Environmental Challenges and Solutions
Series Editor: Robert J. Cabin

Telma Encarnação
Alberto Canelas Pais *Editors*

Marine Organisms: A Solution to Environmental Pollution?

Uses in Bioremediation and in Biorefinery

Environmental Challenges and Solutions

Series Editor

Robert J. Cabin, Brevard College, Brevard, NC, USA

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Editors

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Springer

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Foreword

I am delighted to provide a Foreword to this book *Marine organisms: a solution to environmental pollution?*, edited by Telma Encarnação and Alberto Canelas Pais, with contributions from a number of other well respected workers in the field.

Our contemporary society, with its ready access to the convenience of fast communications, mass transportation, good illumination, home entertainment, etc. has developed over the last three Centuries through advances in science and technology. This has involved a series of industrial revolutions, based on increasing mechanisation, automation and mass production, with the involvement more recently of computer control, artificial intelligence and robotics. This has led to the demand for new materials, such as polymers, alloys and advanced ceramics to replace the traditional ones, such as wood and stone. The supply of the new materials, and their precursors, has been one of the factors responsible for the development of the chemical industry, with its consequent impact on the environment.

Chemicals are vital in almost all areas of our life, including medicine, agriculture, food and drinks, textiles, construction, packaging, personal care and domestic care products. Although most of these chemicals are beneficial for society, uncontrolled or inappropriate use of them can also have serious negative consequences for the environment. High priority must be given as a major goal to minimising such chemical pollution through the development of cyclic systems, or control or elimination of chemical pollutants by using some technique to transform them into innocuous species. The ideal scenario would be complete mineralisation, where pollutants are transformed into water, carbon dioxide and other small molecules.

Possible methodologies for pollutant treatment involve the use of chemical oxidants, such as hydrogen peroxide, photochemistry (using ultraviolet or visible light), frequently with a catalyst such as titanium dioxide, high energy radiation, and the use of various biological organisms. This book focuses on the latter approach using marine-based microorganisms.

Care must be taken in the treatment process that any reaction intermediates are not more toxic than the original pollutants. For detailed study of pollutant degradation,

identification of all species involved and study of their effect on the viability of microorganisms is desirable.

It is sometimes convenient to distinguish between synthetic chemicals, such as aspirin, and naturally occurring ones, like water and oxygen. However, the fact that a substance occurs in nature does not necessarily mean that it is innocuous. Carbon dioxide is a colourless gas, that is essential for processes such as respiration and photosynthesis, However, its presence above a certain concentration can prove fatal. It is also implicated as a greenhouse gas in global warming.

Environmental pollution can be described in terms of the bulk phase involved (water, air, soil…), type of pollutant (pesticides, pharmaceuticals…), chemical category (polycyclic aromatic hydrocarbons (PAHs), heavy metals, polychlorinated compounds…) or by chemical structure (carbon monoxide..). Methods of pollutant treatment frequently show selectivity in terms of chemical structure, and in the case of water treatment may depend on parameters such as pH, salinity and temperature.

The choice of treatment method depends on a variety of factors. Economic parameters are of particular importance, and the use of plants and microorganisms is particularly attractive in this respect. In addition to their Green credentials, since they do not normally require the use of any expensive chemicals, biological processes frequently produce biomass, which is a value added product that can be used as a feedstock for a variety of materials, including pharmaceuticals, biopolymers, fertilisers and biofuels, and may be a source of novel chemicals. This makes such systems particularly attractive, and economically viable, for the treatment of environmental wastes. Their potential is further enhanced by the number and diversity of species available. Although until recently the use of marine sources for bioremediation has been limited, they are attracting increasing interest for various applications in biotechnology. In part this is because they are rich in a variety of interesting species, with a number of actual and potential applications.

In this book, *Marine organisms: a solution to environmental pollution?,* Telma Encarnação, Alberto Canelas País and co-authors present an excellent overview of the potential of marine based species such as microalgae, fungi, bacteria, yeasts and sponges in bioremediation and in the preparation of biobased materials. It makes an important contribution to environmental technology and pollution control, and its multidisciplinary approach makes it attractive to research workers in a wide variety of areas, including biotechnology, microbiology, chemistry, and environmental sciences.

It is well structured and provides an excellent introduction to this rapidly developing field. A succinct introductory chapter is followed by 11 chapters covering various aspects of bioremediation, treatment of specific pollutants, and biomass valorisation using different marine based microorganisms. The book concludes with a chapter on environmental management. A comprehensive and up-to-date bibliography provides rapid access to the current research in this area.

This book fills a gap in the literature on the use of marine microorganisms in the treatment of pollutants. It also acts as a valuable source of information on the application of such systems in the production of value-added biomass, makes an excellent contribution to the fields of biotechnology and bioremediation, and

provides valuable information on how these species can be used for sustainable bioharvesting of novel molecules. These may have interesting properties for applications such as pharmaceuticals, cosmetics, and fine chemicals.

In conclusion, the book, *Marine organisms: a solution to environmental pollution?,* makes an important contribution to the literature and provides an excellent testimony to the authors well recognised scholarly insight in this area.

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Hugh D. Burrows

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Chapter 1
Introduction: Environmental Pollution and Biotechnological Solutions

Telma Encarnação, Maria da Graça Campos, and Artur Mateus

Abstract Progress, wealth, comfort, increased productivity and economic growth are some of the repercussions that emerged from the Industrial Revolutions, providing greater benefits and opportunities. But these advances also brought us great challenges, bringing uncertainties for the future. Our social organization, economic models and lifestyles are altering ecosystems and Earth's patterns, causing environmental degradation, and shaping the face of the Earth. But the same revolutions that brought us challenges can provide us with opportunities to restore the environment. And biotechnology can unlock potential solutions for a more sustainable and resilient future.

Keywords Industrial Revolutions · Environmental pollutants · Biotechnology · Bioremediation · Circular economy · Industry 5.0

The Industrial Revolution marked significant and remarkable milestones in human history. They spurred faster progress in several areas that benefit human life and led to economic, political, and societal changes, changing life in unforeseen ways. The First Industrial Revolution began in Great Britain after the 1750s with the introduction of hydraulic power and steam engine, which led to the mechanization of

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agriculture, manufacturing, and transportation. In 1870, the Second Industrial revolution started with the generation of electricity, shortly after its discovery, incorporating electromotive force in the industry, enabling mass production for the first time. A century later, in the 1970s, the Third Industrial Revolution started with the invention of the transistor and the first microchip; the advances in computer technology after the Second World War triggered further development in the automation of the production process. Since then, a digital revolution has been disrupting traditional industries. A technological fusion of physical, chemical, digital, and biological dimensions is transforming industry, economies, jobs, and society itself, leading us to a New Era, the Fourth Industrial Revolution or Industry 4.0. Although no consensually accepted definition exists yet, the Industry 4.0 concept can be described as the advent of cyber-physical systems (CPSs), the Internet of Things (IoT), and services (MinHwa Lee et al. 2018) which have a significant impact on the efficiency of the production processes and in product development. The COVID-19 pandemic forced a rapid transition to digital technologies, even in sectors that were particularly resistant to the digital transition. The development of the Industry 4.0 technologies such as the Internet, Big Data (BD), blockchain, IoT, Additive Manufacturing (AM), virtual and enhanced reality, and Artificial Intelligence (AI) will probably lead us to radical changes, more human-centered, more sustainable, and more resilient: Industry 5.0. Without a doubt, Industry 5.0 (European Commission 2021) will significantly transform the manufacturing processes and services sectors where collaborative interactions between humans, machines, and systems materialize.

Despite the fact that social inequality may be found in all societies, the industrialized world brought us comfort and wealth in our lives. But this comes with a high price to pay: with all the improvements, a rapid urbanization brought significant challenges to the cities that suffered an increased pressure, such as growing population, lack of potable water, and residues management; a high consumption increased the release of pollutants into the environment. Carbon dioxide, dioxins, phthalates, bisphenols, pharmaceuticals metabolites, pesticides, flame retardants, and so many thousands of chemicals are released into the environment every day and have a dramatic impact on human health and wildlife. Although some damages could become irreversible in a very short time, we still have the opportunity to reverse the trend. We must boost science, technology, and innovation to implement biobased solutions to benefit from the progress that industrial revolutions brought us, reducing the environmental footprint. To achieve that, the entire planet needs to be united in a Green Revolution: civil society, governments, companies, and industries, organizations, all segments of society.

We are living a crisis for resources that will push forward the transition to new and renewable raw materials. Allying this with the emergency of the pollutants mitigation, we will be able to create a structure that allows a sustainable evolution of the planet, both in terms of well-being for humans and the environment.

Among all the tasks that should be done in a very short time, bioremediation, which has already started using plants and microorganisms, allow the production of biomass to be used in different feedstock sources for many industries applications:

for instance, bioplastics, fertilizers, new pharmaceuticals compounds, building blocks, energy, and many other exciting solutions, which unlock new sustainable and eco-friendly possibilities. These challenges also create enthusiastic economic opportunities. These economic outputs should be improved by countries with credible, efficient, and concrete policies that enhance the advancement of sustainable technologies. Science should provide the tools and robust evidence to support decisions before these are implemented into legislation for an ecological transition toward sustainable development.

This book aims to contribute to this fascinating process toward the next industrial revolution. The following chapters will comprehensively discuss bioremediation technologies, to reduce environmental pollution while producing value-added biomass that meets the need for new and better sustainable materials. Various marine microorganisms, such as sponges, microalgae, fungi, bacteria, yeasts, and consortiums of different microorganisms, are all able to biodegrade several families of compounds; for instance, carbon dioxide, PAHs, heavy metals, petroleum sludge, naphthalene and pyrene, pharmaceuticals, persistent organic pollutants. Tailored solutions will be discussed with the primary goal of contributing to zero carbon and zero pollution, and groundbreakingly scientific and technological advances will be critically approached. Legislation and regulatory requirements are also an essential topic of discussion and debate since the release into the market of new developed biobased products can be hindered by legal requirements.

Some Industry 4.0 technologies, such as additive manufacturing and Artificial Intelligence, will be debated in the approached context. The massification application of technologies that result from industry digitization plays and will play an increasingly important role in the development of intelligent solutions. Production and treatment systems will be increasingly monitored and controlled in real time based on machine learning. Waste treatment plants, as well as collection systems, present an increasing application of Digital Twins concepts. Digitization is and should be extended to electromechanical systems for the collection and treatment of waste and water to the point that, in real-time, there is such a collection of data that allows the existence of a digital twin, if possible autonomous and controlled by machine learning (Vitorino et al. 2019). The digital definition of systems (more common on electromechanical systems) must also be followed by the digital definition of products and every tangible material. The increasing of understanding on transforming processes and the increasing of optimization, monitoring and control, allow, the spatial and temporal definition of molecular organization, applied (for example) to additive manufacturing. In this way, this depth increasing of digital control is a precursor to a molecular-level definition of meso parts (from molecules to parts). The products of the future will be designed at the molecular level and implemented with digital, spatial, and temporal control (da Silva et al. 2022).

Can we glimpse the future? Can we glimpse the next industrial revolution, Industry 6.0? It is predicted that possible new technologies may include quantum computing and nanotechnologies. But what about changes in our values and our perceptions? Changes in how we socially relate, work, and connect with Nature for many years separated? Perhaps we can come to say: “Industry 6.0 significantly

transformed the world where collaborative interactions between humans, machines, and Nature materialize."

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Chapter 2
Bioremediation Using Microalgae and Cyanobacteria and Biomass Valorisation

Telma Encarnação, Pedro Ramos, Danouche Mohammed, Joe McDonald, Marco Lizzul, Nadia Nicolau, Maria da Graça Campos, and Abílio J. F. N. Sobral

Abstract Microalgae and cyanobacteria are photosynthetic microorganisms that can be used to bioremediate anthropogenic pollutants from air, water and soil. These organisms can remediate several anthropogenic pollutants, such as carbon dioxide, nitrates and phosphates, heavy metals, pharmaceuticals, pesticides and persistent organic pollutants. The biomass generated in this process can be used as a feedstock source for the production of a multitude of valuable biobased products and applications. Polymers, resins, binders, lubricants, and coatings are some of the promising examples. This chapter provides an overview of the entire process: bioremediation using microalgae and production of value-added products, based

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on a biorefinery concept, focusing on circular economy and sustainability. Essential aspects of legislation and regulations are also approached.

Keywords Biorefinery · Microalgae · Bioremediation · Biomass · Biofuels · Wastewater · Lipids · Biobased · Circular economy

2.1 Introduction

Microalgae is a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms living in different environments. Microalgae include prokaryotic blue-green algae (cyanobacteria) and eukaryotic microalgae (diatoms and green algae). These organisms use light energy and carbon dioxide as carbon source and, through metabolic processes, convert them into various biopolymers such as proteins, nucleic acids, lipids, and polysaccharides, releasing molecular oxygen in the process. They produce a wide range of compounds, some of which have potential commercial value. Depending on species, microalgae and cyanobacteria produce different biomolecules, and, depending on the growth conditions, these biomolecules might vary in composition and concentration. This versatility is reflected in the remarkable number of products and applications that can be developed.

Microalgae and cyanobacteria have been cultivated for decades, but only a limited number of species have been commercially used, mainly for pigments production and food and feed supplements. The most commercially used species are *Chlorella*, *Spirulina*, *Dunaliella*, *Haematococcus, Nannochloropsis*, *tetraselmis* and *Isochrisis*.

Despite their versatility, resilience, and potentialities, a consolidated and accepted microalgae cultivation system is not yet implemented worldwide. Many factors contribute to this reality: the many decades of established and implemented fossil economy, the confidence from investors, the initial costs of the investment, the downstream processing challenges, and the acceptance and willingness of all the stakeholders (political, industry, civil society). In recent years, the number of microalgae production units has increased and represents a significant step towards a sustainable resource for the future. These aspects will be further discussed in the following sections of this chapter.

2.2 Brief History

The importance of microalgae in wastewater treatment was recognised several decades ago. At the beginning of the twentieth century, the first studies focused on nitrogen/phosphorus uptake alongside oxygenation potential. Early studies on wastewater treatment using algal pond systems were developed in the mid-1950s; these systems were used to study algal growth and photosynthetic oxygen production to assist with bacterial degradation of Biological Oxygen Demand (BOD). In the

1960s, researchers began to focus on nutrient removal from sewage and wastewater, such as nitrates and phosphates. At the beginning of the 1970s, a 300 m^2 pilot plant was built and operated for algae wastewater treatment (Borowitzka 2013) and semicontinuous cultures of microalgae began to be studied for their ability to remove heavy metals, such as cadmium, chromium and mercury, from wastewaters.

The concern with environmental degradation by chemicals is not new. Several reports from the late 1960s and during the decade of 1970s state the problem of persistent pesticides in the environment and the impact on human health. And in this period, many reports were published to address the issue of their presence in underground and superficial waters, mainly using microalgae in the process. A notable a paper published in 1976 reported the removal of two herbicides (amitrole and atrazine), from water, by four microalgae species (*Chlorella pyrenoidosa, Scenedesmus quadricauta, Chlamydomonas reinhardtii, and Euglena gracilis*). During the decade of 1980s, many studies on the uptake of nitrogen and phosphorus from wastewater by microalgae were undertaken. The studies on this subject have been constant over many decades (PierreChevalier 1985, Hammouda et al. 1995, Shi et al. 2007, Eroglu et al. 2012, Ferrando and Matamoros 2020, Castellanos-Estupinan et al. 2022).

Much progress has been made in understanding many aspects of microalgal biology and physiology, nutrition, and cultivation conditions. Less research has focused on the uptake and removal of emerging contaminants. But one of the significant limitations of an implemented microalgae system, limiting further expansion, is the high cost of investment and cost-effective harvesting of biomass and extraction of compounds (downstream process).

In the last three decades, researchers have studied harvesting and extraction techniques involving physical (centrifugation, sedimentation, filtration, flotation, etc.) and chemical processes (e.g. flocculation). However, these are energy-demanding processes, and some chemical processes use toxic chemicals (Hoang et al. 2022). Academy and public research institutions, companies, and government agencies have funded research projects focused on the field of microalgae technology. Nevertheless, it still requires much knowledge and innovation, particularly in the engineering field applied to the downstream processing steps.

The history of each field of science is of great importance since one can analyse what was done by the peers in previous decades, what remained to be studied, what failed, and why it was not moved forward. In the case of the removal of pollutants from the environment, although there was some level of concern in previous decades, it was only more recently that public opinion, governments, world organisations, and companies are more willing to protect the environment by restoration and with more ecological alternatives.

2.3 Microalgae Bioremediation: An Effective Approach Towards Environment Restoration

Globally, the emissions of greenhouse gas of which, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), as well as surface and groundwater contamination by organic and metal pollutants, are the major global environmental issues that have captured the attention of scientists, environmentalists and legislators (Sundarrajan et al. 2019). Various physical-chemical methods have thus been proposed. While they are effective, such methods require expensive chemicals and energy-intensive equipment, rendering the treatment costs high and limiting their large-scale application (Danouche et al. 2021).

2.3.1 Application of Microalgae in CO_2 Mitigation

Sequestration of CO_2 can be achieved by using physicochemical and/or biological approaches. Currently, the main abiotic approaches employed for the mitigation of CO_2 include physicochemical adsorption (Song et al. 2019), direct injection into the deep ocean, old coal mines, oil wells, geological formations such as saline aquifers, and CO_2 mineral carbonation. However, some drawbacks are associated with these approaches, for instance, the control of the physicochemical adsorption process is generally difficult, and the sorbent materials are generally expensive and non-renewable. Additionally, the injection of CO_2 into geological formations presents significant challenges in terms of space requirements and potential leakage over time (Zeng et al. 2011). Naturally, microalgae capture photons from the sun's energy to convert CO_2 dissolved in water (as free CO_2, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and carbonic acid (H_2CO_3)) to produce organic molecules through the process of photosynthesis (Eze et al. 2018; Fu et al. 2019). As reported by Zhou et al. (2017), microalgae have a high growth rate and a photoautotrophic efficiency 10–50 times higher than that of terrestrial plants, which makes the capture of CO_2 using microalgae as one of the most promising approaches (An et al. 2021). A number of Chlorophyceae species have exhibited a high capacity for CO_2 sequestration. The most studied species for CO_2 fixation from flue gases belong to *Chlorella* genus, such as *C. vulgaris*, *C. fusca*, *C. sorokiniana*, *C. pyrenoidosa* and *C. kessleri* (Kong et al. 2021). The detailed mechanisms of CO_2 fixation and nitrogen assimilation in microalgae are described in Fig. 2.1.

2.3.2 Application of Microalgae in Wastewater Treatment

Phycoremediation, or the use of microalgae for the remediation of wastewater or contaminated aquatic ecosystems by organic or metallic pollutants, has recently

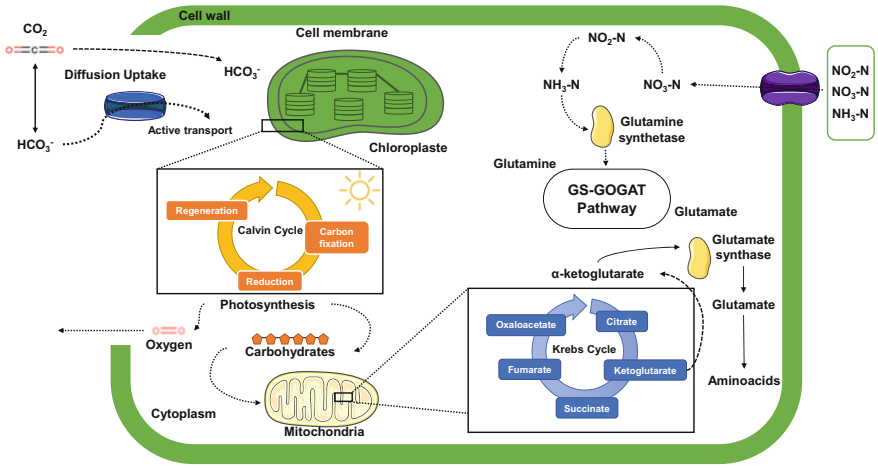

Fig. 2.1 Mechanisms of CO_2 fixation and nitrogen assimilation in microalgae

emerged as a promising, efficient, economical and environmentally friendly strategy compared to other physicochemical processes (Danouche et al. 2020; Singh et al. 2021a, b). The use of wastewater as a culture medium for microalgae is an innovative concept particularly suited to tertiary wastewater treatment. Indeed, microalgae can assimilate a wide range of inorganic contaminants as well as some organic pollutants, besides their capacity to accumulate heavy metals (HMs).

Phycoremediation of Inorganic Pollutants In wastewater treatment systems, microalgae can use several inorganic pollutants during their growth, such as nitrogen, phosphates, chlorides, sulfates and other inorganic pollutants, and may have a major role as intermediates in metabolic activity (Abdel-Raouf et al. 2012). Several studies have highlighted the possibility of using wastewater as a source of nutrients for the cultivation of microalgae, allowing both the elimination of the pollution load and the production of biomass at low cost (Fal et al. 2021). On the other hand, the ability of microalgae to eliminate and detoxify HMs is the result of adaptation mechanisms developed over centuries of evolution in contaminated environments (Ubando et al. 2021). These mechanisms are subdivided into metabolism-dependent and metabolism-independent pathways. Extracellular biosorption of HMs refers to a physicochemical property of the microalgae cell surface that binds to HMs ions independently to the cellular metabolism. However, HMs biosorption into extracellular polymeric substances (EPS) formed by microalgae under conditions of stress is a metabolism-dependent process (Naveed et al. 2019). It has been reported that the biosorption efficiency varies depending on the genus and the species of microalgae (Kumar et al. 2015a, b). For instance, the growth of *C. sorokiniana* and *S. obliquus* in media contaminated with Pb(II), Cd(II), Cu(II) and Cr(VI) was significantly

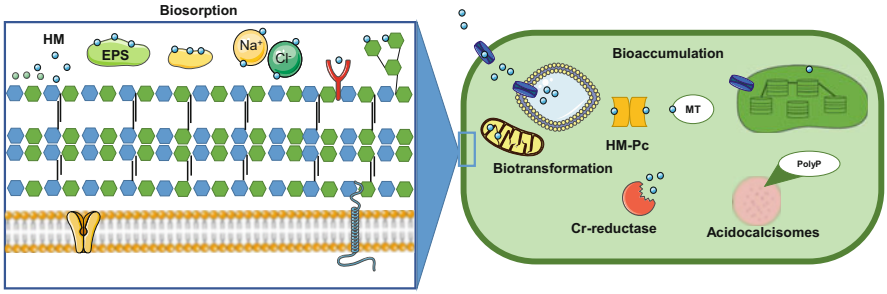

Fig. 2.2 Intracellular and extracellular mitigation pathways for HMs using microalgae

different (Danouche et al. 2020). This can be attributed to the physiology of the strain, in particular, the cell wall composition (Baudelet et al. 2017). In contrast, bioaccumulation is a metabolism-dependent mechanism. It consists of an intracellular accumulation of HMs into the cytosolic compartment through passive and/or active transport across the cell membranes (Chojnacka 2010). According to Pérez-Rama et al. (2002), the bioaccumulation of Cd(II) using *Tetraselmis suecica* was a biphasic process, assisted in the first phase by an adsorption to proteins or polysaccharides, followed by an energy-dependent accumulation to the cytosol. The intracellular mitigation of toxic HMs may involve the chelation by metallothioneins (Balzano et al. 2020), phytochelatins (Gómez-Jacinto et al. 2015), poly-phosphates (Wang and Dei 2006), the compartmentalisation in the vacuole (Shanab et al. 2012), chloroplast (Hanikenne et al. 2009) and mitochondria (Mendoza-co et al. 2005) or the biotransformation via an enzymatic reaction such the biotransformation of Cr (VI) to Cr(III) by strains of *C. vulgaris* through an enzymatic reaction catalysed by the chromate reductase (Lee et al. 2017; Yen et al. 2017). Figure 2.2 depicts the intracellular and extracellular mitigation pathways for HMs using microalgae.

