthetic pigments were generated at a rate of 0.98 mg^{-1} L^{-1} day^{-1} (total chlorophylls) and 0.19 mg^{-1} L^{-1} day^{-1} (carotenoids). This work highlights the potential of a novel microalgae-based thin stillage phytoremediation process with simultaneous cogeneration of high value-added metabolites as a source for bio-commodities, for instance, the high protein content as supplement to animal feed or fertilizer.

Cassava residue proved to be an excellent alternative as raw material for growing several species of microalgae as described in some studies, developing sustainable

efficient and ecological by-products at low cost in the remediation of effluents as the technology for wastewater biotreatment developed. Using a undiluted effluents from ethanol fermentation of cassava powder, cultivation of *C. pyrenoidosa* significantly reduced the organic amount of the residue, without altering ethanol production, and can be reused up to four times, suggesting that the treated wastewater could be recycled in the process of ethanol production entirely and directly (Yang et al. 2008). Whereas by growing *Acutodesmus obliquus* in effluent from cassava industry as a nutrient source for the biomass production had two main roles: first, it removed the nutrients (NO3, PO_4, SO_4, Cl_2, Ca, K, Mg, Na, P, NH_4, and C) for the sustained growth and then produced lipid and carbohydrate for biodiesel and bioethanol production (Selvan et al. 2019).

By using *Spirulina platensis* as cathode biocatalyst for the bio-production of oxygen and the tapioca wastewater containing native microorganisms used as a substrate in the anode chamber, 67% of organic initial amount was reduced with an electrical output generated in the same system with an affordable microalgae biomass production (Hadiyanto et al. 2019).

Conventional filtration and bio-digestion of vinasse, a residue from sugarcane industry, resulted in cleaner residues in which *C. vulgaris* grew better with higher biomass production as a potential strategy to reduce the costs of microalgae production industry (Candido and Lombardi 2017).

Auxenochlorella protothecoides and *C. sorokiniana* grew significantly faster on winery effluents than on minimal media, showing that bacteria and green microalgae provided synergistic growth benefits, which contribute to higher levels of wastewater treatment (Higgins et al. 2018).

Microalgal biomass characteristically has a high protein content which contributes toward high total ammonia concentration in the effluent. Cocultivation of *Tetraselmis suecica* microalgae with oil palm empty fruit bunch and palm oil mill effluent presented the highest specific biogas production, and biomethane yield was achieved with microalgae for anaerobic biomethane production (Ahmad et al. 2014). Growing cyanobacteria in wastewater from olive oil, the produced biofertilizers were applied on a sandy soil to grow celery plant to replace chemical fertilizers (Rashad et al. 2019).

Although the results are still limited, the studies highlight the significant potential and economic merit of recovering energy and nutrients especially from wastewater from high-strength agro-industry because they have a high organic load (Vu et al. 2020). To overcome problems of recovering wastewater from the food industry and agroindustries, it may be necessary to replace chemical treatments with sustainable technologies that use microorganisms for bioremediation.

23.6.3 Agricultural Applications

Aiming to increase the productivity of crops, intensive agriculture has caused increases in production costs due to the need to recover areas degraded by successive

monocropping, unsuitable soil management, and the increasing use of fertilizers and chemicals compounds to protect plants against insects and diseases. This strategy for food production has been replaced somewhat by environmentally friendly alternatives that are taking place in agricultural practices for the production of healthy foods, comprising sustainable agriculture advances in agricultural management practices and technologies (Singh et al. 2011).

23.6.3.1 Soil Restoration

Although naturally formed or artificial cyanobacterial crusts are known as colonizers and protectors of soil surfaces in biological soil crusts, which play a key role in hydrological processes on soil restoration or soil bioremediation, they are not able to prevent desertification process as quickly (Tiwari et al. 2019). However, it has been highlighted that these photosynthetic microorganisms can be used as a tool to change unused lands in cultivated soils for sustainable agriculture, because in addition to fixing N_2, cyanobacteria in soil surface consortia might also act to immobilize and retain nutrients, for example, nitrogen, helping to reduce runoff and increase N use efficiency (Peng and Bruns 2019). The exopolysaccharides produced by cyanobacteria are important constituents for the development of biofilm that are formed on the solid surface, allowing association of microbial communities that act as a reservoir of water and nutrients. Cyanobacterial inoculation into soil to induce protective coating is an alternative approach to be addressed to prevent soil degradation or for soil restoration by improving soil aggregate stability. For instance, inoculation of a cyanobacterium *Schizothrix* cf. *delicatissima* in a sandy soil induced a colonization with the establishment of a thick crust in a very short period of time, without any change in the hydrological properties, suggesting heterogenous distribution of the trichomes and exopolysaccharides on the surface and on the bulk of the crust (Mugnai et al. 2018).

For the development of this sustainable agriculture, the understanding of microbial and plant interactions is mandatory to achieve the goals of having a healthy environment, including soil, water, and air. Most of microbial and plant interactions that are called symbioses are effective as plant growth promoting (PGP), which include microalgae, representing a potentially sustainable alternative for the improvement and protection of crops.

In agriculture, biostimulants are resources that encompass both microorganisms and substances that are applied in seeds or rhizosphere whose function is to start or to accelerate mechanisms or metabolism aiming to enhance plant growth, nutrient use efficiency, tolerance to abiotic factors, and crop quality. The use of both green microalgae and cyanobacteria brings benefits to crops, increasing yields with higher nutritional values, due to metabolites such as phytohormones, polysaccharides, amino acids, and antimicrobial compounds, which are produced by these microorganisms (Rachidi et al. 2020).

These biostimulants, besides plant protection mechanism, play an important role in the colonization of plants and growth of microbial communities in soil. In a review

paper, Chanda et al. (2019) described how microalgae polysaccharides are produced, their biological activities, and their possible application in agriculture as a potential sustainable alternative for enhanced crop performance, nutrient uptake, and resilience to environmental stress.

Stimulating effects of probable active compounds contained in wheat seed-coating formulation with *Enteromorpha* sp. and *Cladophora* sp. and enrichment with mineral stimulated seed germination and the initial plant growth phase (Dmytryk et al. 2015). Microalgae contribute significantly to agricultural activity due to the ability to produce metabolites, for example, phytohormones or bioactive compounds, such as the auxins, indole-3-acetic acid (IAA), and indole-3-acetamide (IAM) from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Charophyceae species (Stirk et al. 2013) and from cyanobacterium *Aphanothece* sp. (Gayathri et al. 2015). Polysaccharides and antimicrobial compounds can promote plant growth directly or indirectly and, thus, become suitable for inoculation and bio-fertilization.