Phycoremediation of Organic Pollutants Although microalgae are classified as autophotrophic organisms, some species have a heterotrophic metabolism, and under certain conditions, some microalgae strains are able to grow in mixotrophic mode. This trophic particularity allows microalgae cells to use the carbons contained in organic pollutants and to ensure the bioremediation of contaminated aquatic ecosystems (Zhou et al. 2017). It has been reported that many microalgae strains have the capability of removing a range of organic pollutants such as polycyclic aromatic hydrocarbon (Semple et al. 1999), synthetic dyes (Bhardwaj and Bharadvaja 2021), pharmaceuticals and personal care products (Hena et al. 2021), pesticides and other emerging contaminants (Maryjoseph and Ketheesan 2020).

Based on the above considerations, we can infer that the benefits of phycoremediation technology are to allow both CO_2 capture through photosynthesis and to remove nutrients and xenobiotics from wastewater. Thus, the resulting biomass can be used as a raw material for several valuable products depending on their composition and the type of pollutant to which it has been exposed. For

example, it can be used for the production of biofuels, animal feed, fertilisers, pharmaceuticals, biosurfactants, proteins, pigments and many other valuable products, that can be extracted from microalgae.

Microalgae production could be integrated into a biorefinery to achieve greater economic potential.

2.4 Microalgae Biomass: Valorisation within a Biorefinery Concept

Like a traditional petroleum refinery, a biorefinery converts feedstock into energy and several chemicals. The process entails different technologies and can be applied for processing different raw materials. There are several types of biorefineries based on biobased feedstock or waste source, end-products and conversion technologies. Potential organic feedstock sources include corn, potato, cellulosic biomass, forestry, agricultural waste, food waste and algae. The variety of possible organic raw materials implies a rich diversity of potential chemicals. Bioethanol, biogas, lignin, secondary metabolites, carbohydrates, lipids and proteins are some of the products that can be obtained using the biorefinery concept (Espinoza Pérez et al. 2017). To obtain this variety, different conversion processing technologies are required. Biorefinery conversion techniques can involve different separation technologies such as thermochemical conversion, chemical conversion and biochemical conversion (Sankaran et al. 2018).

There are three different phases of development of a biorefinery depending upon feedstock and products. Phase I converts a single raw material into one main product using a fixed process. The Phase II biorefinery also processes a single raw material but is capable of producing various products with diverse processing technologies. The biorefinery processes using single raw material sources can lead to food competition, land use issues and environmental impact (Espinoza Pérez et al. 2017; Sankaran et al. 2018). Phase III biorefinery uses a mixture of biomass from different sources, such as whole-crop, lignocellulose and microalgae, that allows the production of many biobased industrial products using different processing technologies. Phase III, the most advanced form of biorefinery, is also an engineering challenge due to the complexity involved. Some of these constraints are related to product separation and purification.

Among the various biomass sources, microalgae are a very promising and remarkable feedstock for the biorefinery process; they do not compete with food, do not require arable land, and can be used for air and water cleaning processes. Large-scale microalgae production requires a high initial investment, especially for the installation of photobioreactors, and production costs; these include high-power consumption, artificial light illumination, the CO_2 feed, the cultivation medium, and nitrates and phosphates. To save water and decrease production costs, microalgae can be used in wastewater treatment stations; they can also be integrated into

different industrial production units such as cement, paper, textile, tannery and dairy. An integrated system requires the full use of waste and exhaust gases.

The biorefinery process consists of the separation of different fractions, such as lipids, minerals, carbohydrates and secondary metabolites. That separation process should not cause damage to the other fractions. Microalgae are rich in lipids, but during the separation process is possible to obtain many other products that can be also transformed into value-added products (pigments, polyunsaturated fatty acids (PUFAs), toxins, and polysaccharides) or may be used to produce bioplastics, for instance. Microalgae biorefinery is divided into several stages which can be categorised into upstream and downstream processes. The upstream processing is determined by the strain selection, carbon dioxide supply, light source and intensity, and nutrients, such as nitrogen and phosphorus sources.

2.4.1 Upstream Production Systems

The production of microalgal biomass, and derived products, is highly dependent on the cultivation production systems. The choice of production system is particularly important for bioremediation applications, as large-scale algae production requires high initial capital investment. Broadly speaking, there are two main types of production system for microalgae; the open pond system or closed systems (Fig. 2.3).

Fig. 2.3 Microalgae cultivation systems

2.4.1.1 Open Systems

Open ponds are systems that allow large-scale production for commercial purposes. Open ponds have various sizes, shapes and types of turbulence. Their construction is often dictated by local conditions and available materials and can be constructed of plastic, bricks, concrete, or adobe. Open ponds include lakes and lagoons, raceway ponds, paddle-wheel-driven open raceway ponds, circular ponds and Inclined and cascaded systems. Open systems generally present some shortcomings in relation to control over process parameters. These systems offer little or no guarantee on the control of the operational variables (temperature and incident light intensity). The contamination by other microorganisms that may occur during the process and the low efficiency of CO_2 utilisation due to lack of agitation of the flow and poor gas exchange in the culture medium compromise the overall algal growth rates.

- *Natural ponds*. It is a naturally selective system, the type of species and strain is closely linked to the soil and climatic conditions of the region, generally being a low-cost, monospecies that can be grown almost all year round. In these systems, the risk of contamination is very high due to its open characteristics.
- *Raceway ponds (also known as high-rate algal ponds)*. These are open-air extensive cultivation systems, in the form of a racetrack, shallow, with mixing undertaken by a paddlewheel that distributes nutrients homogeneously to the microalgae. Generally, they are built with low-cost materials, cement, clay and white plastic to facilitate light capture.
- *Circular ponds*. Since these systems present an inefficient configuration compared with raceway ponds, they are rarely used for commercial purposes. However, in some countries, such as Japan, Taiwan and Indonesia, this process is widely used to produce biomass.
- *Inclined and cascaded systems*. In these systems, the turbulence is generated by gravity and the crop moves from the top to the bottom of an inclined suspended surface. This process is particularly interesting because the flow is highly turbulent, and the thin culture layers improve light absorption, and produce a greater concentration of cells. However, this system has a high evaporation rate, increased sedimentation of the cells under low turbulence mixing regimes and a high energy consumption compared to other pond systems.

The major disadvantages of open systems are essentially the high evaporation rates, the difficulties of temperature control and the high risk of contamination. In general, open cultivation systems produce less biomass per unit area compared to closed photobioreactors.

Although research to develop cultivation systems for microalgae has focused more on closed cultivation systems, today's large-scale industries rely more on open systems for economic reasons; they are cheaper to maintain, easier to operate and require less energy. However, only a small number of algae species can be successfully cultivated in outdoor systems due to contamination, which directly compromises productivity.

Due to the several problems associated with open culture systems, there is a particular interest in closed photobioreactors which, offer more efficiency and more advantages over open culture systems. These advantages include better results in terms of efficiency in photosynthesis; greater capacity for CO_2 removal; versatility in terms of the culture medium; production is not seasonal; the absence or reduction of contaminations; ease of monitoring operational variables such as temperature, pH and CO_2; greater incidence of light on the culture medium. The photobioreactors can be exposed to sunlight or artificial light, the latter offers better control over the process variables.

2.4.1.2 Closed Systems

Closed photobioreactor systems strongly limit any direct exchange of contaminants into the cultivation medium. The algae and cultivation medium flows within the transparent walls of the reactor to reach the cultured cells. Photobioreactors are classified according to their mode of operation and design. Some examples include tubular vertical and horizontal, shaking tank photobioreactors, helical tubular, flat plate photobioreactors, and photobioreactors mixed by air "airlifts".

Although photobioreactors have limitation of contamination as the main advantage, this may not be completely achievable, except in some designs specifically developed for this purpose. They are also more expensive than open systems.

- *Vertical Tubular Photobioreactors*. Since the first invention of photobioreactors in 1950, several models have been developed. The construction cost of this system is very high; however, its maintenance and monitoring are very economical. The vertical stacked system presents a higher concentration of cells and a higher productivity thanks to the gentle and controlled agitation of the mixture which is obtained through the injection of compressed air. Furthermore, vertical tubular photobioreactors are compatible with the majority of microalgae species. The disadvantage of this system is that it has a lower efficiency of sunlight incidence, an issue that can easily be circumvented by applying artificial lighting.
- *Horizontal tubular photobioreactors*. Horizontal tubular photobioreactors were the best solution to solve the problem of sunlight incidence in vertical tubular systems. The horizontal orientation of the tubes increases the capacity to absorb the incidence of light. However, this system also has some difficulties in the O_2 removal and in the CO_2 supply, also the increase of the light absorption capacity implies the growth of the installation area of the tubes.
- *Flat photobioreactors*. These are systems with large, transparent panels, which can be made of glass or polycarbonate, arranged vertically or at an inclined angle. The light intensity is easily controlled due to the possibility of directing the panels in different directions and at different angles. This system has received special attention from the scientific community, which considers it to be a promising system thanks to the light conversion efficiency and productivity per unit area.

- *Airlift photobioreactors*. These are cylindrical tubes with two interconnecting zones, one of which is called a "riser", where the gas mixture is spread, while the other zone, the "downcomer", does not receive the gas. This system stands out for having high levels of mass transfer. The pneumatic agitation caused by the injection of CO_2 at the bottom of the tank increases the velocity of the medium circulation, which in turn increases the efficiency of the microalgae growth.

2.4.2 Downstream Processing

After the cultivation stage, the upstream processing ends, and the downstream processing begins. Typically, downstream processing involves several stages: harvesting of biomass through centrifugation, filtration or flocculation, drying, product extraction, purification and conversion processes. These operation units account for 50–60% of the total costs in the microalgae multi-product biorefinery, with harvesting accounting for at least 20% of these costs ('t Lam et al. 2018; Xu et al. 2020). Microalgae cultures are dilute suspensions, typically varying from 0.5 to 5 g L^{-1}, depending on species, making harvesting a challenge. Common harvesting and dewatering methods encompass centrifugation, filtration, sedimentation, flotation and flocculation. The latter include chemical, biological and electro-flocculation.

After the dewatering process, the microalgae biomass will be subjected to drying processes such as spray drying, solar drying, convective and freeze drying (Chen et al. 2015).

The extraction of high-value products such as lipids, pigments, secondary metabolites, and others can be performed using either wet or dry biomass. The extraction processes vary depending on the products. Some microalgae species can have thick and multilayered walls, silicified, and wall-bound exopolysacharides membranes implying a reduced extraction efficiency. Therefore, appropriated pre-treatment methods are often required before extraction takes place. Cell disruption can be achieved through soaking, maceration, bead-beating, sonification, alkaline lysis, etc. (Catherine Dupré et al. 2020).

After the pre-treatment steps, the biomass can be converted into several products. Extraction methods include conventional solvent extraction, supercritical fluid extraction, enzyme extraction, subcritical water extraction, among others (Catherine Dupré et al. 2020).

2.4.2.1 Production of High-Value Products and Applications

A variety of products and compounds can be extracted and isolated from microalgae biomass: biopolymers, lipids, bioactive compounds, proteins and carbohydrates, which can be applied in a multitude of applications (Table 2.1). The currently existing applications on the market focus on food, feed, nutraceuticals, cosmetics

Table 2.1 Species of marine microalgae, their products and potential applications

Marine species	Compounds	Potential industrial products and applications	Bioremediation/ biodegradation Use	Reference
Chlamydomonas reinhardtii	Spidroin	Biomedical	Cd, Cu, Al and Zn	João Vitor Dutra Molino et al. (2016), Ibuot et al. (2017)
Chaetoceros sp.	Lipids, carotenoids	Nutraceutical, pharmaceuticals, cosmetics, paint, and paper industries, and aquaculture	Bioremediation nitrites, phosphorus, lead	Parvin Molazadeh et al. (2015), Singh et al. (2021a), Tiwari (2021)
Chlorella marina	Lipids, carotenoids	Biodiesel, cosmetics and pharmaceutical industries, feed (colourants and additives), and the healthcare sector	Bioremediation of nitrate, nitrite, ammonia, phosphorus, silicate, chromium, lead, zinc, copper, cadmium, and sulphide, tannery wastewater	Muthukumar et al. (2012), Adam et al. (2015), Kumar et al. (2015a), Cezare-Gomes et al. (2019), Singaram (2022)
Chlorella sp.	PHB	Biomedical, package applications	Bioremediation of nitrogen, ammonia, heavy metals	Da Silva et al. (2018)
Dunaliella salina	Lipids, carotenoids, phytosterols	Biodiesel, nutraceutical industry, aquaculture	Bioremediation of nitrate, silicate, chromium and sulphide, tannery wastewater	Francavilla et al. (2010), Adam et al. (2015), Cesário et al. (2018), Singaram (2022)
Isochrysis galbana	Lipids, carbohydrates, proteins, carotenoids, polyunsaturated fatty acids	Biofuels, food and nutraceutical industry, cosmetics	Biodegradation of phenol, bioremediation of nitrate, silicate, chromium and sulphide	Wang et al. (2019), Koutra et al. (2018), Gomez-Loredo et al. (2016), Ruiz-Dominguez et al. (2020), Adam et al. (2015)
Isochrysis sp.	Lipids, carbohydrates, proteins, carotenoids, polyunsaturated fatty acids, alkenones	Nutraceutical industry, aquaculture, fuels, polymers, phase change materials,	Bioremediation of nitrites and phosphorus	O'Neil et al. (2021), Singh et al. (2021a)

(continued)

Table 2.1 (continued)

Marine species	Compounds	Potential industrial products and applications	Bioremediation/ biodegradation Use	Reference
		sunscreens and cosmetics		
Nannochloropsis sp.	Lipids, carbohydrates, proteins, carotenoids, polyunsaturated fatty acids	Biodiesel, aquaculture	Bioremediation of nitrate, silicate, chromium and sulphide, imidacloprid, paracetamol, ibuprofen, olanzapine. Tannery wastewater	Adam et al. (2015), Encarnação et al. (2020), Encarnação et al. (2021), Singaram (2022)
Rhodomonas sp.	Lipids, carbohydrates	Feed for aquaculture	Bioremediation of para-xylene	Cesario et al. (2018), Li et al. (2020)
Scenedesmus sp.	PHB	Biomedical, package applications	Bioremediation of nitrogen, phosphorus, heavy metals	García et al. (2020)
Spirulina sp.	PHB	Biomedical, package applications	Bioremediation of nitrogen, phosphorus, ammonia, heavy metals	Da Silva et al. (2018), Selvaraj et al. (2021)
Thalassiosira sp.	Lipids	Biodiesel	Tannery wastewater	Singaram (2022)
Tetraselmis sp.	Lipids, carotenoids	Aquaculture, nutraceuticals, cosmetics	Bioremediation of nitrate, silicate, chromium and sulphide	Adam et al. (2015), Schuler et al. (2020)

and pharmaceuticals with several companies commercialising different microalgae products. Few are focused on the chemical industry, and the potentialities are vast and could extend into new areas.

Biopolymers, such as polyhydroxy butyrate (PHB) have been identified in some marine and freshwater microalgae species. They are used in the food industry, pharmaceutical industry, environmental remediation and medical devices. Polyhydroxyalkanoates (PHAs) are biodegradable biopolyesters produced by microorganisms, including microalgae. With properties like those of polyethylene and polypropylene, they can be processed similarly to fossil-based thermoplastics, including injection moulding, extrusion and blow moulding. PHAs are generally produced by heterotrophic bacteria, natural or artificially modified bacteria, such as *Cupriavidus necator*, recombinant *Escherichia coli.*, *Ralstonia* sp., *Halomonas* sp., among several others {Khatami, 2021 #1}(Khatami et al. 2021); it is known that at least 75 distinct genera synthesise PHAs (Reddy et al. 2003). The intracellular levels

accumulated can reach 90% of the cell's dry weight under conditions of nutrient stress (Reddy et al. 2003). At present, more than 160 different monomer units have been identified, and their molecular weight ranges from 50,000 to a million Da (Taguchi and Matsumoto 2021; Vermeer et al. 2022). The variation in the composition of the monomeric units implies a diversity of chemical and physical properties. Microalgae are an alternative to the production of bacterial PHA. PHAs such as PHB can be stored by microalgae and cyanobacteria as reserves of energy material in response to nutritional stress. Excess carbon and nutrient depletion growth conditions lead to the production of these polyesters by these microorganisms. Microalgae species *Chlorella* sp., *Scenedesmus* sp., and *Spirulina* sp. are three species capable of producing PHB (Da Silva et al. 2018; García et al. 2020; Selvaraj et al. 2021). Large quantities of carbon source, such as glucose, are required to produce PHAs, which represents 50% of the total costs of production (Costa et al. 2019). This limitation can be overcome using waste feedstock from industrial waste streams.

Spider silk proteins, such as spider fibroins or spidroins, are another interesting biomaterial that can be obtained from the cultivation of microalgae. The recombinant spider silk proteins have similar properties to those of natural spider silk. This biomaterial has extraordinary properties, such as toughness, strength, elasticity, and biocompatibility, exceeding those of other natural and synthetic materials such as steel, and textile fibres, including Kevlar-like super fibres. Potential applications include biomedical (scaffolds and tissue engineering), hydrogel formation, constructing fibres and electronics.

Genes encoding recombinant spidroin have been expressed in *Chlamydomonas reinhardtii*. (João Vitor Dutra Molino et al. 2016).

Microalgae lipids provide a potential and attractive alternative to crude oil and a source of building blocks for many interesting chemicals. Many microalgae species can produce large amounts of lipids depending on culture parameters, such as light intensity and nitrates concentration. Several reports on lipid production indicate a production range of up to 80% of dry weight (Encarnação et al. 2018, Hess et al. 2018). These microalgal oils are a great source of feedstock chemicals. Lubricants (Farfan-Cabrera et al. 2022), resins (Hidalgo et al. 2019), additives, polyols and polyurethanes (Peyrton et al. 2020), coatings (Decostanzi et al. 2018), plasticisers and surfactants (Pleissner et al. 2015) are some of the potential products.

Regarding biofuel applications, the development has been at a lab and pilot scale. The conversion of microalgae lipids to biodiesel is performed by a transesterification process in which triglycerides react with monoalcohol (methanol or ethanol) in the presence of acid or alkali catalyst to produce fatty acid methyl esters (FAME) and glycerol as a side product. The two most imposing barriers and limitations to the scale-up of this technology to produce biofuels are the downstream processing costs and the bioprocessing.

Companies, industries, and governments should invest in the circular economy, stimulating the transition for a biobased and green economy. The development has been supported by several National, European and International projects. The European Commission has launched ambitious programmes boosting these transitions (Table 2.2).

Table 2.2 Selection of European projects with focus on microalgae. Information related to funded projects can be found on cordis.europa.eu

Acronym	Project title	Programme	Date	Total cost (Private + Public)
ALGAECEUTICALS	Development of microalgae-based natural UV Sunscreens and Proteins as cosmeceuticals and nutraceuticals	H2020 EU	2018–2023	€ 1,129,500
ALGFUEL	Biodiesel production from microalgae	FP7-EU	2011–2013	153,917
BISIGODOS	High value-added chemicals and BIoreSIns from alGae biorefineries produced from CO2 provided by industrial emissions	FP7-EU	2013–2017	€ 5,605,438,85
DEMA	Direct Ethanol from MicroAlgae	FP7-EU	2012–2017	€ 6,388,935,04
D-FACTORY	The microalgae biorefinery	FP7-KBBE	2013–2017	€ 10,074,870,03
INTERCOME	International commercialisation of innovative products based on Microalgae	H2020 EU	2016–2018	€ 2,426,437,75
FUEL4ME	Sustainable biofuel from algae	FP7-EU	2013–2016	€ 5,369,514,10
MAGNIFICENT	Microalgae as a green source for nutritional ingredients for food/feed and ingredients for cosmetics by cost-effective new technologies	H2020 EU	2017–2021	€ 5,685,015,41
SOLENALGAE	Algae biomass: Unlocking new uses as food, feed and fuel	ERC	2016–2021	€ 1,441,875
SUNBIOPATH	Microalgae engineering—Greener biomass and biofuel production	FP7-KBBE	2010–2013	€ 4,366,894,60

2.5 Legislation Framework and Regulations/Policy and Legal Framework

Biobased products are non-food goods, fundamental in a Circular Bioeconomy, which can help to reduce CO_2 and pollution (EuropeanCommission n.d.). They are derived from renewable raw materials. When these are obtained from microalgae biomass used in cleaning processes, as the present chapter discusses, special attention to the legislation applicable to them should be verified. Despite the importance attributed to those products by the Regulatory Entities, and some progress has been made, there is still a long way ahead for better clarification of the rules. For instance, the Legislation for Biobased products in the EU and USA should guarantee standards and measurements which allow the industries to make them available in terms of sustainability. Depending on the type of biobased products, details in quality control still need to ensure better products and to achieve high acceptance by the Market. It would be ideal that the EU and USA, among others, develop harmonised standards for such products to reduce trade barriers and foster a more broad market. However, until now, limited research has been conducted on biobased products derived from microalgae used in bioremediation, and this could drive some constraints on the companies due to some toxic contaminants not yet well evaluated or unknown.

Before we go further in detail on the legislation available, we must highlight some considerations. Regarding biobased materials from microalgae, it is important to identify the products produced and what will be the targeting industry. Thus, it is possible to organise and orient the application of the available legislation. Until now, the well-established bioproducts obtained from algae (macroalgae) were alginates, agar and carrageenans (phycocolloid production), almost exclusively from seaweeds (macroalgae brown and red) and other biopolymers, such as starch, cellulose, chitin and PHA (biodegradable plastic). It is consensual that in these last years, the growth in the production of microalgae introduced a new perspective in the biotransformation and production of different new biomaterials, which require a more detailed look at the possibilities. Moreover, as referred to above in this chapter, the production of high-value products include biopolymers, carbohydrates, lipids, phycobilins, pigments, proteins, polyhydroxyalkanoates, and many more compounds. Resins, coatings, binders, and bioplastics are the main industrial applications as well biofertilisers, food and feed, pharmaceuticals, cosmetics and products for personal care. Even bioplastics which can be converted to produce high-value final products such as medical equipment, prosthetics and scaffolds, will add a new perspective market for these biobased products. Therefore, the legislation available should reflect clear information to be followed, if different from the biobased obtained from plants, for instance. Other interesting microalgae products include the bioactive oligosaccharides (extracted by enzymatic methods), biopigments for food supplements (carotenoids and xanthophylls) or for paints, and cosmetic bioactive compounds for skincare (ex. *Porphyridium cruentum* extract) and γ-linoleic acid or alguronic acid from *Chlorella* extract. For all of the cited products, the legislation

used is the same that should be followed for other similar products from different sources. Fertilisers or soil improvers, feed, energy or fuels, soap, and building or packaging materials are some of the possible examples.

Nevertheless, the main concern for biobased materials production for various applications requires intensive regulatory work in order to protect human health and the environment from harmful fractions of waste, especially if the start material is microalgae previously used for wastewater bioremediation. The legal maximum of residues should be cautious, especially because, in certain cases, some of them are unknown. Despite this, the legislation for Agro-food, Cosmetics and Pharmaceutics have already a list of contaminants that should be avoided, and when admitted, the low amounts of them need to be reevaluated cycling, face to the evolution of data provided by science.

Nevertheless, the companies, when preparing a submission to obtain clearance of the material, should evaluate, for instance, which are the appropriate food simulants to be used to estimate the potential for migration and found to prove to authorities that the substance is stable for an intended application that involves a specific type of food, cosmetic or a pharmaceutical purpose. Generally speaking, it may be necessary to demonstrate the suitable purity of a product with respect to the potential presence of possible contaminants such as, for instance, algal biotoxins and mycotoxins, toxic organic compounds such as dioxins and polychlorinated biphenyls, or inorganic compounds already regulated in various materials as human and veterinary medicine residues, heavy metals, nitrates and pesticides.

For instance, a scientific risk assessment carried out by the Scientific Committee for Consumer Safety (SCCS) in Regulation (EC) No. 1223/2009, in order to address potential risks for human health, lays down a system of restrictions and bans on the use of certain substances in cosmetics based classified as carcinogenic, mutagenic or toxic for the reproduction (CMR), of category 1a, 1b or 2, under Part 3 of Annex VI to Regulation (EC) No. 1272/2008. Substances classified as endocrine disruptors are only banned or restricted automatically in cosmetics if they are also classified as CMR. Actually, some substances classified as endocrine disruptors derived from plants are still found in certain cosmetic products, but this issue should be reviewed for the safety of consumers once scientists continue to link endocrine-disrupting chemicals to various diseases and disorders such as cancer, infertility and obesity.

The legislation here referred to below is representative of the most detailed data that should be consulted before submitting for Market Authorization in EU and in the USA biobased products obtained from microalgae used in the bioremediation of wastewater from different sources (Tables 2.3 and 2.4).

In summary, eco-innovation is a priority on the international agenda, and, despite this, all the regulation available is mainly for biobased products other than microalgae derivatives. The tailored production of microalgae biomass and the respective bio-products still have barriers and constraints, mainly in the understanding of the levels and types of contaminants that sometimes could be under-evaluated in the current legislation. Better and more active involvement of the companies in a green intervention will contribute to a stable policy framework with greater harmonisation and coordination around the world, together with a simplification of

Table 2.3 Lead Market for biobased products from microalgae in *Europe legislation*

General information	https://eur-lex.europa.eu/ http://data.europa.eu/eli/reg/2019/1009/oj https://ec.europa.eu/growth/sectors/biotechnology/bio-based-products_en https://www.biobasedconsultancy.com/en/database https://ec.europa.eu/growth/industry/policy/key-enabling-technologies_en)
Conversion of waste streams into value-added products	https://www.bbi-europe.eu/ https://ec.europa.eu/growth/tools-databases/eip-rawmaterials/en
Materials and articles intended to come into contact with food	https://ec.europa.eu/food/safety/chemical_safety/food_contact_materials/legislation_en Plastics Regulation, (EU) No. 10/2011, for all multi-layer food contact materials (FCM). This regulation includes monomers and other substances and additives (other than colourants) and some polymer production aids that are permissible. For new monomers or additives produced, it is possible to start a petition to the European Food Safety Authority (EFSA), which will review and issue a formal opinion on the safety of the substance and, if approved, will be included in the Plastics Regulation's positive list through an amendment to the regulation. All FCMs in the EU must comply with the safety criteria specified in Framework Regulation (EC) No. 1935/2004: "The principle underlying this Regulation is that any material or article intended to come into contact directly or indirectly with food must be sufficiently inert to preclude substances from being transferred to food in quantities large enough to endanger human health or to bring about an unacceptable change in the composition of the food or a deterioration in its organoleptic properties". All FCMs must also comply with the Regulation, (EC) No. 2023/2006, on good manufacturing practice for materials and articles intended to come into contact with food. Bioplastics from biobased products from algae are required to comply with the same regulations with respect to food and safety as fossil fuel-based plastics, and some concerns related to end-of-life issues remain on the actual agenda.
Fertilising products	Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the Market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003 (Text with EEA relevance) PE/76/2018/REV/1
Pharmaceutics	All products used should be pharmaceutical grade and, in general, fulfil the guidelines required for specific purposes.

Table 2.4 Lead Market for biobased products from microalgae in *US legislation*

General information	Any substance, the intended use of which is reasonably expected to become a component of food must be authorised for such use by the US Food and Drug Administration (FDA) through a food additive regulation, and all the substances must be recognised as safe.
Food-contact polymers/plastic materials and articles intended to contact food	Food additives are listed in the Code of Federal Regulations (C.F.R.), Part 177, "Indirect Food Additives: Polymers", and food packaging material intended to come in contact with food must comply with FDA's Good Manufacturing Practices (GMP). This implies that "*additives may only be used in an amount necessary to achieve their function or purpose and may not contain impurities at levels sufficiently high as to result in the adulteration of food*". In US regulations, the Plastics Regulation includes limits on co-reactants or use levels for starting materials, temperature restrictions, specification of single versus repeated use, and food types for specific substances. www.food.gov.uk/sites/default/files/media/document/bio-based-materials-for-use-in-food-contact-applications.pdf

complex national regulations, which provides more transparency for relevant stakeholders in knowing how sustainable are their products.

Also, national and international programmes and research funding will push forward the development and innovation of these technologies. Moreover, the fact that the alternative products are considered green and biobased is not guaranteed that they have not a similar negative impact on the environment and human health as their conventional counterparts. Therefore, more research is needed to mitigate the potential impacts.

As a final note, the biorefinery concept can transform the linear economy through a biobased circular economy which is an integrated concept that envisages the cascade use of biomass from different sources.

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Chapter 3
Removal of Heavy Metals and Organic Pollutants by Marine Microalgae

Jagannathan Umamaheswari, Ricky Rajamanickam, Sowmya Vilvanathan, Subramanian Shanthakumar, Kadiyala Venkateswarlu, Sudharsanam Abinandan, and Mallavarapu Megharaj

Abstract Marine environment is a predominant player in the overall ecosystem functioning with almost half of oxygen evolution into the atmosphere through the photosynthetic activity of plankton communities. Anthropogenic activities cause pollution at an enhanced pace and pose a major threat to the biological cyclings in the marine ecosystem. Pollutants such as heavy metals and organic compounds in the marine environment are a serious concern as they are associated with complex challenges. Marine microalgae are promising candidates in remediating inorganic and organic pollutants due to their versatile metabolic mechanisms. The present chapter provides a comprehensive understanding of the response of marine microalgae in the removal of heavy metals and organic pollutants. Initially, we present the importance of microalgae and the sources of heavy metals and organic pollutants that reach the marine environment besides highlighting the merits and demerits of the conventional and biological treatment systems used for the removal of these pollutants. Finally, we provide a general perspective on the implication of

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marine microalgae and the associated mechanisms in the removal of heavy metals and organic pollutants.

Keywords Marine microalgae · Heavy metals · Organic pollutants · Bioremediation

3.1 Introduction

The environment is the global ecological life-supporting system that has been affected in complex and accelerating ways because of pervasive and profound human activities. The past few decades witnessed rapid industrial development, population growth, economic wealth, and urbanization, which ultimately disturb the very processes and components of the nature. Marine ecosystems are at serious risk due to the elevated levels of pollutants discharged from industrial and domestic activities (Bergmann et al. 2015; Nelms et al. 2017). The impact of these pollutants on coastal zone has been significantly greater in estuaries due to their residence time than in inland rivers (Saldarriaga-Hernandez et al. 2020). In fact, the occurrence of both heavy metals and organic pollutants in the marine environment is of significant ecological concern. International scientific experts on marine protection define marine pollution as "chemicals introduced by human activities either directly or indirectly into the marine environment affecting the biota and impairment of water quality" (Kuppusamy et al. 2020). Fish inhabiting polluted waters was reported to accumulate metals in the tissues and the accumulation depends on various biotic and abiotic factors (Zeitoun and Mehana 2014). The heavy metals tend to be widely distributed in liver, kidney, and other tissues and potentially get transferred to humans as they are at the top of the food web (Gabriel et al. 2006). Oils are the major sources of organic contaminants released into the marine environment either during processing or accidentally from drilling, production, and storage (Kuppusamy et al. 2020). Consequently, the deteriorated health of the oceans around the world impacted the social and economic status and prompted to bring international options for safer and healthier marine systems (Gelcich et al. 2014).

Several environmental agencies recognized the severity of these pollutants and proposed various policies in reducing the risk toward marine biota. For instance, a list of priority pollutants that should be universally avoided has been prepared as they can cause shorter or longer effects in any ecosystem (Grip 2017; Beiras 2018). The United States Environmental Protection Agency recently updated the priority pollutant list in the Clean Water Act, which includes several heavy metals, organic contaminants such as dyes, phenols, organophosphates, etc. (USEPA 2014). The United Nations Convention on Law of Sea proposed major duties for member states to investigate potential threats in the marine environment (Stelzenmüller et al. 2018). European Commission endorsed the marine strategy framework directive with an aim to manage Europe-bound seas to gain a healthy state following an ecosystem-based approach (Borja et al. 2013; Danovaro et al. 2016). Marine environments contain several biotas together with marine microalgae that serve as the primary producers and can also be used as sensitive bioindicators (Torres et al. 2008). This

chapter highlights the implication of marine microalgae in the removal of heavy metals and organic pollutants.

3.2 Marine Microalgae—An Overview

Marine environments are inhabited by assemblages of several organisms (Tragin and Vaulot 2018). The diversity of marine plankters based on their size is presented in Fig. 3.1. They are easily distinguished based on the nutrition mode: autotrophic organisms, referred to as phytoplankton (microalgae), and grazing organisms, called zooplankton. In addition, marine microalgae are the major primary producers in the marine environment that use solar energy for CO_2 uptake, thus contributing to ocean carbon sink (Huang et al. 2017). These microalgae are generally divided into two

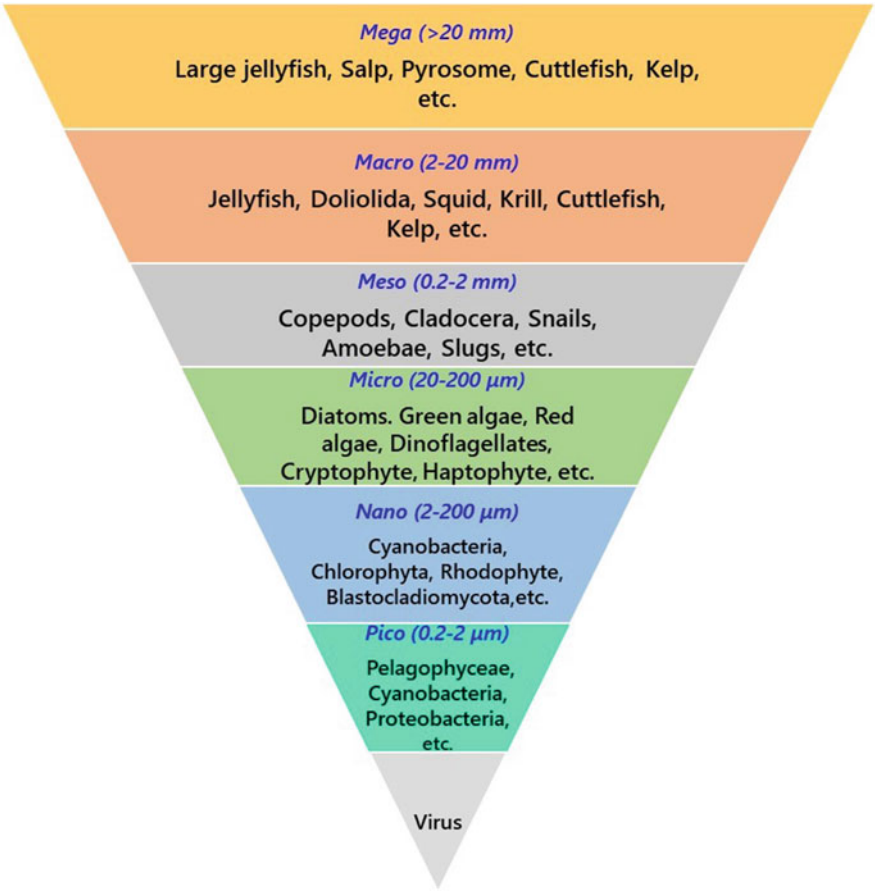

Fig. 3.1 Diversity and classification of marine plankters based on their sizes

lineages such as green and red, with the former being originated by primary and the latter from the secondary or tertiary endosymbiotic process (Nakayama et al. 1998). Chlorophyta is the major algal group in marine waters representing the green lineage, whereas the protists and dinoflagellates fall within the red lineage. Chlorophyta encompasses prasinophytes and chlorophytes, where the abundance is dominated later with *Ulvophyceae*, *Trebouxiophyceae*, and *Chlorophyceae*, all known as the UTC Clade (Leliaert et al. 2012; Fučíková et al. 2014). Chlorophyta consists of chloroplasts surrounded by two membranes with chlorophyll *b* as the major pigment. Parsinophytes comprise eight lineages of different taxonomic levels, and the numbers increase based on the environmental sequences and novel cultures (Tragin and Vaulot 2018). Chlorophyceae alone comprises two thousand species and are well known for several biotechnological applications (Barra et al. 2014). For example, microalgal biomass is reported to yield several primary metabolites such as carotenoids, proteins, lipids, and polyunsaturated fatty acids (Becker 2004; Guedes et al. 2011; Sharma et al. 2012; Christaki et al. 2013). Due to their biomass productivity and surface ratio, microalgae also play a crucial role in biogeochemical cycling of pollutants in marine waters (Van Gestel and Van Brummelen 1996). For example, the cell wall composition of microalgae is reported to have greater capacity for the metal-binding that can be transferred to food chain through grazing (Wang et al. 1998).

Due to the abundance of microalgae in waters, they have been overwhelmingly considered as sensitive bioindicators to monitor pollutants in the marine environment (Levine 1984; Whitton and Kelly 1995; Ali et al. 1999; Volterra and Conti 2000). While thoroughly reviewing the toxic profile of marine algae, Torres et al. (2008) proposed that the widespread abundance of microalgae in the marine environment can be used for seasonal evaluation or the effect of time change in the ecosystem in response to heavy metals and organic pollutants. Owing to the presence of these pollutants, microalgae tend to respond through physiological changes. Reports indicate that among other marine plankton, diatoms are severely affected by pollutants than green microalgae (Harrison et al. 1986; González et al. 2009). In addition, the green marine microalgae are reported to often dominate the bloom of natural population in the marine environment, particularly at increased pollution levels (Bonin et al. 1986; Folgar et al. 2009). Microalgae are known to respond to pollutants through two mechanisms: accumulation and sorption, and they also synthesize phytochelatins that are responsible for metal detoxification (Gekeler et al. 1988; Folgar et al. 2009). Furthermore, the antioxidants and innate enzymes have been shown to detoxify organic pollutants (Sunda et al. 2002; Stahl and Sies 2003; Sharma et al. 2012). Despite their versatile biochemical mechanisms, a detailed understanding of metal and organic pollutant removal by marine microalgae is very limited. The following sections present a comprehensive overview on the role of marine microalgae in the removal of heavy metals and organic pollutants.

3.3 Pollution in the Marine Environment—Sources of Heavy Metals and Organics

Anthropogenic sources of heavy metals and organic pollutants in the marine ecosystem generally result from the direct discharge of wastes, water runoff, and airborne pollutants (Leprovost 2001). These pollutants are carried from inland through sewage, dredged spoil, rainwater, and domestic and industrial waste discharged into coastal waterbodies through estuaries that enter the oceans (Wu et al. 2001; Adeniji et al. 2017). Hydrocarbon pollution is one of the great threats to the marine environment, with estimates of discharge accounting for 1–8 million tons per year (National Research Council Committee on Oil in the Sea, 2003). In addition, around 25,000 ship cargo with 18.5 million barrels of oil per day navigates through gulf waters which can potentially result in minor accidental spills causing a threat to the marine environment (Chitrakar et al. 2019). These crude oil spills affect marine organisms by limiting gas exchange and reducing light penetration (González et al. 2009). Moreover, crude oil spills release several organic pollutants such as benzene, toluene, xylene, and aromatic hydrocarbons that can accumulate in marine biota and sediments, thus acting as a sink affecting the ecosystems (Kachel 2008).

The marine environment is also reported to receive copious and stable inputs of pyrogenic hydrocarbons from coal and oil combustion as well as other organic products such as wood (Ravindra et al. 2008; Page et al. 1999). The predominant source of heavy metals in the marine environment is the industrial effluents discharged into the ocean either through runoff or improper disposal. Three types of heavy metals that cause major environmental problems include toxic metals such as cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn), nickel (Ni), cobalt (Co), etc., precious metals like silver (Ag), gold (Au), palladium (Pd), platinum (Pt), etc., and radionuclides such as uranium (U), thorium (Th), radium (Ra), etc. (Wang and Chen 2009). Human exposure to heavy metals has dramatically risen because of an exponential increase in their use in several industrial, domestic, agricultural and technological applications. Other potential anthropogenic sources of heavy metal pollution are industrial effluents, acid mine drainage associated with mining operations, and coal-based and nuclear power plants. Various industries produce and discharge different heavy metals at varying concentrations into the environment; few of them include electroplating, metallurgy, surface finishing industries, energy and fuel production, iron and steel manufacturing, lead-acid battery manufacturing, fertilizer and pesticide industry, electrolysis, electro-osmosis, microelectronics, leather manufacturing, electrical appliance manufacturing, photography, etc. (Ahmaruzzaman 2011). Natural phenomena such as weathering of rocks and volcanic eruptions also significantly contribute to heavy metal pollution.

3.4 Removal of Heavy Metals and Organic Pollutants by Marine Microalgae

A comparison of conventional remediation techniques such as chemical precipitation, ion exchange, membrane filtration, electrochemical treatment, coagulation, and flocculation with those of bioremediation approaches, in terms of their merits and demerits (Table 3.1), clearly indicates that remediation of the polluted sites following the conventional engineering approaches is challenging both technically and economically. Also, bioremediation that involves the capabilities of microorganisms in the removal of pollutants is the most promising, relatively efficient, and cost-effective technology. The following sections particularly deal with the innate capabilities of microalgae in the removal of heavy metals and organic pollutants from marine environments.

3.4.1 Removal of Heavy Metals by Marine Microalgae

Abundant occurrence of metals in the environment leads to their increased concentration in the organisms over time. Bioavailability most often refers to the availability of contaminants, such as heavy metals or organic pollutants, in an ecosystem. Frequently, it is also used to determine the potential risk of pollutants toward nontarget organisms in any system. Bioavailability in the environment primarily involves physical, chemical, and biological processes. Contaminants or pollutants may be present in varying forms: (i) associated with soil and or sediment particles (bound form), (ii) released from liquid and or gaseous phases (release form), and (iii) associated with living organisms (attached form). A contaminant enters a liquid or gaseous phase once it is released from the bound phase. During this stage contaminant transport will take place through advection, diffusion, and dispersion, which result in the movement of contaminant molecules in the medium (liquid or gas) and thereby reassociation of contaminant or return to the bound state (soil). Meanwhile, the contaminants are carried to the surface of the living organisms (Fig. 3.1). Similar processes occur in the medium and eventually the contaminant reaches the living organisms and enters their tissues through cell membrane. Thus, contaminant transport is an important component of its bioavailability. The contaminants after their entry into the cells are metabolized and/or excreted, causing adverse or toxic effects to living organisms (Fig. 3.2).

3.4.1.1 Biosorption of Metals

Biosorption is the process of removing sorbet (metal ions) from the solvent (water) using biological material called a biosorbent. Marine microalgae have recently gained attention for the development of biosorbent materials due to their high

Table 3.1 Merits and demerits of available remediation technologies for the removal of heavy metals and organic contaminants

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
Physico-chemical treatment methods				
Physical separation	The process involves: Mechanical screening, Gravity separator, Magnetic separation, Electrostatic separation, and Attrition scrubbing	*Merits:* Large-size heavy metals can be easily segregated. Easy maintenance and operation.	*Merits:* Organic contaminants that are clogged onto solid wastes such as wood, plastic, etc. can be removed easily. Gravity separation reduces 50–60% of the suspended solids.	Marino et al. (1997), Dermont et al. (2008), Gupta et al. (2012)
		Demerits: Removal efficiency depends upon the particle size distribution, soil-clay matrix, moisture content, metal magnetic properties, and pollutant hydrophobic properties.	*Demerits:* Regular maintenance is required. Screens are clogged frequently.	
Conventional chemical treatment	Chemical precipitation	*Merits:* Heavy metals are precipitated and removed as sludge. Low cost of precipitant. Simplicity. pH can be controlled.	*Merits:* Effective in removing polymeric electrolytes such as carboxyl, phenol, phosphoryl, and sulfonyl groups.	Liao and Randtke (1986), Matlock et al. (2002), Wingenfelder et al. (2005), Özverdi and Erdem (2006), Chen et al. (2018b)
		Demerits: Removal efficiency depends upon the pH, temperature, and charge of heavy metal ions. Metal hydroxides solubility depends upon the pH conditions (8.0–11.0).	*Demerits:* Usage of large proportions of chemicals for precipitations produces a large amount of sludge. Handling of sludge and usage of chemical is costly.	