For sustainable horticulture and agriculture, the use of microalgae-based products might help to ensure production of food to meet the needs of human with quality and without harm to the environment. For radish (*Raphanus sativus*) plants, filtrates and homogenates of *S. platensis* that were applied for seed soaking and for foliar spray increased the length of plants in comparison to control and commercial product, showing potential as biostimulant products to be used (Godlewska et al. 2019).

In addition, this group has a role in soil nutrient cycle processes, such as mineralization of organic matter and inorganic material, immobilization, and availability of nutrients for plant and microbial community growth (Prasanna et al. 2016).

Some species of cyanobacteria and green microalgae also have the ability to solubilize chemical elements through co-coagulation processes that result in enrichment in food crops, mainly in grains with micronutrients such as iron, manganese, copper, and zinc that are essential for human and animal nutrition (Renuka et al. 2018).

Microalgae have intracellular hormones, though some can produce or excrete hormones in the surrounding environment (Prasanna et al. 2015a, 2016). It has been identified in several genera of microalgae, most all known phytohormones (e.g., auxins, cytokinins, abscisic acid, and gibberellins), jasmonic acid, and ethylene and as well their physiological activities stimulate crops (Ahmed et al. 2010; Gayathri et al. 2015; Hashtroudi et al. 2012; Mazur et al. 2001; Shevchenko et al. 2014; Stirk et al. 2013, 2002).

The growth of microalgae is controlled by the hormonal regulatory system, which might be on growth and biochemical compounds production. For *Desmodesmus* sp., when auxin and cytokine were added to the culture medium, it was observed an increase in biomass production, lipid content with higher levels of palmitic and oleic acids which are preferable constituents for achieving high-quality biofuel (Singh et al. 2020b).

For *Brassica oleracea*, the inoculation of cyanobacterial phytohormones, cytokinins, and indole-3-acetic acid (IAA) has shown to be the best for induction of adventitious roots and shoots on internodal and petiolar segments (Hussain and

Hasnain 2012). Cyanobacteria regulate the dormancy and germination of their own cells and/or other cells via phytohormone cytokinin as it was showed for *Nostoc* sp. germination dormancy cycle (Kimura et al. 2020). By combining two phases, the application of 2,4-dichlorophenoxyacetic acid with abscisic acid in culture of *Phaeodactylum tricornutum* enhanced the accumulation of biomass and lipid more than single phytohormone treatment (Zhang et al. 2020).

23.6.3.2 Biocontrol

The hydrolytic enzymes and biocidal compounds produced by microalgae have an antagonistic effect against many plant pathogens; unicyanobacterial isolates belonging to the genus *Anabaena* inhibit the growth of phytopathogenic fungi, such as *Fusarium moniliforme*, *Alternaria solani*, *Aspergillus candida*, *Drechslera oryzae*, and *Pythium aphanidermatum* (Prasanna et al. 2008).

The biocontrol of fungus and bacterial disease in plants might be due to indirect effects, which help to improve plant immunity after microalgae inoculation. *Anabaena laxa* and *Calothrix* sp. formulation applied in soils with high levels of *Rhizoctonia* spp. revealed significant reduction of cotton plant mortality, which also stimulated the activity of defense enzymes in the plants, such as β-1,3-endoglucanase activity, chitosanases, peroxidase, phenylalanine ammonia lyase, and dehydrogenase, in addition to higher levels of nitrogen and phosphorus available in rhizosphere soil (Babu et al. 2015).

With inoculation of *Calothrix* sp. in rice plants, there was an increase in the activity's peroxidase, polyphenol oxidase and phenylalanine ammonia lyase from root and shoot tissues, also, the activity of nitrogenase enzymes, CMCase, chitosanases, chlorophyll concentrations, growth, and biomass weight were higher in inoculated than non-inoculated plants (Priya et al. 2015). The activities of these enzymes are related to the quality of plants and their resistance. In addition, inoculation promoted growth and increased dry and fresh weight of the plant. The chlorophyll concentrations of rice seedlings increased 77% in the inoculated root tissues and 32% in the leaves compared to the control, and the production of EIA increased by 32% (Priya et al. 2015).

Some species of cyanobacteria that belong to the genus *Anabaena* have been described as producers of biocidal compounds that are secondary metabolites with antifungal action (Prasanna et al. 2008). Inoculation of *Anabaena* sp. in zucchini (*Cucurbita pepo*) against *Podosphaera xanthii* has shown to have both an inducer of systemic resistance and an active antifungal mean, which can be due to multiple mechanisms of enzymes, for example, chitinase with early activation and peroxidase and β-1,3-glucanase with direct antifungal activities in sporulation (Roberti et al. 2015).

23.6.3.3 Biofertilizers and Inoculants

Polysaccharides isolated from microalgae generally trigger a signaling cascade to activate the protection response of plants against salt stress, provide resistance against pathogens, represent a potential biological resource for the protection of agricultural crops, and act as biostimulants. In tomato, the use of polysaccharides from *A. platensis*, *D. salina,* and *Porphyridium* sp. improved significant development of plants compared to control. In addition, it increased the content of carotenoids, chlorophyll, proteins and nitrate reductase, NAD-glutamate dehydrogenase activities in plant leaves (Rachidi et al. 2020).

Applying foliar biofertilizer consisting of a mixture of intact cells of *Microcystis aeruginosa*, *Anabaena* sp., and *Chlorella* sp. under limited fertility conditions increased the activity of the enzymes dehydrogenases, ribonuclease, nitrate reductase, acid and alkaline phosphatase, the amount of nitrogen, phosphorus, and potassium and improved the growth of willow (*Salix viminalis*) plants (Grzesik et al. 2017).

Biofertilizer-based microalgae have shown potential for grain crops similar to a study with wheat. By using biofertilizers formulated with microalgae biomass consortia grown in agro-industry wastewater, nitrogen fertilizer dose can be reduced by 25% and the yields improved (Renuka et al. 2016). Application of cow manure combined with *Spirulina platensis* (*Arthrospira*) or *C. vulgaris* dry biomass in a sandy loam potted soil increased the development and yield of maize plants and resulted in higher content values of N and P in the shoot, and also, N, P, K, Fe, Mn, and Zn levels were improved in the seeds (Dineshkumar et al. 2019).

Application of an eco-friendly biofertilizer from biomass of cyanobacteria that were grown on olive milling wastewater significantly improved sandy soil properties and enhanced celery plant growth (Rashad et al. 2019). The produced biofertilizers were applied on a sandy soil to grow celery plant under different levels (25%, 50%, and 75%) of the recommended chemical fertilizers (Rashad et al. 2019).

Considering this background, both cyanobacteria and eukaryotic microalgae are considered as eligible for applications in the soil as biofertilizers and/or in crop seeds as simple inoculants or co-inoculations with other recommended beneficial microorganisms.

Garcia-Gonzalez and Sommerfeld (2016) showed that some applications of microalgae (e.g., *Acutodesmus dimorphus*) in vivo by cell extracts and by dry biomass can be as potential inoculants or biofertilizers in Roma tomato plants. The mix of microalgae species (*Nostoc commune* and *Nostoc carneum*) in rice contributed to promoting the growth of rice (*Oryza sativa*) seedlings by IAA and exopolysaccharide effects, suggesting that using the combined cyanobacteria biofertilizer with a half of the recommended dose of chemical fertilizer is to decrease production cost without any effects on rice quantity and quality (Chittapun et al. 2018).

The utilization of consortia/biofilms of green algae and cyanobacteria with different agriculturally beneficial microbes as biofertilizer has proved promising

potential (Renuka et al. 2018) that gives an idea what the species concept of nontoxic cyanobacteria can help in most of inoculation strategies, aiming to increasing plant health and grain production. Cyanobacteria species that can fix atmospheric have been used in agriculture as a biofertilizer source, for instance, to increase biomass yield by reducing the use of fertilizer nitrogen and at the same time as conditioners to improve soil physical–chemical properties. Applying microalgae for crop production has shown results comparable to commercial treatments; besides, inoculations with these photosynthetic microorganisms have enhanced levels of carbohydrates and carotenoids in tomato fruits (Coppens et al. 2016).

Inoculants containing *Calothrix* sp. or *Anabaena* sp. in cotton improved N_2 fixation and phosphate solubilization and increased plant growth, possibly due to the release of enzymes by microalgae that degrade inorganic phosphate in the soil, increasing its bioavailability (Prasanna et al. 2015b).

Using *Chlorella* sp., *Anabaena* sp., and *Microcystis aeruginosa* as foliar biofertilizers for willow *Salix viminalis* increased the activity of enzymes assimilating nutrient dehydrogenase, nitrate reductase, acid, and alkaline phosphatase in the leaves which resulted in high shoot biomass similar to conventional fertilizers (Grzesik et al. 2017).

As biofertilizer for maize crop, *Spirulina platensis* and *Chlorella vulgaris* mixed with cow dung manure increased plant height growth, yield characters, biochemical and mineral components, and the germinability of the seeds produced (Dineshkumar et al. 2019).

In tomato, inoculation of *Acutodesmus dimorphus* as aqueous cell extracts in leaf spray and dry biomass as biofertilizer showed increased seed germination, plant growth, and vigor of seedlings, with higher effects when using living cells, and also dry biomass in earlier application had better results due to release of nutrients from biomass for plant uptake (Garcia-Gonzalez and Sommerfeld 2016). The growth of *Arachis hypogaea* and *Moringa oleifera* plants inoculated with an extra cellular products of a cyanobacterium *Aphanothece* sp. was higher than that when using commercial phytohormones, such as 6-benzylaminopurine and indole-3-butyric acid (Gayathri et al. 2015). By combining microalgae consortium, a wide range of horticultural plants have been inoculated, for example, *Anabaena laxa* and *Calothrix elenkinii* on coriander, cumin, and fennel plants (Kumar et al. 2013) and *Scenedesmus subspicatus* and humic acid on onion (Gemin et al. 2019), which in addition to promoting the growth exhibited antifungal activity.

By focusing on the biofortification of food crops to avoid problems of lack of healthy foods, microalgae-based inoculant in consortia or biofilm modes of cyanobacteria, bacteria, and green microalgae has been used as an approach to provide the enrichment of grains with micronutrients, particularly with iron, manganese, copper, and zinc, leading to improved grain quality and reduced production costs (Adak et al. 2016; Prasanna et al. 2015a, b). Inoculation of a consortium consisting of dry biomass of *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Synechocystis* sp. as pretreatment of tomato seeds as well as in foliar spray showed that, overall, seed treatment was found to be more effective than foliar spray (Supraja et al. 2020b).

23.7 Microalgae Supply Chain: Business Opportunity and Challenges

Worldwide, a significant increase in technologies for cultivation of microalgae has boosted the replacement of traditional crops and other raw material in many applications, mainly due to some advantages these microorganisms present, such as photosynthesis and fast biomass production. In a study aimed at prospecting markets for microalgae products, at least six potential major markets are found, for example, bioenergy production, bioplastics, biofertilizers, nutraceuticals, pharmaceuticals, and cosmetics (Rumin et al. 2020), although there are other consolidated opportunities, such as animal supplementation nutrition, biofibers, wastewater treatment, and soil remediation.

The growing market for products that use microalgae as a raw material, such as the dietetic and food, cosmetic, and pharmaceutical industries, has offers for business opportunity.

In the production chain, the main green microalgae (Chlorophyceae) produced aiming for biotechnological industries are *Tetraselmis*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Nannochloropsis*, and cyanobacterium *Arthrospira* (*Spirulina*). With respect to the application of microalgae for the extraction of valuable bioproducts, they are used in different forms such as liquid, concentrates, extracts, powder, or flakes. Microalgal biomass has been used principally in food and feed industries as sources of important by-products, for example: (1) *Chlorella* and *Arthrospira* (*Spirulina*) are sources of functional foods, nutraceuticals, and health supplements (Osorio-Fierros et al. 2017); (2) *Dunaliella* is a rich source of natural β-carotene (Einali et al. 2017); (3) *Tetraselmis* is a source of protein and omega-3 (Riccio et al. 2020); (4) *Haematococcus pluvialis*, a source of astaxanthin produced and marketed in the form of powder, is available in orange- to red-colored powder or flakes for further extraction (Ahmed et al. 2015).