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		Toxic sludge. Metal precipitation is slow with poor settling. Sulfide precipitation results in the production of toxic H_2S gas.	pH-dependent. Weakly adsorbing organic pollutants can retard precipitation.	
	Coagulation and flocculation	*Merits:* Sludge settling is faster. Easy dewatering. Sludge stability. Heavy metal concentrations less than 100 mg L^{-1} can be treated.	*Merits:* Higher molecular weight organic contaminants are eliminated easily.	Randtke (1988), Ayoub et al. (2001), Shen et al. (2015), Sakhi et al. (2019)
		Demerits: The use of coagulants increases settling time. High chemical consumption. A large volume of sludge production with toxic properties. pH-dependent (11.0–11.5).	*Demerits:* Removes only selected group of organic contaminants. Produces large quantity of sludge. Molecular weights less than 500 are not effectively removed. Longer settling time.	
	Flotation	*Merits:* Recovery of heavy metals is selective. Low sludge generation. High separation efficiency. Better removal of small particles. Fewer energy requirements.	*Merits:* Simple in operation. Ability to treat a wide range of organic contaminants. Recovery of organic contaminants is possible. Surfactant cost is low.	Hu et al. (2002), Matis et al. (2004), Salmani et al. (2013)

		Space requirement is low. Less operating cost.	Effective in treating volatile organic contaminants.	
		Demerits: Removal efficiency is dependent on interfacial chemistry and aggregation effectiveness.	*Demerits:* Surfactant needs to be selected based on the organic contaminants present in the wastewater.	
	Adsorption	*Merits:* Low-cost adsorbents can be used (agricultural waste such as coconut coir pith, orange peel pith, plant-derived biomass). Sludge production is very less. Metal recovery is possible. Higher removal efficiency.	*Merits:* A very effective option for tertiary treatment. Various organic pollutants can be handled. Synthetic organic contaminants are effectively treated.	Anirudhan and Sreekumari (2011), Burakov et al. (2018), Alalwan et al. (2020)
		Demerits: Difficulty in adsorbent regeneration Activated charcoal is efficient in treating the pollutant but costly. Efficiency depends upon the adsorbents.	*Demerits:* Regeneration of adsorbent is difficult. Disposal of adsorbent is costly.	
	Ion-exchange	*Merits:* Ion-exchange resins are soluble in water. Valuable heavy metals can be recovered. Cost-effective. Ease of operation.	*Merits:* Operation is simple. Installation is easy. Usage of anion exchange resins effectively removes natural organic matter.	Bolto et al. (2002), Dąbrowski et al. (2004), Bashir et al. (2019)

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		Very effective in treating low-concentration heavy metal ions.		
		Demerits: Highly pH sensitive. Matrix gets easily fouled.	*Demerits:* Treatment efficiency depends upon the resins and type of organic contaminants in the wastewater. Loss of capacity of resins with time.	
Electrochemical	The process involves: Electro-deposition, Electro-coagulation, Electro-flotation, and Electro-oxidation	*Merits:* Ease of operation. In situ generation of coagulant. Sludge production is very less. Can be used as an alternative to conventional coagulation treatment. Very high efficiency of the metal removal process.	*Merits:* Both soluble and insoluble organic contaminants can be degraded. Carboxyl functional group pollutants can be treated effectively.	Chen (2004), Cheng et al. (2007), Sun et al. (2009), Tran et al. (2017)
		Demerits: A high proportion of chemicals are required for coagulation and precipitation. A solid waste disposal problem. The time of precipitation depends upon the metal ions. Poor settling. It is not a cost-effective treatment.	*Demerits:* Chlorinated organics create secondary pollution. Energy consumption is high. Compound-specific removal.	

	Electrodialysis	*Merits:* Highly concentrated heavy metal ions can be treated. Suitable for metal ion concentration below 20 mg L^{-1}. Chromium and copper metals ions can be recovered.	*Merits:* Metal-organic complexes can be separated. Reclamation of treated water.	Kongsricharoern and Polprasert (1996), Aydin et al. (2019), Gurreri et al. (2020)
		Demerits: Corrosion problem. Heavy metal concentration higher than 1000 mg L^{-1} cannot be effectively treated. Periodic maintenance. Operation is not simple.	*Demerits:* Organic contaminants induce fouling. Very efficient only when the concentration of NaCl is low. Energy consumption is high.	
Advanced treatment methods				
Membrane filtration	Ultra-filtration (UF), and Micellar-enhanced ultra-filtration (MEUF) Polymer-enhanced ultra-filtration (PEUF)	*Merits:* More than 90% removal efficiency Ions concentration with 10–112 mg L^{-1} can be treated. pH 5–9.5 can be handled. Smaller space requirement for installation. Recovery of heavy metals.	*Merits:* Usage of chemicals is avoided. No hazardous by-products. High separation efficiency for organic complexes in the wastewater.	Molinari et al. (2008), Landaburu-Aguirre et al. (2010), El Zeftawy and Mulligan (2011), Isawi (2019)
		Demerits: Membrane replacement. Removal efficiency depends upon the concentration of metals and surfactants for MEUF. Heavy metal concentration and type of polymer used in treating effects PEUF. Operating cost is high. Only efficient when the pH	*Demerits:* Membrane easily fouled by natural organic contaminants. The operating cost is high.	

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		condition of the wastewater is above 6.		
	Complexation-ultra-filtration	*Merits:* High separation selectivity. Water-soluble polymer ligands. Lower energy requirements. Higher bonding selectivity. Metal concentrates can be reused. The complexing agent can be easily regenerated and reused.	*Merits:* Simultaneous removal of organic pollutants and heavy metal complexes. Lower energy requirement. Removes trace organic contaminants effectively. The binding capacity of polymeric complexing agents.	Trivunac and Stevanovic (2006), Siyanytsya et al. (2008), Borbély and Nagy (2009), Garba et al. (2019)
		Demerits: Efficiency depends upon complexation agent. Condition of membrane determines the metal removal process. Membrane fouling. The presence of salt affects the membrane surface charge reducing the efficiency.	*Demerits:* Ultrafiltration efficiency decreases when organic pollutant complex increases.	
	Reverse osmosis (RO)	*Merits:* Many types of ions, as well as bacteria, can be removed. Membrane pore size is less than 2 nm. 90–99% removal efficiency.	*Merits:* Trace organic pollutants are easily removed. 97% removal efficiency for ionic contaminants. Membrane availability is plentiful.	Ipek (2005), Yangali-Quintanilla et al. (2011), Rodrigues Pires da Silva et al. (2016)
		Demerits: Separation efficiency depends upon the concentration, pressure,	*Demerits:* Membrane fouling.	

		and water flux rate. High power consumption. Frequent membrane replacement. High-quality feed is required or else the membrane will be fouled by colloidal matters in the wastewater.	Energy consumption is high. Post-treatment is necessary.	
Advanced oxidation methods	Fenton oxidation	*Merits:* Higher degradation efficiency. Flexible in operation.	*Merits:* Enhances biodegradability. Faster mineralization of organic pollutants in the wastewater.	Pérez et al. (2002); Bautista et al. (2008); Nguyen et al. (2021)
		Demerits: Disposal of ferric sludge is not easy. pH-dependent. Catalyst is required in large dosages. Hydroxyl and Carboxyl groups formed in Fenton oxidation can form larger heavy metal ion species.	*Demerits:* Disposal of ferric sludge is not easy. Pre-treatment is required to completely degrade the organic pollutants. pH-dependent.	
	Electrochemical Fenton oxidation	*Merits:* In situ production of H_2O_2. Negligible formation of secondary pollutants. Ability to handle higher concentration of wastewater. A clean and effective way. Does not require additional oxidant.	*Merits:* Very effective in treating recalcitrant organic pollutants. Highly reactive.	Kabdaşli et al. (2010), Anawar and Ahmed (2019), Song et al. (2019), Magro et al. (2020)
		Demerits: Low treatment efficiency. Treatment of heavy metals	*Demerits:* Frequent maintenance is required.	

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		depends upon the current density and pH. Oxidant concentration generated is relatively low.	Wide ranges of organic contaminants cannot be treated. pH-sensitive.	
	Photocatalytic oxidation	*Merits:* Widely used in the removal of heavy metal complexes. UV radiation can be used in the disinfection process as well. Solar energy can be utilized.	*Merits:* Effective polishing step. Taste and odor-causing organic pollutants such as pesticides, geosmin, and methyl tert-butyl ether are effectively oxidized.	Antoniadis et al. (2010), Chen et al. (2017), Brillas (2020)
		Demerits: Depends upon the characteristics of metal ions. Efficient UV illumination in large-scale treatment is inefficient.	*Demerits:* Bulk organic content and high turbidity requires a high UV dosage. Not cost-effective.	
	Ozonation	*Merits:* High removal efficiency. Improvement of wastewater biodegradability.	*Merits:* Lower ozone dose requirement. Very efficient. Aromatic compounds, alkenes, and certain pesticides are effectively removed by ozone.	Clark et al. (1988), Xia and Hu (2018), Honarmandrad et al. (2020)
		Demerits: The low solubility of ozone in water. Energy consumption is high to produce ozone. Low oxidation efficiency.	*Demerits:* Ozone solubility depends upon pH. Alkanes are treated poorly. Expensive treatment technology.	

	Discharge plasma oxidation	*Merits:* High reactivity and can be operated without chemical reagents.	*Merits:* Very effective in treating pesticides 90% removal in 5 minutes. High concentration of pollutants can be treated.	Magureanu et al. (2018), Wang et al. (2017), Liu et al. (2020)
		Demerits: High energy consumption and operating cost. Handling of this technology is complex.	*Demerits:* Degrading efficiency depends upon the molecular structure of the target pollutants. Unstable organic peroxides are formed.	
Biological treatment methods				
Activated sludge	The process involves: Bacteria, Aeration, and Agitation	*Merits:* Sludge is re-circulated back continually. Metals like iron and lead are efficiently removed by the process.	*Merits:* Acid and lipophilic compounds are biodegradable and treated effectively. Biodegradation is the major removal mechanism.	Oliver and Cosgrove (1974), Pagnanelli et al. (2009), Karpińska and Kotowska (2019)
		Demerits: Toxic sludge and disposal of sludge is problematic. Large area and proper maintenance.	*Demerits:* High toxic contaminants cannot be treated. The concentration of pollutants should be low enough to support bacterial life. Trace organic compounds are poorly treated by activated sludge.	
Trickling filters	The process involves: Aeration, Coarse media (stone or plastic), and Micro-organisms	*Merits:* Various types of filter media (gravel media or plastic media) are available for effective	*Merits:* Surfactants polluted water are effectively treated. Energy consumption is less.	Dermou et al. (2007), Ziolko et al. (2009), Katam et al. (2020)

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		treatment. The bacterial population can be easily maintained and controlled. Very effective. Economical process for industrial wastewater treatment. The use of chemicals is avoided.	Biofilm culture (algal-bacteria consortia) enhances the removal efficiency of the filters. Organic matters are easily biodegraded.	
		Demerits: High probability of clogging. High capital cost. Microbial community is affected by toxic metals. Not effective as activated sludge process in treating heavy metals.	*Demerits:* Longer retention time is needed. Toxic contaminants affect the micro-organism population.	
Stabilization ponds	The process involves: Constructed shallow basins, and Micro-organisms	*Merits:* Various types of heavy metal ions can be treated. Very cheap and easy to operate.	*Merits:* A low concentration of organic contaminants is removed effectively. 60% removal efficiency for trace organic pollutants such as caffeine and naproxen.	Polprasert and Charnpratheep (1989), Jasper et al. (2013), Gad and Abdalla (2017), Qaderi et al. (2019)
		Demerits: Slow and inefficient in removing heavy metal ions. Depends upon the interaction of micro-organisms, sunlight, and oxygen. Water source is one of the	*Demerits:* Time-consuming process. Wide range of organic contaminants cannot be treated.	

		limiting factors. Not suitable for all geographic areas only for arid and semi-arid zones.		
Biosorption	The process involves: Adsorption, and Precipitation	*Merits:* Simple in operation and high efficiency. Agricultural by-products can be used as adsorbents (rice husk, pecan shells, maize cob, rice straw, rice husk, coconut shell). Low cost of adsorbents and regeneration of bio sorbent. Usage of dead biomass reduces the need for nutrient supplication.	*Merits:* Simple and cheap compared to advanced treatment technologies. Ability to treat multicomponent organic contaminants. High selectivity of treatment. Less sludge is generated.	Chaukura et al. (2016), Ramírez Calderón et al. (2020)
		Demerits: Removal efficiency depends completely upon the cellulose, lignin, carbohydrate, and silica properties present in the adsorbents. A sudden change in pH and temperature affects the efficiency of the bio sorbent. Decrease in binding sites with usage.	*Demerits:* Disposal of biowaste (spent bio sorbent). Putrefaction of biomass under moist conditions. Limited regeneration capacity of bio sorbents.	
Biopolymers	Biopolymer adsorbent	*Merits:* Widely used in industries capable of reducing metal ions concentration to parts per billion. Low cost and higher removal	*Merits:* Biopolymers such as chitin and chitosan are renewable and biodegradable. Very effective in the treatment of	Vidal and Moraes (2019); Zia et al. (2020)

(continued)

Table 3.1 (continued)

Treatment technology	Treatment process	Heavy metal treatment	Organic contaminant treatment	References
		efficiency. Abundant availability and biodegradability of biopolymers.	phenolic compounds and pesticides (80–90% removal).	
		Demerits: Rapid degradability of biopolymers.	*Demerits:* The selection of biopolymers is based upon the pollutant load in the wastewater. Removal efficiency depends upon the ionic strength of the organic pollutant, pH, contact time, and temperature.	

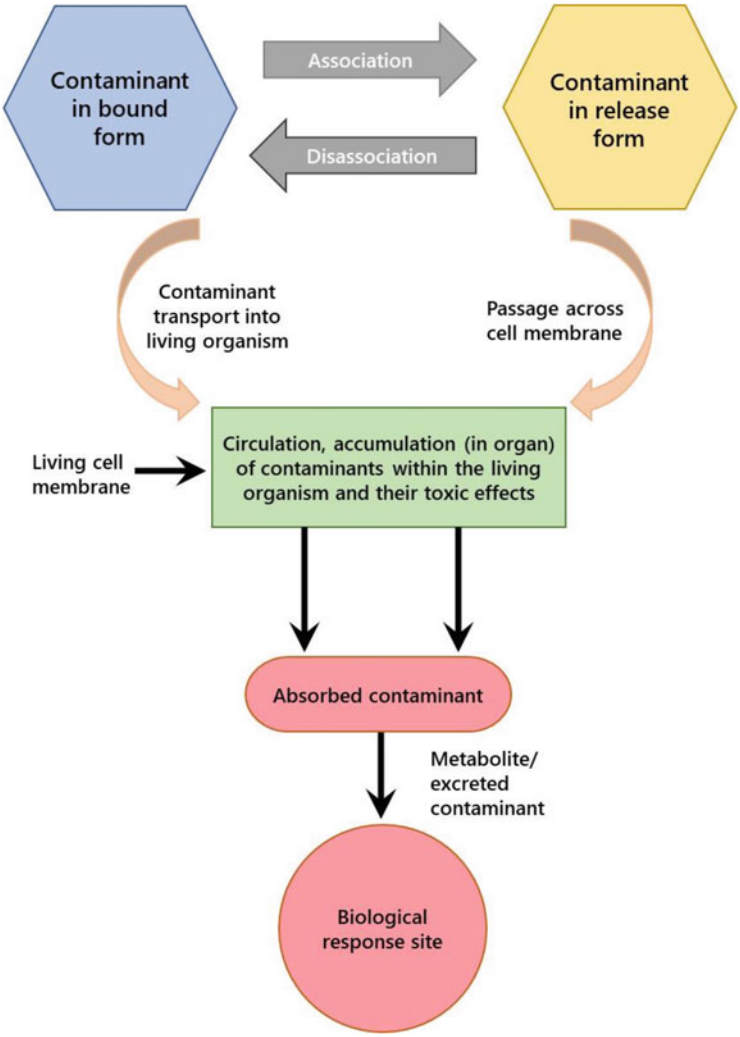

Fig. 3.2 Fate of the contaminants after their entry into the cell system

sorption capacity and availability in seas and oceans. Due to the presence of alkaline metal ions in the composition of algal cell walls the heavy metal ions in the water can be easily treated through a simple ion-exchange process. These sorbents have the metal-sequestering property that can be used to reduce the concentration of heavy metal ions in the solvent from parts per million (ppm) to parts per billion (ppb) level. Biosorption capacity determines the number of metal ions that microalgae can bind on the surface, and it is denoted by q_{max}. Brinza et al. (2007) reviewed the biosorption capacity of some marine microalgal species involving the commonly detected heavy metals found in the wastewater, as shown in Table 3.2.

Table 3.2 The maximum heavy metal biosorption capacity of some marine microalgal species (based on data from Brinza et al. 2007)

Marine microalga	Biosorption capacity q_{max} (mmol g^{-1})				
	Heavy metal				
	Pb	Cd	Ni	Zn	Cu
Chlorella sp.	0.46	0.44	0.31	0.18	0.55
Chlorococcum sp.	0.23	0.15	0.27	0.21	0.36
Cyclotella cryptica	0.42	–	0.14	0.1	0.33
Spirogyra sp.	0.49	0.27	0.12	0.23	0.53
Lyngbya taylorii	0.84	0.32	0.43	0.37	–
Microcystis aeruginosa	0.35	–	0.21	0.23	0.37
Scendesmus sp.	0.45	0.11	–	0.35	0.22

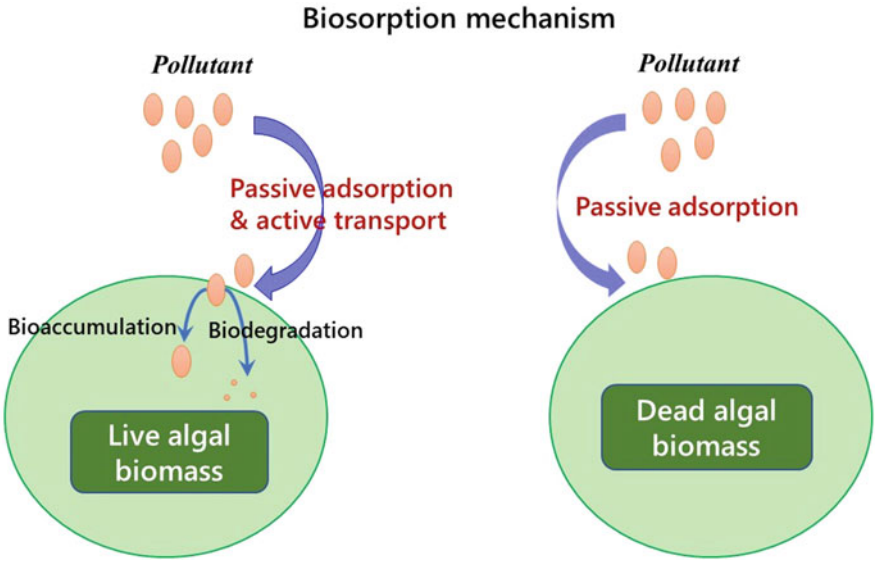

Fig. 3.3 Biosorption of pollutants in marine microalgae

Biosorption is an extracellular process that is carried out in the cell membrane in which the algal biomass binds the heavy metals in the cell wall. The algal cell wall is composed of polysaccharides that contain sulfate. Imidazole, phosphate, hydroxyl, amine, and amino functional groups act as a binding site for the heavy metals to be adsorbed. The biosorption mechanism (Fig. 3.3) can be divided into metabolism-dependent biosorption in which transportation of the pollutant across the cell membrane takes place, followed by intercellular accumulation or detoxification. Metabolism-independent mechanisms involve ion exchange, complexation, chelation, and precipitation process. For example, the cell wall of microalgae is composed of polysaccharides, lipids, and proteins that provide many functional groups capable of attracting both anionic and cationic heavy metal ions exchanging them with the

functional groups present in the cell wall. While investigating the mechanism for removing Cr^{3+}, Cd^{2+}, and Cu^{2+} by *Spirulina*, Chojnacka et al. (2005) found that hydroxyl, carboxyl, and phosphate functional groups were involved in the removal of the metal ions by the ionic-exchange process. Similarly, a microalgal strain, *Tetraselmis marina* AC16-MESO, could remove Cu (90%), Fe (100%), and Mn (50%) after 72-h incubation period, mostly by complexation of metal ions onto functional groups at the cell surface (Cameron et al. 2018). In fact, complexation mechanism is the result of electrostatic attraction between heavy metal ions and organic molecules present on the cell which act as ligands. The complex formation between the metal ion and ligand is due to the covalent bonds. The functional group (phosphonate, carboxyl, and amine) present in the cell wall of *Chlorella miniate* removed Cr^{3+} by the complexation process (Han et al. 2006). Organic acids such as citric, fumaric, lactic, oxalic and gluonic have been found to chelate metal ions resulting in the formation of metallo-organic complexes. Chelation is the advanced form of complexation mechanism in which the metal ion would bond with a ligand in many positions at the same time with higher stability. *Chlamydomonas reinhardtii* removed Hg^{2+} by direct chelation mechanism in which glutathione not only adsorbed the metal ion but also reduced the toxicity of the pollutant in water (Perales-Vela et al. 2006).

Two marine algae, *Chlorella* sp. and *Phormidium* sp., exposed to tannery wastewaters removed Cr concentration by 81 and 90%, respectively, at the end of 15 days incubation period as revealed by metabolic mechanism (Das et al. 2018). When the metal ion solubility decreases, the bioavailability is reduced, resulting in the mechanism of precipitation. Upon exposure to the heavy metal-polluted medium, the algal biomass favored precipitation that was based on pH of the medium. If pH of the medium increases, the active sites on the cell wall attract heavy metal ions. Cu, Ag, and Pb ions were removed by the alga, *Tertaselmis suecica*, by the mechanism of precipitation due to the presence of phosphates on the cellular surface (Ballan-Dufrançais et al. 1991). While growing *Chlorella* sp. in seawater-based medium, nearly 4% of Cd supplemented was found precipitated due to an increase in pH to 8 besides 67% accumulation and 25% adsorption of the metal (Matsunaga et al. 1999).

3.4.1.2 Factors Influencing Biosorption of Heavy Metals

Biotic Factors

Algal Species Marine microalgae can be classified into three broad categories based on the composition of pigment color in green algae (Chlorophyta), red algae (Rhodophyta), brown algae (Phaeophyta) (Davis et al. 2003). Romera et al. (2007) summarized the biosorption capacity of algae related to some heavy metals as indicated in Table 3.3. Brown algae have a higher sorption capacity than red and green algae due to their high alginate content and the presence of functional groups in the structure. But the use of brown and red marine algae has a major drawback due

Table 3.3 Average heavy metal sorption capacity (q_{max}), in mmol g^{-1}, of different algae (data based on Romera et al. 2007)

Phylum	Cadmium	Nickel	Zinc	Copper	Lead
Chlorophyta	0.60	0.51	0.37	0.50	0.80
Rhodophyta	0.20	0.27	–	–	0.65
Phaeophyta	0.90	0.84	0.67	1.01	1.23

to the presence of certain organic compounds such as alginate. Also, the pigments generate secondary pollutants and reduce the biosorption capacity. In green algae the secondary pollutant generation is insignificant but their biosorption capacity is lower as compared to brown and red algae. While studying the impact of biotic factors on Cu adsorption capacity in marine microalgae, Levy et al. (2007) observed that *Dunaliella tertiolecta* was least sensitive than *Minutocellus polymorphus* and was depended on uptake rates across cell membrane rather than the taxonomic status and cell size.

Concentration of Biomass In the biosorption process, the removal efficiency depends upon the biomass concentration because of the greater availability of binding sites on the cell surface. Increased biomass concentration enhances the removal percentage of heavy metals. An increase in biomass concentration of *Ulva fasciata* from 0.5 to 4 g L^{-1} resulted in the improvement of Pb removal efficiency in the range of 42–75%, while the removal efficiency of Cd increased from 43 to 73% with the increase in biomass concentration from 0.5 to 6 g L^{-1} (Nessim et al. 2011). Increased biomass concentration often reduces the biosorbent capacity of microalgae because of the reduction in intercellular distance and cell agglomeration. Kaparapu and Prasad (2018) observed higher biosorption of Cd(II) in *Nannochloropsis oculata* with biomass concentration of 7 g L^{-1} and a decrease in biosorption capacity with increased biomass concentration probably due to the partial biomass aggregation that results in surface area reduction.

Tolerance Algal species are known to grow, adapt, and tolerate hazardous environmental conditions. Heavy metal tolerance in algae depends upon the algal species. However, members of Chlorophyceae are generally known to tolerate Cu^{2+}, Zn^{2+}, and Cd^{2+}. Strains of *Chlorella* sp. isolated from mercury-contaminated sites tolerated higher Hg^{2+} concentration than the isolates from uncontaminated habitats (Gaur and Rai 2001). Pérez-Rama et al. (2010) observed 87% of Cd accumulation in a marine microalga, *T. sueccia*, and was related to phytochelatin synthesis. Folgar et al. (2009) reported that *Dunaliella salina* was tolerant to higher concentrations of Cd due to the intracellular metal-binding ligands.