For biotechnological applications of microalgae, there is a huge opportunity for the discovery of novel bioactive metabolites, including the identification of compounds with potential antimicrobial, antifungal, and antitumorigenic, and already, there are many species for this purpose, such as *Tetraselmis chuii*, *C. sorokiniana*, and *Chondrus crispus* (Barkia et al. 2019).

Similar to human food and nutrition, microalgae applications for animals have a great challenge to animal production. Animal feed needs to become less dependent on expanding arable land and less impact on the environment; in this context, microalgae have been used as a source of protein, for example, Spirulina to poultry that benefits to color and flavor to kind of animal meat (Altmann et al. 2020).

The challenges for microalgae supply production chain have been on industrial integration with an analogous system to the concept of ecological symbiosis, where the interaction between different industries has higher advantages than operating alone which results in ecological industrial parks. In these conglomerates of enterprises, waste from industry A becomes the input for the production of microalgae in industry B; the raw material produced in industry B will be inputs for the production

of coproducts in other industries. A good example of the environmental application of microalgae is bioremediation using the integrated agro-industry-biorefinery, as described by Kumar et al. (2020) for the dairy effluent treatment system based on the production of microalgae biomass in a large-scale plant to reuse water instead of the drinking water that is currently used for the growth of microalgae.

From the perspective of microalgae supply chain in a circular economy, the concepts are to reduce and to reuse resources for a longer period of time which can reduce greenhouse gas emissions and significantly reduce the volume of waste (Nagarajan et al. 2020). Feasibly, the most significant impact on the microalgae production chain is evidence of circular zero-residue process using these microorganisms for efficient water decontamination, biofuel production, and carbon dioxide fixation (Serrà et al. 2020). Take into consideration that there is significant interest in recycling water from hydroponic plant cultivation, shifting to a sustainable production system where residues become nutrients in new processes, products or materials can be repaired, reused, updated, or re-inserted in new cycles with the same or better quality instead of being discarded. In cocultivation mode, microalgae have been studied for growing the cyanobacterium and raising chrysanthemum nursery (Bharti et al. 2019) with tomato plants (Barone et al. 2019; Supraja et al. 2020a) and by utilizing coproduction of *Chlorella vulgaris* with arugula, purple kohlrabi, and Lettuce (Huo et al. 2020).

The option for a sustainable growth of the microalgae supply production chain, aiming to reduce the release of waste into the environment close to zero is the integration of industrial production plant units in shared areas in partnership with other agro-industry, the eco-friendly industrial parks.

Regarding the production of renewable energy from microalgae, biorefineries are the key to the integration of the state of the art in global scenarios; however, the intricate process design is triggered mainly by the lack of suitable technologies especially in cell disruption and extraction of specific compounds. One of the challenges for the microalgae production chain is still in large-scale biomass production, requiring investment in the line of research, development, and innovation.

23.8 Conclusions

The variety of products accessible from the primary and secondary metabolism of diverse algal species clearly demonstrates the importance of these versatile microorganisms as cellular factories. Microalgae have recently attracted considerable interest worldwide, due to their renewable, sustainable, and economical sources of extensive application potential in the renewable energy, biopharmaceutical, nutraceutical industries, biofuels, bioactive medicinal products, and food ingredients. Several microalgae species have been investigated for their potential as value-added products with remarkable pharmacological and biological qualities.

When the goal is to share microalgae production chains with another agro-industry production chain in eco-parks that generates nutrient-rich wastewater,

growing microalgae in wastewater to replace conventional method of treatment of an industrial effluent requires only few investment and operating cost which is a promising future of microalgae cultivation.

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Chapter 24
Marine Resources with Potential in Controlling Plant Diseases

Felipe R. Garcés-Fiallos, Felipe M. de Quadros, and Mateus B. de Freitas

Contents

Abstract Seaweeds are aquatic photosynthetic organisms with high potential of use in agriculture as fertilizers, biostimulants, and pesticides. Despite this, seaweeds (especially macroalgae) and their polysaccharides still have been little explored. In plant diseases management, algal extracts can control several diseases in different plant species. Seaweed extracts also act as biostimulants, increasing plant growth and yield as well as tolerance to abiotic stresses. This is because seaweed extracts contain several metabolites that induce changes in morphophysiological and biochemical processes in plants. Among these metabolites, polysaccharides are one of the most abundant organic molecules in marine habitats. Laminarans, carrageenans,

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and ulvans are the main seaweed-derived polysaccharides studied and used in agriculture. Several methods have been used to extract different metabolites from algae, including cultivation, fermentation, and processing. Overall, the extraction is carried out using water, alkali, or acid although the use of other more eco-friendly and efficient methods has become more and more frequent. Considering the benefits obtained in plant growth and protection, it is plausible to assume that the use of products derived from seaweed in agriculture will increase over time.

Keywords Algal polysaccharides · Algal extracts · Biostimulant · Resistance induction · Plant disease management

24.1 Introduction

Seaweeds are aquatic photosynthetic organisms with high potential for the animal and human food industries as well as in various textile and pharmaceutical industrial sectors, and in agriculture as fertilizers, biostimulants, and pesticides. In agriculture, reports indicate that the oldest use of seaweeds was as fertilizer (Righini and Roberti 2019), although current scientific studies have shown that they are also effective in plant growth (biostimulant effect) and protection (Paulert et al. 2009).

Currently, the search by the world market for products both efficient and friendly to human health and the environment is increasing. Thus, the algae and their polysaccharides are flawlessly introduced into this scenario. Despite the potential, seaweeds (especially macroalgae) have been little explored as physiological stimulants and/or resistance inducers to biotic stresses (Stadnik and de Freitas 2014). Among the most studied seaweeds are those belonging to Phylum/Class Chlorophyta (green algae), Phaeophyceae (brown algae), and Rhodophyta (red algae) (Righini and Roberti 2019). Extracts or polysaccharides produced from these algae have been the most studied in diseases management of agriculturally important crops all over the world (Stadnik and de Freitas 2014).

Application of algal extracts or their polysaccharides can induce defense responses in plants. Within this context, it is known that extracts obtained from macroalgae such as *Ulva* sp. and its preventively applied polysaccharides control fungal diseases in different plant species. For example, aqueous extracts of *Ulva* spp. reduce the severity of anthracnose (*Colletotrichum lindemuthianum*) of beans by 44% (de Abreu et al. 2008), alternariosis (*Alternaria porri*) of small onion by 70% (Araújo et al. 2012), and of the powdery mildew in vine (*Erysiphe polygoni*), cucumber (*E. necator*), and beans (*Sphaerotheca fuliginea*) by 77%, 80%, and 90%, respectively (Jaulneau et al. 2011). Likewise, preventive applications of *Ulva* spp. apart from protecting barrel clover plants (*Medicago truncatula*) from the fungus *C. trifolii* can activate genes related to the biosynthesis of phytoalexins and pathogenesis-related proteins and cell wall (Cluzet et al. 2004). These aspects

and recent advances related to the use of extracts and the most studied macroalgae polysaccharides such as carrageenans, fucans, laminarans, and ulvans to protect plants are discussed in this chapter.

24.2 Potential of Marine Algae for Plant Disease Management

Despite the existence of studies on the potential of marine algae in plant disease management, its use in this type of situations is still minimal. Extracts or derivatives of various marine algae induce acquired systemic resistance in various plant species subsequently infected with necrotrophic, hemibiotrophic, and biotrophic pathogens (Table 24.1).

24.3 Algal Extracts

Seaweeds have been used in agriculture as fertilizers since antiquity, mainly in coastal areas of the northern hemisphere. However, the first methodology for preparing algal extract for using in agriculture was developed in the 1940s (Craigie 2011; Mukherjee and Patel 2020). Since then, several seaweed extracts have been made commercially available.

Currently, most commercial algal extracts are prepared from brown seaweeds including *Ascophyllum nodosum*, *Durvillaea* spp., *Ecklonia maxima*, *Fucus serratus*, *Laminaria* spp., *Sargassum* spp. and *Turbinaria* spp. However, extracts prepared from green (*Enteromorpha* spp. and *Ulva* spp.) and red (*Kappaphycus* spp.) seaweeds are also available (Craigie 2011; Shukla et al. 2019; Mukherjee and Patel 2020).

Extracts can contain a large group of unique metabolites from seaweeds including amino acids, aminopolysaccharides, betaines, humic substances, hormones and hormone-like substances, lipids, oligo- and polysaccharides, macro- and microelements, phenolic compounds, peptides, proteins, and vitamins (Yakhin et al. 2017; Mukherjee and Patel 2020). These compounds can provide a myriad of biological effects when applied onto plants or incorporated into the soil. For instance, algal extracts can enhance nutrient absorption, plant and root growth, fruit setting, and resistance against biotic and abiotic stresses (Craigie 2011; Yakhin et al. 2017; Mukherjee and Patel 2020).

Extracts from brown algae are able to control several diseases in different plant species. For instance, drenching wheat seedlings with a liquid extract from *A. nodosum* reduced the severity of *Fusarium* head blight caused by *Fusarium graminearum* and the number of infected spikes by up to 30% (Gunupuru et al. 2019). Additionally, the extract also reduced mycotoxin content in wheat kernels by

Table 24.1 Classification of algae cited in the literature with potential for plant disease management

Phylum/Class	Algal species	Extract/ polysaccharide and experimental conditions	Host plant	Pathogen	Disease intensities[a]	Effect on the host	References
Chlorophyta (green algae)	*Codium isthmocladum*	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Reduced	Not analyzed	de Abreu et al. (2008)
	Enteromorpha sp. and *E. lingulata*	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
	Ulva spp.	Ethanolic extract/growth chamber conditions	Barrel clover	*Colletotrichum trifolii*	Reduced	Induction of gene *PR10* and expression of defense-related genes involved in the biosynthesis of phytoalexins, pathogenesis-related proteins and cell wall proteins	Cluzet et al. (2004)
	Ulva fasciata	Ulvan/green-house and out-door pot conditions	Bean	*Uromyces appendiculatus*	Reduced	Not analyzed	Delgado et al. (2013)
	Ulva fasciata	Ulvan/green-house and out-door pot conditions	Bean	*Pseudocercospora griseola*	Reduced	Not analyzed	Delgado et al. (2013)
	Ulva fasciata	Ulvan/green-house conditions	Bean	*Fusarium oxysporum* f. sp. *phaseoli*	Reduced	Not analyzed	de Borba et al. (2019)

	Ulva fasciata	Ethanolic extract/field conditions	Bean	*Pseudocercospora griseola*	Reduced	Not analyzed	Garcés-Fiallos et al. (2020)
	Ulva lactuca and *U. fasciata*	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
Phaeophyceae (brown algae)	*Ascophyllum nodosum*	Ethanolic extract/green-house and field conditions	Strawberry	*Podosphaera aphanis*	Reduced	Increase of total phenolic and flavonoid content and induction of defense-related enzymes, such as phenylalanine ammonia-lyase (PAL), poly-phenol oxidase (PPO), and peroxidase (POX) activity.	Bajpai et al. (2019)
	Ascophyllum nodosum	Alkaline extract/green-house and field conditions	Tomato	*Alternaria solani* and *Xanthomonas campestris* pv. *vesicatoria*	Reduced	Increased of fruit yield and levels of activity of PPO, PAL, POX, chitinase and glucanase enzymes, of accumulation of higher levels of phenols, and upregulation of JA/ethylene pathway.	Ali et al. (2016)
	Ascophyllum nodosum	Stella Maris®/ laboratory conditions	*Arabidopsis thaliana*	*Pseudomonas syringae* and *Xanthomonas campestris*	Direct inhibi-tory effect on both bacterial growth	Induction of expression of WRKY30, CYP71A12 and PR-1 genes	Cook et al. (2018)
	Petalonia sp.	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)

(continued)

Table 24.1 (continued)

Phylum/Class	Algal species	Extract/ polysaccharide and experimental conditions	Host plant	Pathogen	Disease intensities[a]	Effect on the host	References
	Sargassum stenophyllum	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
Rhodophyta (red algae)	*Acanthophora spicifera*	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Reduced	Not analyzed	de Abreu et al. (2008)
	Bostrychia sp.	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
	Bryothamnion triquetrum	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
	Centroceras clavulatum	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
	Cheilosporum sagittatum	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)

	Gracilaria tepocensis	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Reduced	Not analyzed	de Abreu et al. (2008)
	Hypnea spinella	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)
	Lithophyllum sp.	Ethanolic extract/green-house conditions	Bean	*Colletotrichum lindemuthianum*	Unaffected	Not analyzed	de Abreu et al. (2008)

[a]Disease reduction compared to control (systemic effect)

30%. The reduction in disease severity as well as mycotoxin content can be due to resistance induction in wheat plants since an increase in the expression of defense-related genes such as *PR1*, *PR2*, *PR3*, and *Glu2* and enzymes such as phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase was detected (Gunupuru et al. 2019).