Surface Area to Volume Ratio The ratio of surface area to volume in microalgae influences the sequestration of heavy metals in the solution. The take-up nutrients, in terms of per biomass, are faster in microalgae than macroalgae because of the size, growth, metabolism, and biochemical composition (Hein et al. 1995). Khoshmanesh et al. (1997) reported that the uptake of Cd was similar in an algal species having different sizes. The microalgal culture with a specific surface area of 2.20 m^2 mg^{-1}

cells showed higher uptake of Cd ions as compared to the culture with a specific surface area of 0.98 m^2 mg^{-1} cells.

Abiotic Factors

pH Biosorption of heavy metals in solution depends upon pH conditions due to the functional groups that dissociate at certain pH levels in the algal biomass. The maximum sorption of heavy metal ions by the marine microalgal biomass was obtained at a pH range between 4.0 and 6.0. The observed percentage removal efficiency for Cr, Cd, As, Pb, and Hg at pH 6.0 were 98.30, 92.50, 96, 92.20, and 80, respectively (Kumar et al. 2020; Leong and Chang 2020). When the pH value is lower than 6.0, the hydrogen ion concentration does not compete with the metal ions, and during adsorption of heavy metals no vacant active sites are created in the algal biomass (Gupta et al. 2011). If the pH value is greater than 6.0, the metal species are hydrolysed and are no longer available for the biosorption process (Romera et al. 2007). Kaparapu and Prasad (2018) reported that the biosorption of Cd(II) at pH >7 was reduced in a marine microalga, *Nannochloropsis* sp., and at pH 2–4 there was a competition between metal ions and metal-binding sites located on algal cell surface. The reduced Cd biosorption at higher pH was attributed to the maximum immobilization of positive charges. When the initial pH was maintained at 7.8, the cells of *T. suecica* were metabolically active and increased in cell number from 30 to 40 mg g^{-1} within 48 h, suggesting that the live cells are more suitable for biosorption than dead cells (Pérez-Rama et al. 2010).

Temperature Temperature plays a vital role in the biosorption process as it influences the process in both positive and negative ways depending upon the range in temperature (Khambhaty et al. 2009). The solubility of metal ions was found to be higher at elevated temperatures, but an increase in temperature decreases the biosorption capacity of the biomass. The maximum biosorption of Cu^{2+} ions attained at 37 °C was 90% in *Spirulina* species but the biosorption capacity was reduced to 82% at 60 °C and then gradually decreased with further increase in temperature (Al-Homaidan et al. 2014). The biosorption efficiency of *N. oculata* biomass increased with contact time up to 90 min and remained constant (Kaparapu and Prasad 2018).

Contact Time The efficiency of biosorption process depends upon the contact time between the algal biomass and the heavy metal ion. It was observed that the optimum contact time for the maximum adsorption of 80–90% of Cu, As, Cd, Cr, Pb, and Hg by various marine algal species was within 60–90 min (Al-Homaidan et al. 2014; Leong and Chang 2020). Initially, many active sites are available on the cell surface for the adsorption of heavy metals, and there will be a reduction in active sites with time, resulting in a gradual decline in the removal capacity of biomass that requires regeneration of algal biomass. The amount of biosorbed Cd in *D. salina* biomass was greater after 24-h contact time and was subsequently reduced due to the enhanced sorption onto the cellular surface (Folgar et al. 2009). It has been reported that

biosorption yield of Cd(II) decreased with increased temperature at an optimal contact time due to the following reasons: relative increase in leaching tendency of ions from solid phase to bulk phase, and weakness of active sites for biosorption in the sorbed phase (Kaparapu and Prasad 2018).

3.4.1.3 Desorption of Heavy Metals and Biomass Regeneration

Desorption of heavy metals is the process of recovering valuable metal ions from the algal biomass by adding eluent. The eluent restores the biosorbent to its original state for the reuse of biomass in the process. Mineral acids, complexing agents, and organic acids are used as the eluents as they are non-damaging to the sorbent, and they ensure the metal-binding capacity of microalgae. Desorption of Cr^{3+}, Cd^{2+}, and Cu^{2+} from biomass of *Spirulina* sp. by nitric acid resulted in 98% removal of the metal ions (Chojnacka et al. 2005). In fact, *Chlorella vulgaris* remains unaffected even after five cycles of biomass regeneration using 0.1 M EDTA as eluent to recover Cd metal ions, and the adsorption capacity loss was less the 5.8% (Kumar et al. 2018). Both HCl and EDTA are the most used eluents for desorbing algal biosorbents. However, HCl decreases biosorption capacity of algal biomass after every wash, and the use of EDTA is not eco-friendly as it dissolves alginate upon every use which can lead to secondary pollution. Therefore, it is essential to screen the desorbing agents for efficient metal ion recovery.

3.4.1.4 Heavy Metal Detoxification by Marine Microalgae

The ability of microalgae to adapt and survive in habitats contaminated with heavy metals and organic pollutants depends on genetic adaptation which enables them to develop defence mechanisms to resist and adapt the harsh environmental conditions (Nayaka et al. 2017). This mechanism of defence allows microalgae to develop some tolerance and resistance toward the pollutant that can detoxify the pollutants inside the cell. The defence mechanism involves the production of short-chained polypeptides such as phytochelatins (PCs) and metallothioneins (MTs) that are abundant in sulfhydryl and carboxyl groups and can bind to the pollutants (Cobbett and Goldsbrough 2002). The bound pollutant further moves in for internal detoxification process which involves conjugation of the pollutant with the polypeptides and further compartmentalization of the pollutants by transporting them into the vacuoles (Qin et al. 2006). Folgar et al. (2009) reported that metal complexing ligands in *D. salina* were rich in cystine although most of the known are GSH and PCs. They observed that levels of cystine synthesis led to maximum Cd accumulation intracellularly. In another study, *D. salina* was shown to be resistant to As which exhibited higher levels of lipid peroxidation with a differential expression of 65 proteins involved in energy metabolism, protein synthesis and folding, ROS scavenging, and amino acid synthesis (Ge et al. 2016). Wang et al. (2017) reported variation in thiols such as cysteine, glutathione, and PCs in *D. salina* exposed to arsenite and

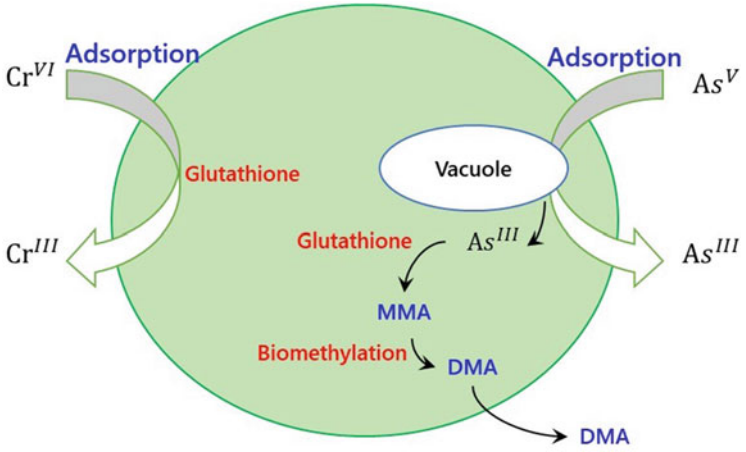

Fig. 3.4 Biotransformation mechanism for heavy metal detoxification in microalgae. MMA, Monomethylarsonic acid; DMA, Dimethylarsinic acid (Source: National Research Council 2003)

demonstrated that transformation of arsenite-induced several PCs initially and later decreased under various phosphate regimes. The synthesis of PCs varied with As (V) and As(III) which affected GSH levels, suggesting that the conversion of GSH to PCs is essential for arsenite mitigation (Wang et al. 2017). The biochemical mechanisms involved in heavy metal detoxification by microalgae (National Research Council 2003) are presented in Fig. 3.4. Sathasivam and Ki (2019) observed higher levels of phytoene synthase (PSY), phytoene desaturase (PDS), and β-lycopene cyclase (LCY-B) in *T. suecica* exposed to copper.

3.4.2 Removal of Organic Pollutants by Marine Microalgae

Human attempts to produce various organic compounds to protect many lives and support economic advantages significantly resulted in acute and chronic toxicity of some of these chemical substances making the biota deteriorate rapidly (Adeola 2004). Although these organic compounds are susceptible to degradation at a very slow process, they tend to persist in the environment or accumulate inside the biota (Subashchandrabose et al. 2013). Organic pollutants that are widely distributed in marine environments and prone to biodegradation by marine microalgae include phenolics, pesticides, persistent organic pollutants (POPs), and hydrocarbons (Dsikowitzky et al. 2011).

3.4.2.1 Pesticides

Pesticides including insecticides, fungicides, and herbicides are often detected in marine waters due to the urban or agriculture runoff causing serious threats to the marine biota. Atrazine sensitivity, in terms of 96-h growth inhibition, for the estuarine phytoplankter, *D. tertiolecta*, in nutrient-replete media was 159.16 μg L^{-1} and was influenced by the duration and nutrient-limited conditions (Flood et al. 2018). Chen and Jiang (2011) reported enhanced catalase activity in *D. salina* when exposed to trichlorfon and dimehypo at lower concentrations of 0.025 g L^{-1} and 0.0005 g L^{-1}, respectively. In a toxicity study involving treatment of *D. salina* with dimethylphenol and dinitroaniline, Zhu and Jiang (2009) observed that the EC_{50} values were significantly higher when exposed to a single pesticide compared to their combination. However, increased concentrations led to significant inhibition in the growth of the microalga that was attributed to the effect on osmosis of cell membrane allowing toxicants to react with internal parts and damage membrane lipids. Thakkar et al. (2013) exposed *D. tertiolecta* and a brown tide alga, *Aureococcus anophagefferens,* to various concentrations of metachlor and observed a significant increase in cell size with glutathione production as detoxification mechanism. Although 40–50% of sublethal concentration of tributylin (TBT) could be removed by *N. oculata* and *Dunaliella parava* during 2–6 days of incubation, the former microalga adsorbed most of the added anti-fouling agent while the latter degraded it to mono-butyltin and di-butyltin (Taha et al. 2009). DeLorenzo and Serrano (2003) determined the toxicity of atrazine, chlorpyrifos, and chlorothalonil individually and as mixtures on *D. tertiolecta* and observed that atrazine and chlorothalonil concentrations at 25 and 33 μg L^{-1} decreased growth rate, while chlorpyrifos was toxic only at >400 μg L^{-1}. In another study, the effect of herbicides such as diuron, irgarol, atrazine, and ametryn was tested toward *D. tertiolecta* in four different scenarios of increased temperature and salinity and reported that increasing temperature reduced growth but enhanced the contents of chlorophyll and starch and lipids (DeLorenzo et al. 2013).

3.4.2.2 Hydrocarbons

Water soluble fraction of crude oil containing mono- and diaromatic hydrocarbons affected *D. tertiolecta* within 24 h though photosynthesis impairment and cell division inhibition occurred. Despite the well-known tolerance of *Dunaliella* species, the exponential phase measured in terms of photosynthesis was reduced while lag phase showed growth inhibition, suggesting that duration of exposure influenced the overall growth (Siron et al. 1991). Fabregas et al. (1984) reported stimulation in the growth of *T. suecica* upon exposure to low hydrocarbon concentrations in crude oil whereas the dispersant did not exhibit any selective toxicity. Dunstan et al. (1975) observed that low concentration (10 mg L^{-1}) of oil had no effect on the growth of *D. tertiolecta*. Similarly, low concentration (0.05%) of light diesel and an oil

dispersant (0.005%), either alone or in combination stimulated the growth of *Chlorella salina* and impaired respiration (Chan and Chiu 1985). Photosynthesis in *D. tertiolecta* exposed to oil samples from tanker spill was significantly affected within 60 min, while survival of the cells was slightly affected (Carrera-Martinez et al. 2011). Jiang et al. (2002) exposed microalgal strains to four PAHs, viz., toluene, naphthalene, 2-methylnapthalene, and phenanthrene, and reported that *C. vulgaris* and *Platymonas subcordiformis* were least sensitive compared to other tested species.

Exposure of *Chlorella salina* to phenanthrene significantly increased the toxicity with an EC_{50} value that ranged from 1.893 to 0.23 mg L^{-1}, and a decrease in pH from 9 to 6 was also significantly toxic suggesting that the acidification of sweater greatly influenced the effect of organic compounds (Chen et al. 2018a). Bretherton et al. (2018) observed that marine alga, *D. tertiolecta*, was resistant to oil and dispersant and referred to it as "robust" because chlorophyll was not affected during lag phase and was followed by biomass accumulation. Moreover, short-term exposure of *D. tertiolecta* to petroleum and diesel oil impacted the growth and photosynthetic performance and reported to recover during long-term incubation (Romero-lopez et al. 2012). Recently, Salinas-Whittaker et al. (2020) observed that *D. tertiolecta* exposed to water-soluble fraction (WSF) from fuel oil/diesel mixture increased physiological and biochemical response in unsaturated acyl chain of fatty acid suggesting the uptake of hydrocarbons. Mohammady et al. (2005) exposed *Nannochloropsis salina* to various concentrations (0–100%) of diesel fuel oil aqueous extract and observed a decrease in cell bioavailability leading to cell division and enhanced membrane permeability. Both the limitation of carbon and hormesis phenomenon, as evaluated by stable isotope analysis, were prevalent in *Platymonas helgolandica* when it was treated with water accommodated fraction of fuel oil (Liu et al. 2020). Dissolved crude oil at lower concentration (20 mg L^{-1}) stimulated the growth of *Dicrateria* sp. but growth was inhibited with increased exposure time. However, consortia of marine microalgae involving *Dicrateria* sp. on biotreated seawater showed enhanced cell density that ranged from 4.0×10^5 to 1.7×10^6 cells mL^{-1}. Chao et al. (2012) reported that four fuel oils, viz., F120, F180, F380, and F20 were toxic to a marine alga, *Chlorella* sp., due to the concentration of several PAHs. Hing et al. (2011) demonstrated that *C. salina* was able to tolerate diesel concentrations at steady state and was only affected when the concentration exceeded 170 mg L^{-1}. Very recently, Marques et al. (2021) reported that *N. oculata* was able to grow in petroleum-contaminated water exhibiting a PAH removal efficiency of 94%. In particular, the percentage removal of several organic compounds such as naphthalene, benzopyrene, and acenaphthylene was 89–99% due to their intracellular biodegradation by oxidoreductase enzymes.

3.4.2.3 Other Organic Compounds

Phenol is an organic compound that results from the transformation of aromatic compounds via degradation, oxidation, and synthesis. Besides being enriched in coal

tar, phenol is also produced as a by-product from several industrial processes as well as during organic matter decomposition (Michalowicz and Duda 2007). Mofeed and Abdel-Aal (2015) found that exposure of *D. salina* to various concentrations (50–-200 μmol L^{-1}) of phenol significantly affected antioxidant enzyme activities. Phenol at a concentration of 72 mg L^{-1} led to programmed cell death in marine microalgae by inducing changes in ultrastructure with shrinkage of the nucleolus and vacuole enlargement (Duan et al. 2017). During treatment of real refinery wastewater containing phenol and its derivatives such as *o*-cresol and *p*-cresol, marine alga, *Nannochloropsis* sp., removed >80% of both the cresols as compared to freshwater *Chlorella* sp. (Surkatti and Al-Zuhair 2018). The biodegradation was reported to occur in two steps: split in methyl group resulting in its conversion to methanol and further breakdown of phenol produced as an intermediate (Papazi et al. 2012). Bisphenol A, with production estimates of approximately two million tons, is well distributed in the environment and known for its endocrine disruption potential (Burridge 2003). While reporting the first toxicity data of chlorophenols on *D. tertiolecta*, Ertürk and Saçan (2012) reported that toxicity of chlorophenols decreased between 48 and 96 h due to the increase in pH of the medium or acclimation response of the marine microalga to the toxicants. Regardless of the exposure time, the toxicity was greater with an increasing number of chlorine atoms, while *ortho*-substituted chlorophenol was lesser than *meta* and *para* congeners (Ertürk and Saçan 2012). POPs are widely distributed due to domestic and industrial activities that are reported to reach marine environments (ter Schure et al. 2004; Lema et al. 2007). Polybrominated diphenyl ethers (PBDEs), the flame retardants, enhanced oxidative stress in *D. salina* with increased activities of superoxide dismutase, catalase, and glutathione reductase and decreased glutathione peroxidase activity (Zhao et al. 2017). Similarly, exposure of *D. salina* to dibutyl phthalate at 100 mg L^{-1} decreased glutathione peroxidase and superoxide dismutase (Wei et al. 2021).

3.5 Conclusions

Besides highlighting the advantages of the use of marine microalgae for the removal of heavy metals and organic pollutants, we presented the inherent drawbacks of the conventional treatment processes. Marine microalgae respond to heavy metals in several ways such as biosorption and bioaccumulation; however, there is a very clear paucity of data on organic contaminant removal and the associated mechanisms. Furthermore, it is very clear that marine microalgae can offer sustainable approach in the treatment of heavy metals and organic pollutants for safer marine ecosystem and biomass production from microalgae after detoxification. Thus, this chapter presents the overall understanding of the potential of marine microalgae in the removal of heavy metals and organic pollutants.

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Chapter 4
Algal-Bacterial Consortiums, from Fundamental Interactions to Environmental Applications

Ignacio de Godos Crespo, Alfonso García Álvaro, César Ruíz Palomar, Félix Gaspar Gonzalo Ibrahim, and Raúl Muñoz Torre

Abstract Microalgae cultures offers solutions for pollution control at water reuse. The ability to nutrient and heavy metal up-take along with disinfection is a considerable more sustainable solution than conventional wastewater treatment processes based on mechanical aeration. Since microalgae have evolved together with bacteria in natural aquatic environments a whole range of interactions take place between both organisms. Ecology descriptions and population characterizations of the phycosphere are key elements for the design of microalgae-based units addressed to pollution control and for the understanding of the biochemical transformations that take place during cultivation. Negative interactions occur as consequence of resources competence or parasitism. Mutualism have the basis on substrate exchange and release of promotors. These and other interactions take place during the environmental applications such as wastewater treatment, gas treatment, or water reuse affecting the final performance.

Keywords Algal-bacterial processes · Photosynthetic biodegradation · Ecological interactions · Bioremediation · Biomass

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4.1 Microalgae Biomass Culture

Microalgae cultures are a highly productive pathway to convert solar energy and inorganic substrates (CO_2, water and ions) in high value biomass and oxygen. The process of algae production and processing has been done for millennia in different parts of the globe. For instance, the cyanobacteria *Nostoc* was produced in China and *Arthrospira* in Chad and Mexico (Hamed 2018). Presently, microalgae have several applications apart of human and animal nutrition: cosmetics and the production of high value molecules (essential fatty acids, pigments, stable isotope biochemicals) (Pulz and Gross 2004). Proteins, lipids, sugars, minerals and vitamins and other biocomponents are contained in microalgae biomass. For decades, commercialization of *Haematococcus* is taking place in Japan and Israel while *Chlorella* is produced in Germany and Portugal. The ability to accumulate lipids of algae biomass has been extensively explored as source of biodiesel for decades, although implementation at industrial scale has not been materialized (Grima et al. 2003). Carbon dioxide fixation with intense algae cultivation has been researched using flue gas and other polluted gas streams (de Godos et al. 2014a). A summary of the microalgae culture applications is depicted in Fig. 4.1.

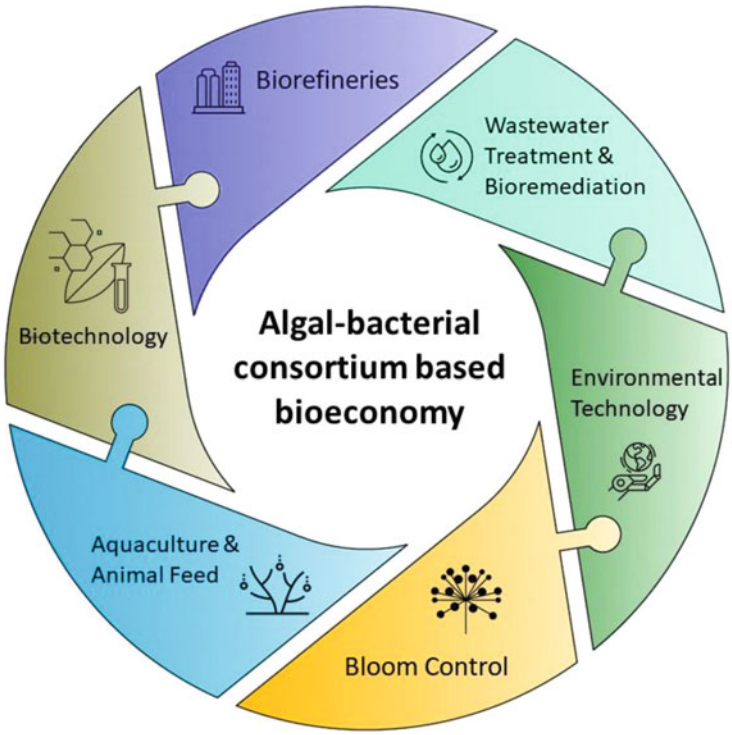

Fig. 4.1 Applications microalgae biomass

Microalgae production process can be summarized in chemical reaction as follows:

$$CO_2 + 0.148\, NO_3 + 0.014\, H_2SO_4 + 0.012\, H_2PO_4 + 0.751\, H_20 \rightarrow CH_{1.715}O_{0.427}N_{0.148}S_{0.014}P_{0.012} + 1.437\, O_2 \quad (4.1)$$

Therefore, carbon, nitrogen, and other elements are assimilated from culture broth and while oxygen is released along with the generation of biomass. This primary metabolism of microalgae is highly efficient in wastewater treatment applications (de Godos et al. 2017). The oxygen released by polycultures of different species of algae provides biological oxidation of organic matter while the high rates nitrogen and phosphorous assimilation results in high quality effluents. This ability to remove pollution from water has been proposed 60 years ago when wastewater treatment systems began to be implemented in western countries (Oswald and Ramani 1978). However, bacteria based systems consisted in aeration tanks such as activated sludge have been much more successful solution with millions of installations worldwide. Recent concern about sustainability have attracted interest toward systems based in carbon and nutrient recovery. In this sense, microalgae based systems offers assimilation of key elements (nitrogen, phosphorus) and neutral or even negative greenhouse gases emissions. Since the process is driven by sunlight, electricity consumption during the process is considerably lower than aeration systems. Beside this, the possible synergies between environmental applications and other algae uses, such energy production, carbon dioxide capture or bioproducts is attracting more interest around this technology (Murry et al. 2019).