Aqueous extracts from *Laminaria digitata*, *Fucus spiralis*, and *Cystoseira myriophylloides* applied either via seed soaking or foliar spray reduced by 55% leaf alteration and stunting indexes induced by *Verticillium dahlia* in tomato (*Solanum lycopersicum*) plants (Esserti et al. 2017). Moreover, browning index of host vessels caused by the pathogen was reduced by more than 90% independently of the application method used. Considering that tested seaweed extracts increased activities of the host defense-related enzymes peroxidase and polyphenol oxidase, disease control can be associated with the induction of resistance. Additionally, extracts of *F. spiralis* and *C. myriophylloides* increased the number of flowers and fruits and fresh weight of fruits in inoculated plants when sprayed onto leaves (Esserti et al. 2017).

Increase in yield after the application of seaweed extracts may vary depending on the presence or not of stressful conditions. For instance, no increase in plant growth or yield was recorded in tomato (*S. lycopersicum*) grown under saline stress or reduced nutrient availability and treated with two *A. nodosum* extracts (di Stasio et al. 2018). Nonetheless, these extracts enhanced nutritional value by increasing the accumulation of antioxidants, essential amino acids, and minerals in tomato fruits (di Stasio et al. 2018).

Extracts prepared from green seaweeds are also able to promote plant growth and to induce modifications in metabolism. For instance, incubation of tomato (*S. lycopersicum*) seeds with polysaccharide-enriched extracts (PEEs) of *Ulva rigida* and *Codium decorticatum* under in vitro conditions increased both germination percentage and speed by 15% (Mzibra et al. 2020). Under greenhouse conditions, PEEs of *U. rigida* and *Codium tomentosum* increased the length and shoot and root dry weights of tomato plants when applied via soil drenching. On the other hand, when applied via foliar spray, those same extracts increased only the number of leaves (Mzibra et al. 2020). Additionally, an increase in the chlorophyll content was observed only in plants treated via soil drenching. Therefore, it seems reasonable to assume that the method used to deliver seaweed extracts to plants plays an important role in defining the upcoming events.

Applying green seaweed extracts via soil drenching may present other advantages such as decreasing the population of the plant-parasitic nematode *Meloidogyne* spp. Ethanolic extracts from *Ulva fasciata* under in vitro conditions reduced egg hatchability of *M. incognita* by 70% (Ghareeb et al. 2019). Additionally, the same extract is also effective in killing second-stage juveniles of *M. incognita* also in vitro. In this situation, a 12-hour exposure to *U. fasciata* ethanolic extract is sufficient for a killing rate of up to 85% (Ghareeb et al. 2019). Under greenhouse conditions, soil drenching with *U. fasciata* extract reduced the number of galls, egg masses, and eggs per plant as well as the second-stage juveniles by 75%. The extract also increased the length and fresh weight of shoots and roots and the enzymatic activity of peroxidase and

polyphenol oxidase in tomato (*S. lycopersicum*) plants (Ghareeb et al. 2019). In these circumstances, *U. fasciata* extracts can act either directly (nematicidal activity) or indirectly (induction of defense responses) against *Meloidogyne* spp.

Red seaweed extracts can also stimulate plant growth. For instance, incubation of tomato (*S. lycopersicum*) seeds with PEEs prepared from *Gigartina* spp., *Chondracanthus acicularis*, *Gelidium crinale*, and *Schizymenia dubyi* increased the germination percentage by 15% and speed by 50% (Mzibra et al. 2020). Interestingly, those PEEs also increased the dry weight of shoot and roots and chlorophyll content of tomato plants treated via soil drenching or foliar spray, but the effect was more evident when using the former one (Mzibra et al. 2020).

24.4 Algal Polysaccharides

Polysaccharides of algal origin are one of the most abundant organic molecules in marine habitats (Stadnik and de Freitas 2014; Zheng et al. 2020). However, our knowledge regarding marine biodiversity, composition, and potential applications has only scratched the surface (Stadnik and de Freitas 2014). Without the intention of exhausting the subject, we are going to discuss the latest published works about biological activity of the main studied seaweed-derived polysaccharides (i.e., laminarans, carrageenans, and ulvans).

Laminarans or laminarins are storage polysaccharides extracted from brown algae mainly belonging to the genus *Laminaria* (Stadnik and de Freitas 2014). These β-1,3-glucan polymers are able to induce several metabolic changes in plants leading to the activation of defense responses against both biotic and abiotic stresses (Stadnik and de Freitas 2014; Wu et al. 2016; Xin et al. 2019; Zheng et al. 2020). For instance, laminarans increased fresh weight and tolerance to salt and heat stresses in *Arabidopsis thaliana* plants when amended to the growth medium (Wu et al. 2016). Further analysis revealed that laminarans upregulated the expression of host genes related to different pathways including abiotic, biotic, and oxidative stresses, metabolism, ion response, and transcription factors (Fig. 24.1) (Wu et al. 2016).

Laminarans are also able to protect plants against herbivorous insects under both controlled and field conditions. For instance, foliar spray of laminarans increase defenses of tea plants (*Camellia sinensis*) against the green leafhopper (*Empoasca* (*Matsumurasca*) *onukii*) (Xin et al. 2019). In this case, laminarans seem to activate MAPK cascades and WRKY transcription factors and to increase the content of hydrogen peroxide, salicylic acid, and abscisic acid, and the activity of defense-related enzymes such as chitinase, phenylalanine ammonia-lyase, and polyphenol oxidase. Additionally, plants treated with laminarans seemed to be more and less attractive respectively to the egg parasitoid wasp of leafhopper (*Stethynium empoascae*) and to the leafhoppers themselves. Under field conditions, parasitism of leafhopper eggs by *S. empoascae* was 55% higher in plants previously treated with laminarans (Xin et al. 2019).

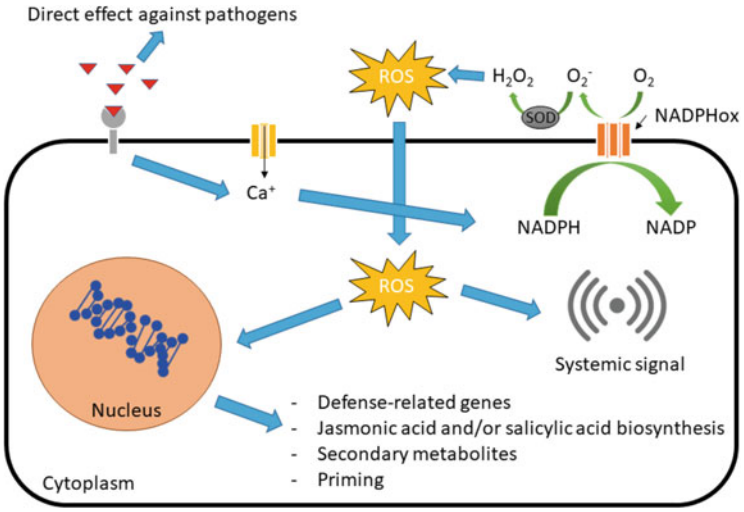

Fig. 24.1 General schematic illustration showing defense responses induced by algal polysaccharides in plants. Polysaccharides can affect directly the pathogen and/or be recognized by receptors. This recognition triggers a calcium influx, which leads to the activation of membrane-bound NADPH oxidase (NADPHox). Superoxide produced in the apoplast by NADPH oxidase dismutates spontaneously and by the action of superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2) which in turn is able to enter the cytoplasm and activate several defense responses. Adapted from Stadnik and de Freitas (2014) and Zheng et al. (2020)

Carrageenans are the main polysaccharides found in several species of red seaweeds. These linear galactans are composed by repeating disaccharides of α-1,4-D-galactose linked to each other by alternated bonds of α-1,3 and β-1,4. Carrageenans can be organized in three mains groups depending on the sulfate content. Thus, kappa-, iota-, and lambda-carrageenan have, respectively, one, two, and three sulfate group in each repeating disaccharide (Stadnik and de Freitas 2014; Zheng et al. 2020).

Carrageenans are able to protect plants against several pathogens by both direct effect and activation of defense responses (Fig. 24.1). For instance, foliar spray of kappa-carrageenan delayed the appearance of symptoms caused by *Colletotrichum gloeosporioides* in pepper plants (*Capsicum annuum*) by 6 days and reduced the disease final severity by 25% (Mani and Nagarathnam 2018). The protection can be due to an increase in the expression of defense-related genes such as *PR1*, *PR5*, *PDF1.2*, and *NPR1*. However, a direct effect cannot be ruled out since kappa-carrageenan under in vitro conditions reduced the growth of *C. gloeosporioides* by affecting the integrity of the plasma membrane of the fungus (Mani and Nagarathnam 2018). In another work, lambda-carrageenan reduced the severity by 70% of *Zymoseptoria tritici* in wheat (*Triticum aestivum*) leaves (Le Mire et al. 2019). In those plants, lambda-carrageenan upregulated the expression of several defense-related genes belonging to both salicylic acid- and jasmonic acid-dependent

signaling pathways. In this situation, however, no direct effect against the pathogen was observed under in vitro conditions (Le Mire et al. 2019).

Ulvans are water-soluble heteropolysaccharides obtained from green seaweed belonging to the genus *Ulva*. In general, ulvans are composed by rhamnose, xylose, glucose, uronic and iduronic acids, and sulfate (Stadnik and de Freitas 2014; Zheng et al. 2020). These sugars are organized in two main repeating disaccharides, the ulvanobiuronic acids type A (β-D-GlcA (1 → 4) α-L-Rha 3S → 1) and type B (α-L-IdoA (1 → 4) α-L-Rha 3S → 1) (Stadnik and de Freitas 2014).

Ulvans are able to induce defense responses in several plant species against different pathogens (Fig. 24.1). For instance, ulvan spraying reduced the lesion diameter of *Alternaria brassicicola* and *Colletotrichum higginsianum* by 60% and 35% in *A. thaliana* plants, respectively (de Freitas et al. 2015). In another work, it was demonstrated that ulvans require an active NADPH oxidase in order to induce defense responses at least in *A. thaliana* plants (de Freitas and Stadnik 2015). Ulvans are also able to induce a priming effect (Paulert et al. 2010). In this situation, pretreated plants are able to activate faster and stronger defense responses upon pathogen attack (Paulert et al. 2010). Seed soaking with ulvans increased the emergence of bean (*Phaseolus vulgaris*) seedling by 40% in soil infested or not with *Fusarium oxysporum* f. sp. *phaseoli* (de Borba et al. 2019). Additionally, when sprayed onto leaves, ulvans reduced the area under disease progress curve of *F. oxysporum* f. sp. *phaseoli* by 30% (de Borba et al. 2019).

Ulvans are also able to protect fruits against postharvest diseases. When applied onto papaya fruits, ulvans reduced the incidence and lesion diameter of *C. gloeosporioides* by 40% and 75%, respectively (Chiquito-Contreras et al. 2019). Considering that ulvans did not affect spore germination of the fungus under in vitro conditions and that it increased the activity of defense-related enzymes such as peroxidase, catalase, and superoxide dismutase, the disease control observed could be due to induced resistance (Chiquito-Contreras et al. 2019).

24.5 Extraction Techniques

Recently, several studies have been carried out on techniques to obtain seaweed extracts. The technologies for production and preparation of seaweed extracts are diverse and may include cultivation, extraction, fermentation, processing and purification, hydrolysis, and high-pressure cell rupture treatment. In general, the extracts are made using water, alkali or acid, or by physically disrupting the seaweed by low temperature milling to give a "micronized" suspension of fine particles (Craigie 2011). Another alternative used is through cell disruption using a high pressure, where the soluble cytosolic components are recovered in the filtered liquid (Stirk and van Staden 2006).

Several biologically active metabolites are produced by seaweed, where carbohydrates and lipids are the main that make up (Chiaiese et al. 2018). Proteins and amino acids, such as tryptophan and arginine, are key for significantly increasing the

defense to pathogens, growth and yield of cultivated crops because they are precursors of phytohormones (Colla et al. 2017). The amount and quality of these metabolites in extracts depend on the extraction technique and the seaweed species used.

24.5.1 Extraction Using Water

This method has the advantage of requiring few chemicals. The usual method is based on hot extraction with tap water or with distilled water at 70–90 °C for 2–6 h (Flórez-Fernández et al. 2018). Dried and pulverized seaweeds are mixed with water. Thus, seaweed compounds are extracted by water and the solid residues are separated by filtration. Extracts using this method are rich in phytohormone-like activity (Shukla et al. 2019).