Wrongly termed microalgae cultures are rather a co-culture of microalgae and bacteria in most of cases (except for axenic cultures of algae performed in conditions of microbiological isolation). The interactions between algae and bacteria could affect positively or negatively the biomass production and biochemical transformations that take place. A wider range of culture conditions can be found since microalgae grow in illuminated water bodies and wet surfaces all over the world. As consequence of the photosynthesis, microalgae create a particular environment with high concentrations of dissolved oxygen and elevated pH, particularly during central hours of the day when the light intensity reach its maximum (Arbib et al. 2017a). Organic materials are released as consequence of physiologic activity and algae cells decay. This particular environment presents appropriate conditions for the development of a wide range of organisms. In this sense, bacteria, which exhibits an extensive variety of metabolic routes from photo and chemoautotrophic to heterotrophic growth using different organic substrates, grow and interact with microalgae in natural and artificial environments (Rosenberg 2013). These interactions are still not well studied from a biotechnological point of view. At this point it must be notice that only a few thousand microalgae strains are kept in collections, only few hundred are researched for their possible application and just a handful are cultivated in industrial quantities, while more than 50.000 species are believed to exist. In case of environmental applications such as wastewater treatment, the knowledge about these

interactions is even more limited since cultures are not usually studied form the microbiological point of view (Ferrero et al. 2012). Microbiology characterizations and ecology studies have been barely explored. Recent progress in molecular identifications and the interdisciplinary approach of the research projects have shed light on the importance and impact of the symbiotic bacteria in the microalgae growth (Barreiro-Vescovo et al. 2021). This chapter reviews the ecological interactions between algae and bacteria and their importance in microalgae environmental applications.

4.2 Evolution of Microalgae and Bacteria

Half of the global net primary productivity is produced by microalgae in water bodies, with oceans as the major habitat of these organisms (Parlevliet and Moheimani 2014). These photosynthetic organisms beside with cyanobacteria grow in the illuminated region of the aquatic habitat and receive the name of phytoplankton. Bacterioplankton is the term applied to bacteria living in the same habitat. The metabolic activity of both communities impacts the global geocycles of oxygen, carbon, nitrogen, phosphorous, and other elements. The very intimate interactions between both groups of microorganisms have shaped the actual atmosphere and geosphere composition. Three thousand eight hundred million years ago, when the atmosphere was anoxic and mainly formed by N_2, CO_2, NH_3, and CH_4, simple living organisms have appeared (Sleep 2010). Prokaryotes existing in hydrothermal vents using chemoautotroph reactions are believed to be the first living organisms according to the reconstruction of the ancestral sequences. Photoautotrophic organisms arose 3500 million year ago, first without oxygen production and later with reactions that produce oxygen and final by-product. Photoxygenic organisms transformed the atmosphere increasing the levels of O_2 until the actual values of 20,9% (v/v) (Rasmussen et al. 2008). Cyanobacteria were responsible for this oxygenation environment and later to the formation of the photosynthetic eukaryotes, such microalgae and plants, through the process named primary endosymbiosis. This hypothesis established that heterotrophic eukaryotic organisms engulfed cyanobacterium and engaged it as an organelle. Consequently, eukaryote organisms acquired photosynthetic metabolism (Yoon et al. 2004). Although bacteria were proposed as the early host cell which received the cyanobacterium, most recent studies are suggesting that an archaea-type organism was responsible for this evolution event. Phylogenic studies indicate that three different lineages appear as a consequence of endosymbiosis: red algae, green algae, and glaucophytes (Ramanan et al. 2016). Subsequent endosymbiosis events, involving bacteria, archaea, protest, and cyanobacteria, resulted in a diversification of the phototrophic organism. More recent studies indicate that apart from the endosymbiotic events, horizontal gene transfer for each group of organism took and take place and facilitate the adaptation of an organism to the different environments (Schönknecht et al. 2013).

4.3 Ecological Interactions Between Organisms

Algae are the primary producers while bacteria present decomposing and producing activity depending on the substrate and light availability. Therefore most of the ecological interactions can be found between both organisms and most of them are present in the planktonic habitat (Ramanan et al. 2016). Occurrence of these interactions during microalgae culture in environmental applications affects positively when microalgae biomass production is enhanced resulting in high rates of pollutant up-take. However, negative interactions can negatively impact algae growth rate limiting the treatment capacity. At this point it must be stressed that microalgae growth rate (and biomass productivity) is mainly impacted by external factors such as temperature, light intensity, reactor configuration, and hydrodynamic factors (Fig. 4.2). Since bacteria are simultaneously co-cultured with the microalgae, and also affected by these external variables, microbial interactions can often go unnoticed or their effects may be attributed to the external variables. In this context, the improvement of culture techniques must take into consideration all the possible interactions: mutualism, parasitism, commensalism, and others.

Mutualisms interactions, where both parts take advantage of the coexistence, have been described between microalgae and bacteria in natural environments or lab-controlled experiments. These interactions can be facultative, obligate, or

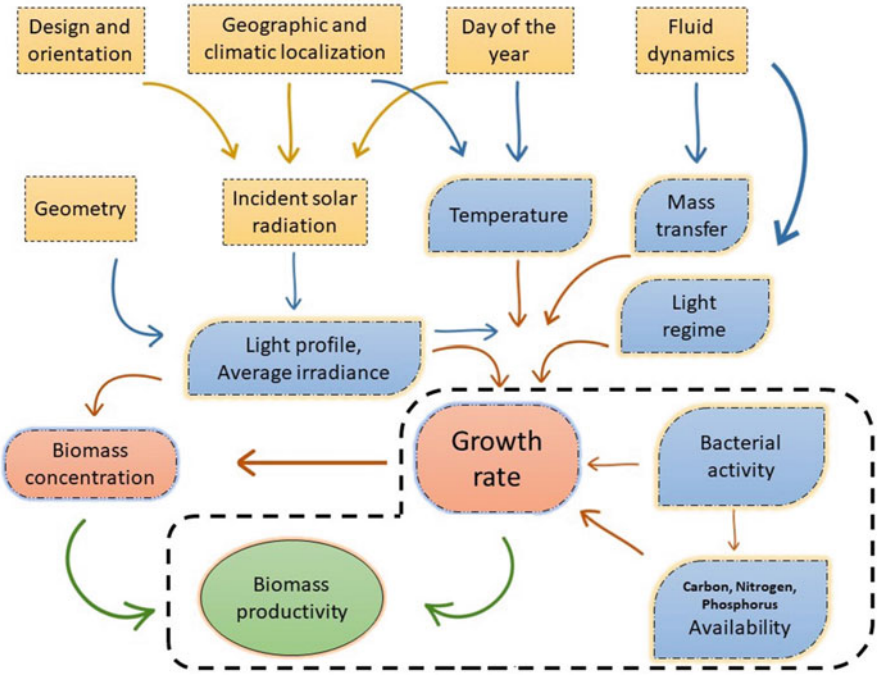

Fig. 4.2 Diagram of the main factors affecting microalgae cultures

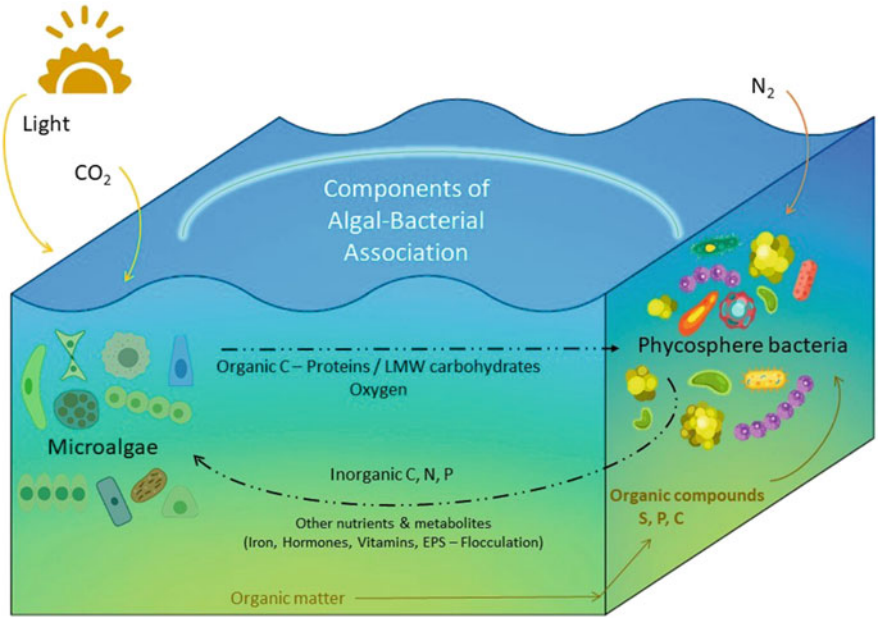

Fig. 4.3 Interactions between microalgae and bacteria

opportunistic. The main mutualistic interaction is the aforementioned gas exchange where microalgae can provide the oxygen that heterotrophic bacteria require for the breakdown of the organic materials, while bacteria concomitantly release the carbon dioxide, nitrogen, and phosphorus needed by microalgae during photosynthesis (Fig. 4.3). This is of important relevance in eutrophic environments with high concentrations of organic matter and it has been extensively used for the treatment of polluted water streams such as domestic, industrial, or livestock wastewaters. However, the mutualistic interactions are not limited to the inorganic components exchange. Exchange of organic compounds has been described in both directions. Vitamin B12 is produced by some bacteria and assimilated by microalgae. In some cases, microalgae even repress the expression of genes associated to Vitamin B12 synthesis when symbiotic bacteria provide this supplement (Croft et al. 2005). Mutualistic interactions have been described between green algae and bacteria that are also responsible for the very well-known growth promotion of plants. That is the case of *Rhyzombium*, *Mesorhizobium* and *Azospirillum, w*hich enhance microalgae growth and the other way around (Luz et al. 2004). Although some cyanobacteria are capable of atmospheric nitrogen fixation, green algae do not present this metabolic route. *Rhyzobium* provides organic nitrogen in oligotrophic ecosystems where this element is limiting the primary production. Besides this, this interaction is not limited to nitrogen since carbon can be also exchanged concomitantly. Organic compounds are supplied to the bacteria (*Rhyzobium* or others) while inorganic carbon as CO_2 or HCO_3^- is supplied in return. These bacteria promote algae growth

by the release of compounds such as indol acetic acid. It is worth noting that similar interactions have been also described between multicellular algae (Dao et al. 2018).

Negative interactions have also been described between microalgae and bacteria (parasitism interaction). Algicidal activity of some bacteria, for instance genera *Alteromonas*, *Pseudomonas*, *Bacillus*, *Dietzia*, *Janibacter*, has attracted the attention of its potential use as a controlling agent of algal or cyanobacteria blooms, also named Harmful algal blooms (Coyne et al. 2022; Croci et al. 2006). These natural phenomena occur across the world and considerably affect the aquaculture production systems placed in coastal waters. The occurrence of algal blooms has increased over the last decades likely due to two main factors: increasing pollution of water bodies and global warming. Species of dinoflagellates cause severe physiological and biochemical damage to fish. Algae toxins are accumulated by bivalves and human intoxication has been described after mollusk consumption. Worldwide, these algae toxins are responsible for more than 60,000 cases of intoxication per year, and the human mortality rate reaches a value of 1.5%.

Occurrence of bacteria with the capacity to kill microalgae during harmful blooms is being studied and proposed as a control system. Some bacteria species present physiological and biochemical mechanisms to attack microalgae. These mechanisms are similar to the existing in bacteria attacking higher plants. Chitinases, glucosidases, cellulases, and other enzymes found in the disruption of plant cell walls have been reported in phycosphere bacteria. It is worth mentioning that this parasitic interaction and similar enzymatic mechanism has been documented in other organisms such as marine fungi and mollusk (Nikolaeva et al. 1999). Algae cell decay results in the release of intracellular materials that are used as nutrients by bacteria and fungi. Consequently, competition for existing nutrients with microalgae results in reduced growth rates of phototrophs and outcompeting their existence in the environment. Enzymes present in these organisms (bacteria, mollusk, and fungi) are useful for many applications in algal and industrial biotechnology (Dahiya et al. 2006). Development of these industrial applications is based in the knowledge of the interactions and the study of the ecology of species succession, parasitism, and competition. Parasitic bacteria can easily be found attached to the algal cell wall or in the associated mats contributing degradation of the cell wall. Some marine bacteria living in association with microalgae are able to produce substances that block the sodium channels in algae resulting in algae decay. In the case of microalgae cultures used for environmental purposes, predatory events have been described on several occasions but the organisms involved are rather protozoa or animals than bacteria (Martínez et al. 2021).

Negative interactions between microorganisms are also based on competition. Substrate competition between microalgae and bacteria has been described in natural environments and bioreactors. While heterotrophic bacteria create a mutual interaction based on exchange of inorganic (CO_2, NH_4^+, PO_4^{3-}) and organic substrates, autotrophic bacteria could be characterized by competitive interactions. In this sense, ammonia-oxidizing bacteria use the same substrates than microalgae, inorganic carbon, and ammonia, for their primary metabolism. As consequence, the

coexistence of nitrifiers and microalgae is hampered (de Godos et al. 2014b). In case of natural environments, a wide range of conditions can be found and the presence of both groups of microorganisms has been reported. In the case of artificial environments, such as photobioreactors treating wastewater, conditions are created for maximizing microalgae productivity. For instance, the design of photobioreactors provides elevated irradiance inside the culture media, creating an environment similar to surface of water bodies. That is the case of high-rate algae ponds treating wastewater of different natures: domestic, agro-industrial, or livestock. Limitation of ammonia-oxidizing bacteria has been reported under these conditions (de Godos et al. 2010). It is important to highlight that this competitive phenomenon impacts the performance of wastewater treatment facilities positively or negatively depending on the bioprocess configuration. In this sense, most of the photobioreactors treating wastewater are designed for a maximum ammonia assimilation by microalgae (Arbib et al. 2017b). Although microalgae can use nitrate, nitrite, and ammonia as nitrogen sources, the last one is assimilated in first place since oxidized forms involved more energy investment by cells. Therefore, ammonia oxidation can result in the accumulation of NO_2^- and NO_3^- and consequently effluents will not achieve the discharge limits (De Godos et al. 2016). However, other reactor configurations based on nitrification and denitrification rely on high rates of nitrification (de Godos et al. 2014b). In that case, sufficient inorganic carbon must be present in the influent.

4.4 Environmental Applications of the Microalgae-Bacteria Consortia

Effluents resulting from various human activities such as farming, households and industrial production contains pollutants that can potentially endanger ecosystems and reduce possibilities of water reuse. If not treated properly this leads to water ecosystem destruction and involved human health risks (UN-Water 2021). Conventional water treatment are focused in organic matter, phosphorus and nitrogen removal (Jenkins 2014). Besides this, organic and inorganic substances including important amounts of elements such as sulfur, arsenic, chlorine, magnesium, calcium, and metals could be present in wastewater (Metcalf and Eddy 2003). Pathogenic organisms present in wastewater include a wide variety of microorganisms, including protozoa, viruses, and bacteria (Chambonniere et al. 2021), which are the origin of waterborne diseases like cholera, hepatitis, typhoid, tuberculosis, and dysentery. These diseases cause more than one million deaths yearly (UN-Water 2021). Both organic and inorganic components of polluted effluents, such as nitrogen and phosphorous, and carbonaceous compounds, are assimilated into microalgae biomass and thus, the concentrations of these compounds are reduced reaching the discharge limits (Barreiro-Vescovo et al. 2020). The main advantage of using microalgae-bacteria consortia instead of only bacteria systems is energy and

equipment saving involved in oxygen supply. Besides this, microalgae utilize any nitrogen source present in the wastewater: organic molecules, ammonium, nitrite, and nitrates (Taylor et al. 2012). Unlike, conventional water treatment facilities based on activated sludge, microalgae assimilate the nitrogen allowing for reutilization. Moreover, algae treatment units comprise primary and secondary treatment in one basin (normally a shallow pond mixed). Therefore, no transition between multiple operational conditions is required in order to remove inorganic nitrogen and phosphorous, simplifying the facilities and reducing the energy consumption of the treatment due to pumps and water elevation (de Godos et al. 2017).

Microalgae-based treatment is not limited to organic matter and nutrient removal. In fact, heavy metals and emerging contaminants like pharmaceutical and personal care products are efficiently removed during algae cultivation (López-Serna et al., 2022; de Godos et al. 2010). Heavy metals present a high binding affinity with the cell wall of microorganisms (including algae and bacteria) (Hwang et al., 2016). Metals that are usually found in domestic wastewater: include mercury, copper, zinc, nickel, arsenic, lead, cadmium, chromium, and manganese (Metcalf and Eddy 2003). Presence of these elements can be caused by oxidative damage in microorganisms cells by the stimulation of production of reactive oxygen species inside cells (Shahid et al., 2020). Experiments conducted by Shahid et al. (2020) demonstrate that microalgae exposed to high concentrations of copper presented higher levels of lipid peroxidation as a consequence of reactive oxygen species formation and a decrease in the level of pigments such as chlorophyll-a, chlorophyll-b, and carotenoids. Metal and heavy metal elimination mechanisms taking place during algae-bacteria cultures include: absorption, detoxification by the low-weight proteins called metallothioneins which induce complexation by chelates and polysaccharides and alkaline precipitation due to high pH values. Active and passive mechanisms have an effect on metal ions uptake by cells. The passive uptake or biosorption consists in the intermolecular binding that takes place when the cellular structure entraps heavy metal ions at binding sites (Kumar et al., 2015). Metals ions are bonded to sulfate, carboxyl, and phosphate groups existing in cell polysaccharides, On the other hand, during active uptake, heavy metals are included in metabolic cell pathways. While active sorption is limited to living cells, passive removal includes the elimination of non-living biomass. At this point it must be stressed that dead biomass (of algae and bacteria) can account for an important proportion of total biomass existing in wastewater treatment process). During the first stage, metal cations undergo adsorption onto the cell surface and the active transportation of metal species inside the cells takes place in the second phase. Transportation is a complex and irreversible process that requires energy consumption. Active process of metal uptake involves the following steps: transport across the cell membrane, formation of complexes, cation exchange, physical adsorption, and precipitation metabolism of the cell. During the cation exchange process, metal ions that are bounded to the cell surface can be replaced with another cation present in solution of similar charge. Complexation involved the formation of secondary compounds formed as a result of the linkage between organic molecules and metal ions acting as ligands. Electrostatic interactions and covalent bonds stabilize these

complexes. It is worth mentioning that the precipitation of metals occurs in both active (dependent of metabolism) and passive processes.

Disinfection or pathogen removal is a key process in water reutilization. In this sense, algae systems provide a tertiary treatment since pathogenic bacteria face harsh conditions inside cultures (Neil 2014). Microalgae cultivation is normally carried out in shallow ponds specifically designed to support a high algal productivity by optimizing sunlight exposition of the culture broth. However, elevated biomass concentration increases light attenuation and reduces the significance of the light decay and inactivation mechanisms of bacteria a virus. Nevertheless, the negative impact of light reduction may be counteracted by the intense mixing, provided by paddle wheels or similar, that enables intermittent light exposure of moving cells toward the surface (Chambonniere et al. 2021). In addition, the high microalgae photosynthesis rates increase the magnitude of diurnal pH peaks (values higher than 10) and dissolved oxygen concentration, which can easily double the saturation levels. These conditions enhance sunlight-mediated pathogen deaths. Presence of toxic algae metabolites has been suggested; however, limited information is available in this topic.

4.5 Communities in the Phycosphere

Associated bacteria and other organisms growing in environments dominated by microalgae is defined as phycosphere. In some cases, specific microbiota has been described in cultures treating effluents or cultures devoted to biomass production. Other studies have found wider distributed organisms community found in the association. At this point, it must be stressed that techniques employed in microbial population determination finally impacts the amount of organism detected. In this sense, first studies based on characterization by selective media cultivation introduce a significant bias in population determination (Ferrero et al. 2012). Molecular tools techniques developed at the end of the last century have emerged as the fundamental basis of microbial determination and therefore microbial ecology research. These techniques have provided new insights that have helped improve the understanding of biological processes and consequently the design and operation of bioreactors (Manoylov 2014). In contrast to the high biodiversity observed in bacterial communities associated with rhizospheres of higher plants, phycosphere is characterized by a significantly less diverse microbial community. Bacterial communities are dominated by the phyla: Bacteroidetes and Proteobacterial, classes alpha, beta, and epsilon. Archea species have not been detected in the reported studies (Sapp et al. 2008). Important metabolic features impacting the treatment performance have been detected: chemoautotrophs involved in organic matter oxidation, chemoautotrophs responsible for ammonia or nitrate oxidation, predatory organisms that could impact the concentration of pathogen. Recently the characterization followed by metatranscriptome analysis, study of the genes activated, have indicated activity in genes that are involved in the interaction of bacteria with the microalgae. These

genes are related to infection-related secretion pathways, biosynthesis of exoenzymes, modifying important metabolic pathways such as the synthesis of carbohydrates, lipids, and flagella.

4.6 Conclusions

Human activities deeply impact the Earth's equilibrium between organisms and geosphere, atmosphere and hydrosphere. Alteration of biogeocyles as a consequence of resource exploitation has been reported for many of the elements of the periodic table. In this scenario application of nature-based solutions with assimilatory capacity of key elements present in polluted effluents is becoming a necessity. Microalgae cultures present the ability to up-take some of the elements which present elevated concentrations in polluted wastewaters, such as nitrogen and phosphorous, but also more dangerous elements and compounds such as heavy metals and emerging pollutants. Since microalgae and bacteria have undergone a parallel evolution that has shaped the inferences between them, positive and negative, culture of microalgae with environmental purposes must be an account with a solid knowledge of the possible interactions and the consequences for the stability. In this sense population characterizations and ecological analysis should be considered in technical operations and scientific research.

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Chapter 5
Biodegradation of Environmental Pollutants by Marine Yeasts

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Abstract Organic contaminants are among the main pollutants of ecosystems because of their presence in domestic, agricultural, or industrial effluents. Indeed, many organic xenobiotics such as aromatic hydrocarbons, pesticides, synthetic dyes, etc., are not easily biodegradable in the environment and can therefore accumulate in ecosystems causing various toxic symptoms in exposed organisms, including humans. Yeast-assisted biological treatment has emerged as a promising new strategy for the biodegradation of such hazardous contaminants. Firstly, this chapter provides an overview of the applications of yeast in the biodegradation of organic contaminants. Subsequently, synthetic dyes were chosen as a model of organic pollutants to highlight the enzymes involved in their biodegradation process using various yeast strains. Indeed, the main oxidases involved are laccase, tyrosinase, lignin peroxidase, and manganese peroxidase. While the main reductases are Azoreductase, NADH-DCIP reductase, and malachite green reductase. The last section highlights the effects of physicochemical conditions on the effectiveness of mycoremediation.

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5.1 Introduction

Yeasts are a group of polyphyletic fungi composed of basidiomycetes and ascomycetes species that have the particularity of living in a single-celled state. The environmental role that yeasts play is identical to that of other fungi. In fact, they act as saprophytes that transform plant and animal organic matter into biomass and its by-products (Kutty and Philip 2008). These ecological properties have been exploited by man since antiquity and continue to be developed even in the present day in a range of applications such as the fermentation processes (Beer, Wine, Sake, Soy Sauce), in food and feed ingredients (enzymes, flavors, pigments, amino acids, organic acids), the biocatalysis (pharmaceuticals, chiral chemical intermediates, and biotransformation), the biocontrol (food and feed safety, crop protection, and probiotics), as well as in fundamental research in biology and biomedical (Molecular biology, pathway engineering, systems biology mechanisms, drug metabolism and resistance, etc.) (Johnson and Echavarri-erasun 2011).