24.5.2 Acid Hydrolysis

Seaweed biomass are treated with acid (for example: sulfuric or hydrochloric acid). It has been reported that acid hydrolysis removes the complex phenolic compounds and increased the depolymerization of polysaccharides. This method is used for the extraction of sulfated polysaccharides (Flórez-Fernández et al. 2018; Shukla et al. 2019).

24.5.3 Alkaline Hydrolysis

One the most industrially used method, it uses alkaline solutions (for example: NaOH or KOH) for seaweed compounds extraction (Shukla et al. 2019). The process by alkaline hydrolysis breaks down complex polysaccharides into smaller, lower molecular weight oligomers (Shukla et al. 2019). Alkali treatments also act on polyphenols to produce a complex array of reaction products, which are dependent on the hydroxylation pattern of the original polyphenol. The extracts produced by this method are intensely colored due to their high content of humic-like polyphenols or phlorotannins (Craigie 2011).

24.5.4 Extraction Using Enzymes

This is an efficient method that no solvents are required for extraction. Complex molecules in the seaweed cell walls are degraded by enzymes, increasing the extraction efficiency. Several carbohydrate-degrading enzymes and proteases such as Viscozyme, Celluclast, Termamyl, Ultraflo, carrageenanase, agarase, xylanase, Kojizyme, Neutrase, Alcalase, and Umamizyme are commonly used for the extraction of bioactive compounds from seaweeds (Shukla et al. 2019). Another advantage of this method is that water-insoluble chemical components are converted by hydrolytic enzymes in water-soluble products, eliminating the problem of water solubility of bioactive compounds (Flórez-Fernández et al. 2018).

24.5.5 Pressurized Liquid Extraction

This method uses high pressure and temperature for bioactive compounds extraction from seaweeds. The high pressure and temperature of solvents above their boiling point increase the solubility of complex algal molecules, also increasing the mass transfer rate. This method has been used for extraction of sulfated polysaccharides to increase yields, preserving the sulfate content and enhancing the antioxidant properties (Flórez-Fernández et al. 2018). Pressurized liquid extraction is a faster extraction method compared to other methods (Shukla et al. 2019).

24.5.6 Eco-Friendly Methods

Extractions assisted by microwave (EAM) or ultrasound (EAU) are characterized as eco-friendly by an increase in the efficiency of solvent extraction. In EAM, the solvent is heated by microwave, facilitating the metabolites extraction (Flórez-Fernández et al. 2018). On the other hand, the EAU method uses ultrasound waves of high frequency (greater than 20 kHz), transmitting through solid, liquid, and gas media by rarefactions and compression (Shukla et al. 2019). Both methods facilitate the extraction of lipid (Yap et al. 2014) and phenolics (Shukla et al. 2019).

Today, aqueous preparations from seaweed extract are prevalent in marketplace. These preparations vary widely in color, odors, viscosities, solids, and particulate matter contents depending on the extraction method. In some instance, the product can contain mixture of components derived from different sources and production methods (Yakhin et al. 2017). In other cases, macro- and micronutrients are added in preparations, since the seaweed extracts have natural chelating properties which prevent trace metal ions from precipitating (Mukherjee and Patel 2020). Though, the methods of manufacture are rarely published, being held as proprietary information.

Overall, commercial extracts are manufactured from the brown seaweeds *Ascophyllum nodosum*, *Laminaria* spp., *Ecklonia maxima*, *Sargassum* spp., and *Durvillaea* spp., although other species such as *Fucus serratus*, *Enteromorpha intestinalis*, *Ulva lactuca*, and *Kappaphycus alvarezii* have been used (Craigie 2011).

24.6 Application Methods

Several applications techniques of seaweeds extracts have been adopted in agronomic and horticultural crops. Each technique depends on the seaweed product, formulation, and purpose. The main modes of application include (a) seeds treatments with seaweed formulation against seed pathogens (citation); (b) soil drench for treatment against soil pathogens (de Borba et al. 2019); and the method most utilized (c) foliar application (de Freitas and Stadnik 2012). The foliar application seems to be effective when applied under high relative humidity conditions and when leaf stomata are open in order to increase the permeability and uptake of the product (Chiaiese et al. 2018) (Table 24.2).

24.7 Concluding Remarks and Perspectives

Despite the fact that seaweed extracts and purified polysaccharides have been used in agriculture for a long time, mankind only exploited the surface of their potential. Seaweeds possess a myriad of unique molecules with several biological properties. So far, extracts and polysaccharides obtained from a few species of brown algae are the most commercially abundant to be used as fertilizers and biostimulants in agriculture. At the research level, on the other hand, extracts and polysaccharides obtained from green, red, and other species of brown algae have been showing potential as both biostimulants and resistance inducers in several plant species. However, it takes a long time for making a product available in the market due to various factors including funding, regulations, and problems in securing a continuous supply of seaweed.

Chemically modified polysaccharides are proving to present special biological activities. These molecules may activate stronger or even different signaling pathways than its original counterparts. Modifications include sulfation, hydrolysis, exposure to radiation, and formulation with nanoparticles. This promising field is still in its childhood and it is receiving a lot of attention in the past few years.

Considering the biodiversity of marine ecosystems and the encouraging results obtained so far, it seems reasonable to assume that the next generation of products dedicated to promote both plant growth and protection may come from the sea.

Table 24.2 Summary of benefits of the aqueous-based extraction techniques

Extraction method	Advantage	Disadvantage
Conventional	Simplicity	Low yield and selectivity; long extraction time; high temperature and energy consumption; generation of impurities
Enzyme-assisted extraction	Moderate temperature; selective breakage	Slow process; enzyme recycle and cost limited enzyme recycle; enzyme cost
Pressurized liquid	Increased solubility and higher yield; selective solubilization of components by controlling severity; hydrolysis of polymeric fractions; lower extraction time	Cost of equipment; degradation of susceptible components
Microwave-assisted extraction	Fast and uniform heating; compact equipment; fast start-up; short time; less solvent and energy	High energy consumption; thermal degradation of some components
Ultrasound-assisted extraction	Low cost; simplicity; selectivity; effective mixing; fast start-up and response; low operation temperature and time	Localized heating and need to cool; radical species formation; noise pollution

Adapted from Flórez-Fernández et al. (2018)

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