In addition to these applications, the exploitation of this ecological principle of yeasts in the biodegradation of different organic materials has prompted scientists to evaluate their capacity in the biodegradation of different carbon-based xenobiotics such as organic solvents, humic substances, phenolic compounds, petroleum, surfactants, pesticides, pharmaceuticals, and dyes, etc. (Aksu 2005). Numerous studies have reported the ability of yeast to biodegrade a variety of hazardous contaminants, including aromatic hydrocarbons (Deeba et al. 2018), phenol compounds (Filipowicz et al. 2020), pesticides (Han et al. 2019; Isia et al. 2019), fungicides (Kucharska et al. 2020), insecticide (Chen et al. 2012) and herbicides (States and States 2011) and synthetic dyes (Danouche et al. 2021c, 2022).

In this chapter, we first reviewed existing studies dealing with the topic of biodegradation of organic pollutants using halotolerant yeast strains. Then, we approached a comprehensive analysis of the enzymatic process involved by various yeast strains in the biodegradation of synthetic dyes as a model of organic pollution. Finally, we discussed the involvement of physicochemical factors in enhancing the mycoremediation capacity of yeast species.

5.2 Biodegradation of Organic Pollutants by Yeast

Biodegradation process is defined as an energy-dependent mechanism by which organic substances are decomposed into simpler and smaller by-products through the action of various enzymes (Kaushik and Malik 2009). This bioprocess is called mineralization when the products of the biodegradation are more straightforward elements, such as H_2O, CO_2, NH_3, CH_4, H_2S, or PO_3. This same process is defined

as biotransformation when the organic compounds are not completely mineralized (Danouche et al. 2021b). Numerous halophilic microorganisms belonging to bacteria, fungi, and microalgae have shown the ability to decompose a wide variety of organic hazardous substances under high salt conditions (Castillo-carvajal et al. 2014). In recent years, the biotechnological application of fungi (mycoremediation) has become a model example for bioremoval of organic contaminants. It has been reported that various species of fungal could be used for the biodegradation of organic chemicals including aromatic and aliphatic hydrocarbons, industrial dyes, and other organic contaminants, released into the aquatic environment from various industrial and agricultural sectors (Aleu and Collado 2009; Sen et al. 2016). Despite the advantages of yeast strains over other species of the fungal kingdom, including rapid growth, high plasticity, and the ability to adapt to adverse growth conditions (Jafari et al. 2014; Sen et al. 2016), only a few studies have been conducted on the use of halotolerant yeast strains for the biodegradation of organic pollutants.

Aliphatic and Aromatic Hydrocarbons The yeast species described as hydrocarbon degraders are mostly from the genera of *Yarrowia, Candida, Pichia, Debaryomyces, Sporidiobolus, Metschnikowia, Lodderomyces, Rhodosporidium, Leucosporidium, Rhodotorula, Stephanoascus, Sporobolomyces, Trichosporon*, and *Cryptococcus* (Csutak et al. 2010; Kumari and Abraham 2011; Jain and Bajpai 2012; Gargouri et al. 2015; Deeba et al. 2018). Recently, Hashem et al. (2018) reported that other yeast strains of *Meyerozyma guilliermondii KKUY-0214, Yamadazyma mexicana KKUY-0160, R. taiwanensis KKUY-0162, P. kluyveri KKUY- 0163, R. ingeniosa KKUY-0170,* and *C. pseudointermedia KKUY-0192* were approved for their ability to degrade both aromatic and aliphatic hydrocarbon.

Phenolic Compounds Pollution with phenolic compounds can occur in the soil as well as in water bodies, due to their presence in discharges from industrial, agricultural, or domestic activities (Anku et al. 2017). Species of the genus *Candida* are documented as yeast strains with the highest capacity to decompose a diverse range of phenolic compounds. For example, a yeast strain of *C. rugopelliculosa* was reported to be able to decompose various phenolic compounds such as phenol, bisphenol A, nonylphenol, 4-methylphenol, 4-ethylphenol, 4-tert-butylphenol, 4-tert-OP, 4-tert-, and isooctane (Huang et al. 2017). The phenol was also reported to be degraded by other strains of *C. tropicalis* (Gong et al. 2021), *C. tropicalis* PHB5 (Basak et al. 2019), *C. subhashii* A011, *C. oregonensis* B021, *Schizoblastosporion starkeyi-henricii* L012 (Filipowicz et al. 2020), and *R. kratochvilovae* HIMPA1 (Patel et al. 2017).

Pesticides, Fungicides, Insecticide and Herbicides Various agricultural practices lead to the release of organic contaminants into the soil and surface or ground water. Selected yeast strains can be used as biodegradation agents for these xenobiotics. The pesticides like diazinon or pendimethalin can be biodegraded with *Saccharomyces cerevisiae* (Ehrampoush et al. 2017) or *Clavispora lusitaniae* (Han et al. 2019) respectively. The biodegradation of fungicides such as propiconazole was illustrated through the use of yeast strains of *Aureobasidium pullulans, Rhodotorula glutinis, and Cryptococcus* sp. (Kucharska et al. 2020). Regarding the insecticide,

Chen et al. (2012) showed that *C. pelliculosa* was efficient in the biodegradation of Bifenthrin. Also, *C. xestobii* was documented to have a high biodegradation capacity of Metolachlor and Alachlor herbicides (States and States 2011).

Synthetic Dyes Biodegradation of synthetic dyes by yeast has also been documented in the literature using different yeast strains (Danouche et al. 2021b). The most commonly used yeast for the degradation of synthetic dyes are strains belonging to the phylum Ascomycetes, such as *Saccharomyces*, *Candida,* and *Pichia* species. While only a few studies have involved basidiomycetous yeast strains, namely *Trichosporon* and *Pseudozyma* (Pajot et al. 2014). In the following section, we will focus on synthetic dyes as a model of organic pollutants because of their different chemical characteristics that make them resistant to biodegradation in natural ecosystems, as well as because of their toxicity toward exposed organisms, including humans (Danouche et al. 2021a). In the remainder of this chapter, an in-depth review of the enzymatic mechanisms involved in the biodegradation of these chemicals by yeast is presented in detail.

5.3 Yeast Enzymes Implications in the Biodegradation of Synthetic Dyes

Synthetic dyes can be degraded enzymatically by yeast cells in either the extracellular or in the intracellular compartment. The most studied enzymes for the biodegradation of dyes by yeast are the oxidases, which are the class of enzymes that use oxygen (O_2) as an electron acceptor to catalyze the redox reaction, generating H_2O or H_2O_2 as products. They contain a metal or a Flavin-type coenzyme on the active site (Phale et al. 2019). In addition, it has been shown that some reductases are also involved by some yeast strains. The reductases indicated for the biodegradation of synthetic dyes are Azoreductase, NADH-DCIP reductase, and Malachite green reductase (Danouche et al. 2021b).

Laccase (Lac: EC 1.10.3.2) Synthetic dyes biocatalysis with Lac can be achieved by direct biodegradation of the dye molecule by a nonspecific radical mechanism. This enzymatic pathway has the advantage of avoiding the formation of toxic by-products, such as aromatic amines, which are usually obtained as a result of specific cleavage of the azo bond of various dyes via reductases or chemical catalytic processes (Dave et al. 2015). Several studies have documented the involvement of Lac in the biodegradation of the synthetic dye by yeast strains of *Sterigmatomyces halophilus* SSA-1575 (Al-Tohamy et al. 2020), *Galactomyces geotrichum* GG (Guo et al. 2019), *Cyberlindnera fabianii* (Danouche et al. 2021c), *T. akiyoshidainum* HP2023 (Martorell et al. 2017a), *T. multisporum*, and *T. laibachii* (Pajot et al. 2007).

Tyrosinase (Tyr: E.C. 1.14.18.1): referred also to as monophenol monooxygenase or polyphenol oxidase. It is an oxidase with copper coenzyme, which can be employed for the detoxification of wastewater containing phenol or other organic pollutants (Kim and Uyama 2005). The catalytic reaction of the dyes

with Tyr occurs in two successive steps, a first catalyzing reaction is the o-hydroxylation of the monophenols to the corresponding catechols (monophenolase activity), next a second oxidation of monophenols to the corresponding o-quinones (diphenolase activity) (Duckworth and Coleman 1970). On their involvement in the biodegradation of synthetic dyes by yeast cells, it has been identified in only a few yeast species. (Danouche et al. 2021c) reported their involvement in the biodegradation of the azo dye Acid Red 14 with a yeast strain of *C. fabianii*, in addition to other yeast strains including *G. geotrichum* MTCC (Waghmode et al. 2012a, b), *S. cerevisiae* MTCC 463 (Jadhav et al. 2007), *C. krusei* strains (Charumathi and Das 2011), *Candida* sp. MM 4035, *T. porosum* MM 4037, *C. satwnus* MM 4034, *Barnettozyma californica* MM 4018 (Martorell et al. 2012).

Lignin Peroxidase (LiP: EC 1.11.1.14) LiP is an extracellular enzyme whose enzymatic substrate is nonspecific, this particularity confers it the capacity to degrade various aromatic phenolic and non-phenolic compounds (Chowdhary et al. 2018). For example, the biodegradation of sulfonated azo dye with LiP can be accomplished in two consecutive one-electron oxidations of the oxidized LiP forms by H_2O_2 in the phenolic ring, where the corresponding carbonium ion bearing the azo bond contributes to the formation of quinone and phenyldiazine by nucleophilic attack by H_2O. The phenyldiazine product is then oxidized by O_2 to a phenyl radical and the azo bond is removed as N_2, and then the phenyl radical extracts hydrogen from its surroundings to produce a stable aromatic compound (Chivukula et al. 1995). The catalytic activity of LiP was investigated during the biodegradation of various synthetic dyes by basidiomycota yeast strains of *T. laibachii and T. multisporum* (Pajot et al. 2007), as well as, by ascomycota yeast strains of *S. halophilus* SSA-1575 (Al-Tohamy et al. 2020), *G. geotrichum* (Guo et al. 2019), *P. occidentalis* (Song et al. 2018a), *S. cerevisiae* (Jadhav et al. 2007), *C. krusei* (Charumathi and Das 2011), *Diutina rugosa* (Bankole et al. 2017), and *C. samutprakarnensis* (Song et al. 2018b).

Manganese Peroxidase (EC 1.11.1.13) MnP is a substrate-specific oxidase that oxidizes Mn^{2+} to Mn^{3+} from the surface of the enzyme and subsequently oxidizes phenolic substrates such as model lignin compounds or other organic contaminants (Zhou et al. 2013). Yeast used MnP for the biodegradation of synthetic dyes as well, it was revealed in yeast strains of *C. fabianii* (Danouche et al. 2021c), *P. occidentalis* (Song et al. 2018a), *D. polymorphus, C. tropicalis* (Yang et al. 2008), *T. multisporum,* and *T. laibachii* (Pajot et al. 2007).

Azoreductase (AzoR: EC 1.7.1.6): are a class of enzymes that catalyze the reduction reaction such as the reduction of azo bonds (-N=N-) of azo dyes and nitroaromatic and azoic drugs (Misal and Gawai 2018). The AzoR can be classified according to their structures, or according to flavin dependence. The flavin-dependent class of AzoR can also be divided based on their coenzymes like NADH, NADPH (Saratale et al. 2011; Solís et al. 2012). The involvement of AzoR in dye biodegradation by yeast has been reported in some research employing

yeast strains of *Issatchenkia occidentalis* (Ramalho et al. 2004), *C. fabianii* (Danouche et al. 2021c), *C. krusei* (Charumathi and Das 2011), *S. cerevisiae* MTCC 463 (Jadhav et al. 2007), and *T. beigelii* NCIM-3326 (Saratale et al. 2009a).

NADH-Preferring 2,6-Dichloroindophenol Reductase ***(NADH-DCIP: EC 1.6.99.3)*** NADH-DCIP reductase is an oxidoreductase that reduces 2,6-dichloroindo-phenol (DCIP) with NADH as an electron donor (Nishiya and Yamamoto 2007). Some studies have shown an increase in the NADH-DCIP reductase activity during the biodegradation of various azo dyes with strains of *P. occidentalis* G_1 (Song et al. 2018a), *C. samutprakarnensis* (Song et al. 2018b), *D. rugosa* (Bankole et al. 2017), *P. kudriavzevii* CR-Y103 (Rosu et al. 2018), and *T. beigelii* NCIM-3326 (Saratale et al. 2009a).

Malachite Green Reductase NADH is used as an electron donor by this reductase to transform malachite green into leucomalachite green. It has been first reported by Jadhav and Govindwar (2006) that in *S. cerevisiae* MTCC 463 was used for the biodegradation of green malachite. Next, Jadhav et al. (2008b) demonstrated their implication in the biodegradation of methyl red by *G. geotrichum* MTCC 1360. Also, Charumathi and Das (2011) reported the increase of MG-reductase activity in *C. krusei* used for the biodegradation of Basic Violet 3, as well as, in *S. cerevisiae* used for the biodegradation of Malachite green (Biradar et al. 2016).

Regardless of the mechanisms involved, the performance of the microorganisms in the bioremediation of organic pollutants can be influenced by various environmental factors, including nutrients, pH, temperature, etc. It is therefore critical to emphasize the impact of these parameters on the yeast's ability to eliminate such contaminants.

5.4 Factors Controlling Mycoremediation Performance

Yeast cells are sensitive to the environmental conditions in which they grow. Determining the impact of these physiochemical factors on the efficiency of the removal of synthetic dyes is therefore crucial in order to make the mycoremediation process faster, more efficient, and more practical for large-scale applications. There are two ways to perform this optimization, either using a single-factor optimization approach or based on statistical methods of optimization by the design of the experiment (Gönen and Aksu 2009; Mahmoud 2016).

Carbon and Nitrogen Sources The effect of carbon and nitrogen sources on the bioaccumulation or the biodegradation capacity of dye by yeast strains has been the subject of several studies. It has been reported that at constant sucrose content, the concentrations of both Remazol Black B and Remazol Blue dyes inhibited the growth of *C. tropicalis*, with constant dye concentration, the growth efficiency and the bioaccumulation capacity increased with sucrose concentration up to 15 g L^{-1} (Aksu and Dönmez 2005). This combined effect was also analyzed using

a statistical approach of response surface methodology. Okur et al. (2014) found that the optimal values for dye uptake by *C. tropicalis* correspond to sugar concentration of 5.1 g L^{-1} and a dye concentration of 499 mg L^{-1} of initial dye concentration. Also, Gönen and Aksu (2009) found that the optimum combination predicted by response surface methodology (RSM) confirmed that *C. utilis* was able to bioaccumulate Remazol turquoise blue-G with a maximum uptake yield of 82.0% in 15 g L^{-1} sucrose and 50 mg L^{-1} dye concentration. Additionally, (Das et al. 2010) confirmed that *P. fermentans* MTCC 189 was able to accumulate Basic violet 3 up to 69.8% in 10 mg L^{-1} of dye-containing medium and 24 g L^{-1} sugar extracted from sugarcane bagasse through RSM analysis. Concerning the carbon sources effect on the biodegradation efficiency of dye by yeast cells, it has been reported that the addition of carbon sources, especially glucose at a certain level, stimulates the biodegradation of dyes (Chang et al. 2000; Waghmode et al. 2011). Indeed, glucose is an essential element in several mechanisms, it provides an energy source for yeast growth, it regenerates the redox mediators NADH and FADH, and also it serves as a substrate for the production of H_2O_2, which acts in turn as a co-substrate for MnP and LiP (Swamy and Ramsay 1999; Jafari et al. 2014). In the same way, the supplementation of the medium with nitrogen sources such as peptone, yeast extract, urea, or others favors the regeneration of NADH which is used as an electron donor for the reduction of azo dyes by the different enzymes (Bras et al. 2001).

Dye Concentration The initial dye concentration can significantly influence their removal efficiency. A higher dye concentration gradually decreases the percentage of their decolorization. This can be attributed to the toxicity of the dyes toward the yeast cells, or to a lower biomass production making the decolorization operation inefficient (Saratale et al. 2011). It has been reported by Das et al. (2010) that the bioaccumulation of Acid Blue 93, Direct Red 28, and Basic Violet 3 by *P. fermentans* MTCC 189 decreased as the initial concentration of these dyes increased from 10 to 30 mg L^{-1}. Likewise, Dönmez (2002) and Aksu (2003) described that the increase in the initial dye concentration inhibited the growth and caused a long lag period of *S. cerevisiae and C. tropicalis*. Other studies indicate that the presence of dyes at high concentrations may inhibit azoreductase activity during enzymatic biodegradation of the synthetic dye, due to the binding of dye molecules to the active site of enzymes (Jadhav et al. 2008a; Saratale et al. 2009a).

Temperature Temperature is one of the crucial factors that influence the metabolic pathways, the enzymatic activity, and the physicochemical interaction of dye molecules with the cell wall. According to the available literature, no study has been devoted to the question of the effect of temperature on the bioaccumulation of dyes using yeast cells. Therefore, it is very important to consider this factor as a research question for future studies. Regarding the effect of temperature on the ability of yeast to biodegrade dyes, many studies have been conducted on the activation energy of the involved enzymes (Chequer et al. 2013; Miranda et al. 2013). The performance of decolorization increased with increasing temperature to the optimum temperature, then a reduction in activity occurs at higher temperatures (Tan et al. 2013, 2014, 2016). The decrease in the ability of yeast strains to remove the dye molecules can be

attributed to the denaturation of the enzymes involved or to the resulting loss of cell viability (Saratale et al. 2009b).

pH pH of solutions is one of the most influential factors on the ability of yeast cells to bioremediate various organic pollutants, including synthetic dyes, it can modify the physicochemical properties of the dye molecules, as well as the physicochemical properties of the yeast surface where the cell-dye molecule interaction initially occurs (Fu and Viraraghavan 2002). At low pH, the surface of yeast cells becomes protonated with a positive charge, which promotes the binding of anionic dyes. On the other hand, at higher pH values, the cell surface acquires a negative charge, resulting in the electrostatic attraction of cationic dyes (Charumathi and Das 2012). The pH may have an inhibitory effect on the transport of dye molecules across the cell membrane, which is considered the first stage of the intracellular biodegradation or bioaccumulation process (Khan et al. 2013). In addition, the initial pH can affect the physiology of the yeast cells and the enzymatic activity. Most yeast strains show better decolorization efficiency under neutral or acidic conditions. The optimal value for the bioaccumulation of Remazol Blue, Reactive Red, and Reactive Black by the yeast strain *C. tropicalis* (Dönmez and Aksu 2002) as well as for Remazol Red RB, Remazol Black B, and Remazol Blue by *S. Cerevisiae* (Aksu 2003) was observed was 3.0. Likewise, Das et al. (2010) investigated that the maximum bioaccumulation rate of Basic Violet3, Direct Red 28, and Acid Blue 93 by growing cells of *P. fermentans* MTCC 189 was at pH 5.0.

Shaking Agitation allows a uniform distribution of oxygen and nutrients in the medium. It also facilitates the exchange of gases produced during the fermentation process of dyes by yeast cells (Yu and Wen 2005; Pajot et al. 2007; Yang et al. 2008; Martorell et al. 2017b). Differing opinions have been expressed regarding the effect of oxygen on the decolorization process, with some researchers considering that during the dye reduction reaction by the reductase, oxygen behaves as a stronger electron acceptor than the dye molecule, thus preventing the azo dye reduction reaction (Kalyani et al. 2008). On the other hand, other research indicates that oxidizing enzymes, such as laccases, require oxygen to oxidize aromatic molecules, including dyes (Thurston 1994). Therefore, it is necessary to optimize the level of agitation that regulates the concentration of dissolved oxygen in the medium throughout the yeast decolorization process to ensure an effective treatment.

5.5 Conclusions

On the basis of the research discussed in this chapter, it is appropriate to consider mycoremediation as a cost-effective, eco-friendly, and efficient approach for the biodegradation of organic contaminants. However, an efficient mycoremediation process requires the optimization of physicochemical conditions, notably the initial concentration of the pollutant, the supply of nitrogen or carbon sources, as well as growth conditions such as pH, temperature, and agitation. On the other hand, the

effectiveness of this kind of bio-processes should not be limited to emphasizing the degradation of the considered pollutant molecule; instead, it requires characterization and evaluation of the toxicity of the obtained by-products, since they can have more harmful effects than the original molecule. Lastly, we strongly recommend the application of this emerging biotechnology in wastewater treatment at pilot or large scale in order to prove its potential application.

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Chapter 6
Sustainable Direct Digital Manufacturing Using Marine Resources

Luís André R. Marques, Sara Biscaia, Anabela Massano, Rafael M. Tavares, and Artur Mateus

Abstract For over 60 years, global apparent food fish consumption has increased considerably generating a large volume of preconsumer and postconsumer residues, which consists mainly of shells and bones. Usually, fish by-products are used directly as feed in aquaculture or fertilizers. However, other applications have been gathering attention recently, such as the production of biofuel and biogas, pharmaceuticals, cosmetics and many others. Nowadays, this biowaste represents a promising source of biomaterials from marine discarded materials and this approach is very attractive due to their abundant availability, accessibility and low-cost source.

Direct Digital Manufacturing (DDM) is currently a main subject in the manufacturing industry placing many advantages, such as a high degree of geometric freedom for design and reduction of material waste, when compared to conventional manufacturing techniques. Therefore, DDM is seen as an energy-efficient technology. The use of biobased and biodegradable polymers in DDM technologies has been an emerging field in recent years, mainly in biomedical areas, due to the increasing interest in sustainable products and solutions, instead of using limited resources such as fuel-based polymers.

Thus, the valorization of bioactive compounds from fish by-products is of great interest due to their high market value, also can satisfy the demand of low-cost biomaterials, reduce marine pollution and can be used as alternative materials for DDM technologies. The reuse of these waste resources to produce biomaterials through sustainable processes can be a way to create new companies and job opportunities.

Keywords Fish waste · Biomaterials · Direct Digital Manufacturing · Fused Filament Fabrication · Direct-Ink Writing · Inkjet 3D Printing · Stereolithography

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Abbreviations

3D	Three-Dimensional
ABS	Acrylic Butadiene Styrene
AM	Additive Manufacturing
ASTM	American Society for Testing and Materials
CAD	Computer-Aided Design
CIJ	Continuous Inkjet
DDM	Direct Digital Manufacturing
DIW	Direct-Ink Writing
DOD	Drop on Demand
FDM	Fused Deposition Modelling
FFF	Fused Filament Fabrication
GelMA	Gelatin-methacryloyl
HA	Hydroxyapatite
ICT	Information and Communication Technology
ISO	International Standard Organisation
PEGDA	Polyethylene Glycol Diacrylate
PET	Polyethylene Terephthalate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PLA	Polylactic Acid
SL	Stereolithography
SLA	Stereolithography Apparatus
β-TCP	Tricalcium phosphate

6.1 The Use of Fish Waste as 3D Printed Biomaterials

Annually, millions of fish waste are produced worldwide, despite the percentage of food losses and waste varies mostly according to culture (Yan and Chen 2015; de la Caba et al. 2019). The European Market Observatory for Fisheries and Aquaculture Products (EUMOFA) reported that in 2018 the total world catches and aquaculture production was of 97 million tonnes and 115 million tonnes, respectively (Fig. 6.1).

On the other hand, for over 60 years, global apparent food fish consumption has increased considerably. In the period 1961–2017, the average annual growth rate of total food fish consumption was 3.1%. Annual per capita fish consumption varies from 9 kg to more than 24 kg due to the influence of cultural, economic and geographical factors, including the proximity and access to fish landings and aquaculture facilities (Fig. 6.2) (FAO 2020).

The consumption of fish generates a large volume of preconsumer and postconsumer residue, the largest proportion of which consists of shells and bones, which could correspond to 50–70% of the product content (de la Caba et al. 2019).

Continent	Volume (1.000 tonnes)	% catches	% aquaculture
Asia	155.005	32%	68%
Americas	24.462	84%	16%
Europe	18.183	83%	17%
Africa	12.408	81%	19%
Oceania	1.634	87%	13%
Total	**211.692**	**46%**	**54%**

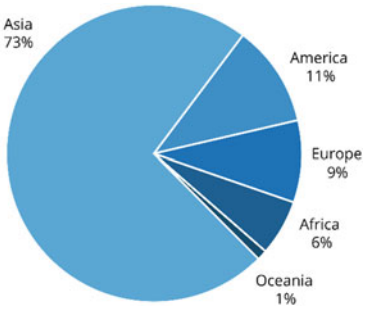

Fig. 6.1 World catches and aquaculture production by continent in 2018 (adapted from European Market Observatory for Fisheries and Aquaculture Products (EUMOFA) (2020))

Region/economic grouping	Total food fish consumption (million tonnes live weight equivalent)	Per capita food fish consumption (kg/year)
World	152.9	20.3
World (excluding China)	97.7	16.0
Africa	12.4	9.9
North America	8.1	22.4
Latin America and the Caribbean	6.7	10.5
Asia	108.7	24.1
Europe	16.1	21.6
Oceania	1.0	24.2
Developed countries	31.0	24.4
Least developed countries	12.4	12.6
Other developing countries	109.5	20.7
Low-income food-deficit countries	23.3	9.3

Fig. 6.2 Total and per capita apparent fish consumption, 2017 (adapted from FAO (2020))

Considering the most consumed species in the world in 2018, around 34% of them present external shells (i.e. squids, clams, mussels, shrimps) and the remaining have bones in their composition (i.e. salmon, tunas, colds) (Fig. 6.3) (FAO 2020; Nisticò 2017).

Global food loss and waste is a serious issue concerning the focus of Sustainable development goal target 12.3, which aims at halving wastage by 2030 (FAO 2020). The current management of fish waste is the disposal in public waters or onto landfills. These can cause noxious odours as a consequence of the decay of remaining organic matter or of the microbial decomposition of salts into gases, and consequently, this causes the appearance of animals, such as insects, that feed of organic matter (Chierighini et al. 2011; Hamester et al. 2012). Regarding bivalve

Fig. 6.3 Share of main groups of species in fish trade in terms of value, 2018 (adapted from FAO (2020))

shells waste, the major problem of discharge into the sea is the residue accumulation in the ground, which over the years causes the increase of sediments, a harmful factor for cultivation promoting the decrease in oxygen in the water, hindering the growth of microalgae, which are mainly responsible for the nutrition of some bivalves, which hinders their growth in their natural habitat (Chierighini et al. 2011; Shavandi et al. 2018).

Another scenario for fish waste, is their use as feed or raw material since the correct waste management must involve trying to avoid or reduce its production and minimize the risk to human health and the environment (Yao et al. 2014). The reduction of waste production can be done through the reuse of products and, when this is not possible, recycling should always be used for another type of recovery, using only waste disposal as the last alternative (Monteiro 2014). Typically, fish by-products are used directly as feed for aquaculture, silage or fertilizers (FAO 2020;

Nisticò 2017). However, in the last decades, other applications have been gaining attention, since presently can be treated with more efficiency as a result of improved processing technologies (FAO 2020). Fish by-products have also been used in the production of biofuel and biogas, dietetic products, pharmaceuticals, natural pigments, cosmetics, alternatives to plastic, and constituents in other industrial processes. Despite this, more research and innovation are still needed in this area, this biowaste needs to be valued through the development of economically sustainable routes in order to eliminate the related environmental problems (Nisticò 2017; Yao et al. 2014).

Fish bones are an excellent source of collagen, gelatine and calcium and other minerals such as phosphorus (Jafari et al. 2020; Maschmeyer et al. 2020; Terzioğlu et al. 2018). Calcium phosphates present in fish bone have been the focus of many researchers due to its potential to take the advantage of producing a high-quality bioengineering material for several applications such as drug delivery, tissue engineering and environmental remediation (FAO 2020; Terzioğlu et al. 2018). Crustaceans and bivalves also offer several applications for their by-products, which not only benefit the economy of industrial processors through the recovery of bioactive molecules from the waste but also help to reduce environmental pollution caused by the slow natural degradation rate of their shells (FAO 2020; Nirmal et al. 2020). Chitin, a polysaccharide extracted from crustacean shell waste, has gained much attention due to their non-toxicity and variety of bioactivities (Chierighini et al. 2011; Shavandi et al. 2018; Borić et al. 2020). Its derivative, chitosan, has been successfully 3D printed using extrusion-based processes for bone tissue engineering (Sanchez-Rexach et al. 2020). The shells of bivalves, such as mussels and oysters, can be turned into calcium carbonate or calcium oxide, two highly versatile chemical compounds with wide biomedical applications (FAO 2020; Ismail et al. 2021).

Altogether, represent promising sources of biomaterials from marine discarded materials and this approach is very attractive due to their abundant availability, less processing time, low-cost source, easy accessibility and simple way of handling (Govindharaj et al. 2019). In addition, the reuse of these waste resources to produce biomaterials through sustainable processes can be a way to create new companies and job opportunities. So, the valorisation of bioactive compounds from these by-products is of great interest for their high market value, beyond can satisfy the demand of low-cost biomaterials for commercialization, can promote cleaner production, reduce marine pollution and can be used as alternative materials for DDM technologies (Govindharaj et al. 2019; Caruso et al. 2020).

6.1.1 Biomaterials from Fish Waste

6.1.1.1 Calcium Carbonate

Calcium carbonate ($CaCO_3$) is an exceptional material, especially in industrial and biomedical fields. Compared with other inorganic materials, $CaCO_3$ has shown

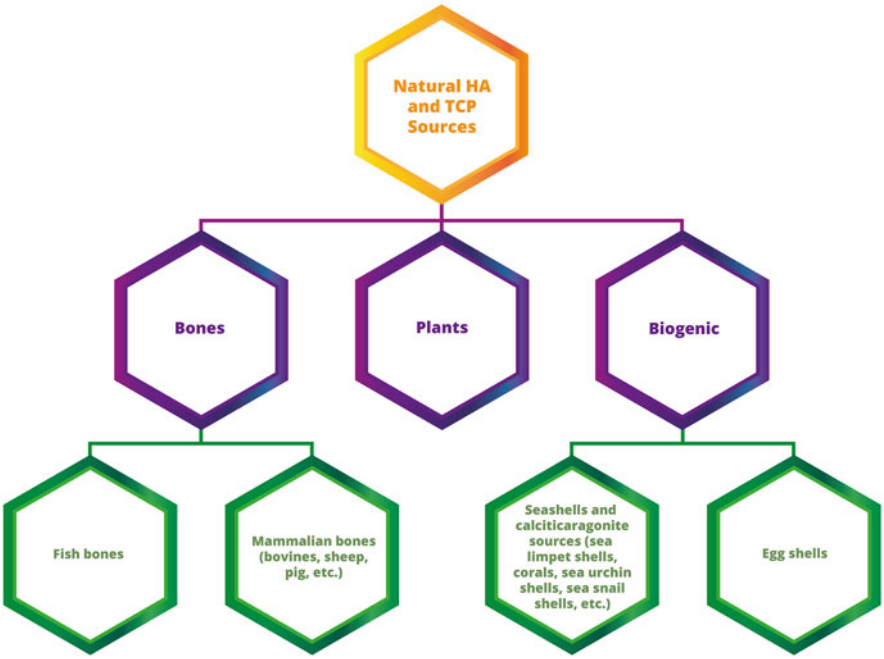

Fig. 6.4 Natural sources of HA and TCP (adapted from Terzioğlu et al. (2018))

promising potential for biomedical applications because of its biocompatibility, biodegradability and high mechanical strength (Barhoum et al. 2015a; Sambudi et al. 2015). Furthermore, $CaCO_3$ particles can be used as filler materials (Abdolmohammadi et al. 2012; Barhoum et al. 2015b), coatings (Barhoum et al. 2014), pharmaceuticals (Saveleva et al. 2018; Mohd Abd Ghafar et al. 2017) and bone tissue engineering (Luo et al. 2018; Didekhani et al. 2020). $CaCO_3$ can have mainly four polymorphs: calcite, vaterite, aragonite and amorphous $CaCO_3$ and can be extracted by mining or chemically synthesized in the laboratory (Mohd Abd Ghafar et al. 2017; Hoque et al. 2013). Calcite is the thermodynamically stable phase of $CaCO_3$ and vaterite and aragonite are the metastable phases of $CaCO_3$ (Ma et al. 2016). The amorphous phase is unstable, it rapidly crystallizes near ambient temperature and pressure within a few seconds to a few minutes and acts as a seed for crystal growth of the other polymorphs (Mohd Abd Ghafar et al. 2017; Boyjoo et al. 2014). Calcite, vaterite and aragonite have typical morphologies/shapes respectively rhombohedral, spherical and needle-like (Boyjoo et al. 2014).

Fish bones are rich in $CaCO_3$, making it an alternative source of low-cost $CaCO_3$ for the synthesis of calcium phosphate bioceramic used in bone regeneration (Corrêa and Holanda 2019). Various fish bones have been used as a starting material to produce HA and β-TCP as listed in Fig. 6.4.

Regarding a traditional method to prepare calcium phosphates from fish bones, the thermal calcination method concerns some aspects such as bones source,

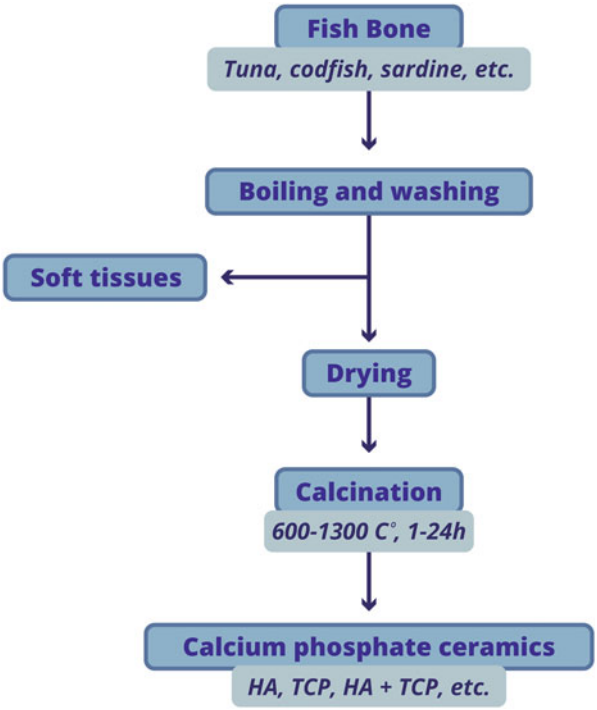

Fig. 6.5 Calcium phosphate extraction from fish bones (adapted from Terzioğlu et al. (2018))

extraction method, calcination temperature and time that can influence the final properties of calcium phosphates, particularly, the morphology, purity degree, particle size and distribution, surface properties and Ca:P ratio (Terzioğlu et al. 2018; Naga et al. 2015; Piccirillo et al. 2013) (Fig. 6.5). Alternative methods have been studied to produce calcium phosphates, such as alkaline hydrolysis (Venkatesan et al. 2015), hydrothermal (Goto and Sasaki 2016) and laser ablation (Terzioğlu et al. 2018; Boutinguiza et al. 2007).

Hydroxyapatite (HA) has been prepared from fishbones using an alkaline hydrolysis method. This biomaterial, shows excellent results in bone remineralization processes, with regard to osteoblast cell proliferation (Shavandi et al. 2018). Côrrea and Holanda (2019) synthetized HA from fish bone waste by wet precipitation method and demonstrate that this approach has great potential for producing nanopowder of biphasic calcium phosphate, particularly for the regeneration of damaged bone tissue in orthopaedics. Another study by Naga et al. (Naga et al. 2015) aiming to obtain hydroxyapatite powder from fish bone skeletons by thermal treatment and preparation of highly porous 3D ceramic scaffolds by polymeric sponge method revealed the mechanical properties of scaffolds very near to the strength of trabecular bone.

Regarding the extraction of calcium carbonate from shellfish, studies revealed the chemical composition of various seashells in 92–99% of this mineral with about 5% of organic matter. The dry shell weight of oysters, mussels, molluscs, cockles and

Table 6.1 Chemical compositions of shells (adapted from Yao et al. (2014))

Oxides	Oyster	Hard-shelled mussel	Clam	Short-necked clam
CaO	51.06	53.70	53.92	53.58
MgO	0.51	0.33	0.22	0.20
SiO_2	2.00	0.20	0.46	0.66
Al_2O_3	0.50	0.13	0.20	0.40
Fe_2O_3	0.20	0.03	0.04	0.04
P_2O_5	0.18			
K_2O	0.06			
SrO	0.09			
SO_3	0.60			
Na_2O	0.58			
TiO_2	0.02			
Mn_2O_3	0.02			
lg. loss (%)[a]	44.16	45.61	45.16	45.12

[a]Including CO and organic materials lost by heating

scallops ranges from 0.01 g to 58.33 g or approximately 52–80% of the whole animal. From a chemical point of view, calcing or pyrolyzing could convert calcium carbonate into calcium oxide, so it is possible to use shell waste as an alternative source to obtain these compounds (Yao et al. 2014). Several studies with the purpose of extracting calcium carbonate from different seashell types reported calcium oxide contents rather than calcium carbonate contents (Table 6.1). The results revealed that the removal of organic material from seashells may be difficult and heating to high temperatures results in forms with a higher degree of purity (Owuamanam and Cree 2020).

Islam et al. (Islam et al. 2013) reported a study using an environmentally friendly method for the synthesis of calcium carbonate nanoparticles from cockle shells and demonstrate that this extraction method has great potential in industry for the large-scale production of calcium carbonate nanoparticles for biomedical applications. Luo et al. (Luo et al. 2018) prepared polycaprolactone and oyster shell powder scaffolds by additive manufacturing using a 3D printing system and demonstrated no significant cytotoxicity effect of the prepared scaffolds towards MG-63 cells and favourable biocompatibility for bone tissue engineering.

6.1.1.2 Chitin and its Derivatives

Chitin can be found in many organisms, such as fungi, plankton and the exoskeletons of insects and crustaceans.

The main marine sources of chitin and the commercial production of chitosan especially rely on chitin from crab, shrimp, prawn and krill species (Raghavankutty et al. 2018).

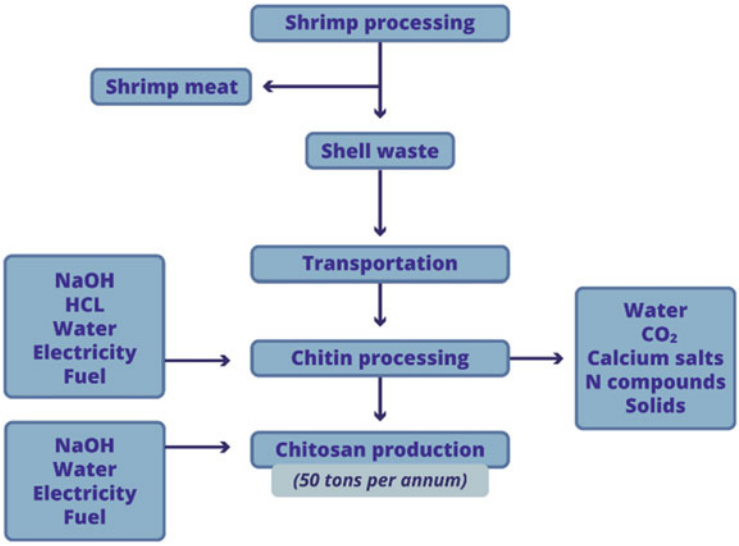

Fig. 6.6 Waste input and output from Chitosan Industries (adapted from Mathew et al. (2021))

Chitosan is composed of N-acetyl-d-glucosamine and d-glucosamine units and is polycationic in nature (Raghavankutty et al. 2018). Currently, the deacetylated form of chitin, chitosan and their derivatives has also been developed for a broad spectrum of applications, such as industrial chemistry, such as cosmetics, textiles, water treatment and biomedicine (Yao et al. 2014). Films, hydrogels, microspheres and nanoparticles are the major forms of chitosan in biomedical and pharmaceutical applications (Raghavankutty et al. 2018). Biocompatibility, biodegradability, antimicrobial activity, wound healing, and anti-tumour activity are some of their properties that contribute to its inevitable importance in fields already mentioned (Raghavankutty et al. 2018).

The industrial-scale production of chitin and chitosan require harsh chemicals like HCl for demineralization (to remove calcium carbonate) and NaOH (to remove the protein) (Mathew et al. 2021). The extraction of chitin from crustaceans requires approximately 6.3 kg of HCl and 1.8 kg NaOH, which turns the extraction very expensive. Furthermore, approximately 1.4 tonnes of water are required to completely remove the chemicals from the crustacean wastes, as well as high temperatures for longer durations (Mathew et al. 2021). Figure 6.6 explains the input and outputs derived from the chitosan factory.

Chitosan hydrogels have been successfully 3D printed using extrusion-based processes for engineering bone tissue. Inorganic molecules have also been mixed with chitosan for the purpose of improving its bioactivity to resemble bone (Sanchez-Rexach et al. 2020).

6.1.1.3 Collagen and Gelatin

Collagen is the major structural protein of fish skin and bones, representing 30% of the total protein content, and it is the main component of the extracellular matrix (ECM) in connective tissues (skins, tendons, ligaments and bones) (Jafari et al. 2020). Collagen is classified according to its structural features; there are at least 28 types of collagen and in fish is possible to obtain types I and II. Collagen and gelatin are derived from the same macromolecules, being gelatin a partially hydrolysed form of collagen in a denaturated state (Caruso et al. 2020). The use of collagen from marine sources has attracted increasing attention due to their cost-effective process, high collagen content, biocompatibility similar to conventional bovine and porcine collagen and high absorption by the human body (Jafari et al. 2020).

Collagen and gelatin have a wide range of applications in food, cosmetic and pharmaceutical industries. In biomedical applications, they also have been explored as drug and gene carriers, bone-filling materials and wound dressings (Shavandi et al. 2018; Jafari et al. 2020).

Regarding the various extraction methods used to prepare collagen from marine sources, the conventional protocol includes the highlighted steps shown in Fig. 6.7. The type of fish collagen must be considered to use more adequate extraction parameters, such as the extraction medium pH and the temperature of the process, since they can influence the final properties of the extracted collagen (Shavandi et al. 2018; Raghavankutty et al. 2018).

Collagen can be used in several forms, such as injectable solutions and dispersions, that can be processed further using Direct Digital Manufacturing technologies. Govindharaj et al. (Govindharaj et al. 2019) reported the extraction of collagen from marine eel skin as a potential blue biomaterial and incorporated into alginate hydrogel to fabricate scaffolds using extrusion-based 3D printing technology. Sanz et al. (Sanz et al. 2021) described the extraction, characterization and methacrylation

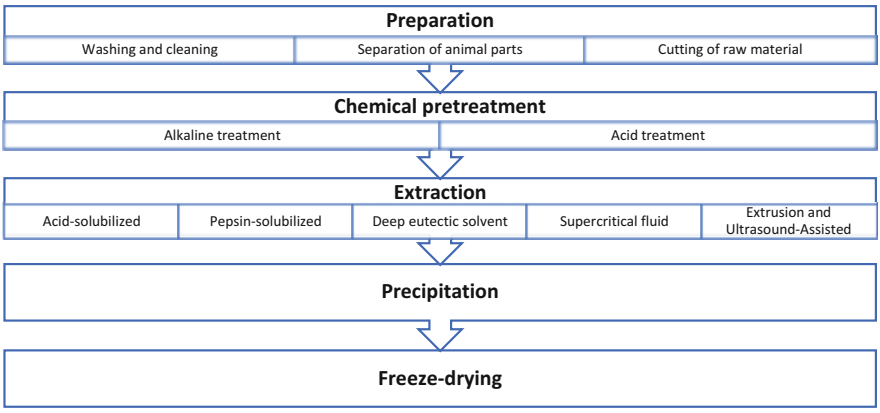

Fig. 6.7 Collagen extraction procedure from fish by-products (adapted from Jafari et al. (2020))

of Red Snapper collagen for 3D Coaxial Printing of neural and skeletal muscle cell cultures as a model for neuromuscular junction formation.

6.2 Direct Digital Manufacturing: General Overview

Direct Digital Manufacturing (DDM) is currently a main subject in manufacturing industry. Therefore, it is important to discuss the evolution of this term. Additive Manufacturing (AM) is a continuously growing technology seen as a viable and reliable solution to process a wide range of different materials. AM is defined by the International Standard ISO/ASTM 52900:2021 as "the process of joining materials to make parts from 3D model data, usually layer upon layer, as opposed to subtractive and formative manufacturing methodologies" (ISO/ASTM52900:2021 (en) 2021).

AM techniques, commonly also referred to as 3D printing, were introduced in the market in the late 1980s, with the development of stereolithography (SLA) process by Charles "Chuck" W. Hull (Hull 1986). Since then and until now, with a constant increase in interest in this area, many technological advances have been developed. Therefore, the term AM is also evolving to DDM, referring to the interconnection of AM technologies and information and communication technology (ICT), such as computer software and communication through a network, making possible the creation and production of final products and end-use components. This technology introduces a change in the production paradigm, since the products will be derived from a 3D CAD model and could be produced closer or at the customer location utilizing an AM equipment (Chen et al. 2015).

According to the International Standard ISO/ASTM 52900:2021 (ISO/ASTM52900:2021(en) 2021), DDM technologies are divided into seven different categories (Fig. 6.8), such as:

- Material Extrusion – a process in which material is selectively dispensed through a nozzle or orifice.

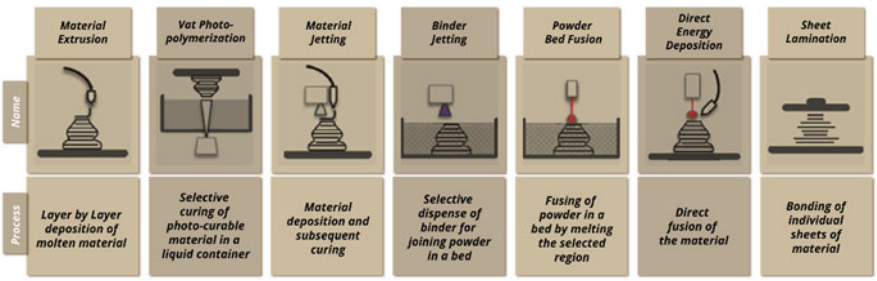

Fig. 6.8 Classification of additive manufacturing processes according to ISO/ASTM 52900:2021 (adapted from Dilberoglu et al. (2017))

- VAT Photopolymerization – a process in which liquid photopolymer in a vat is selectively cured by light-activated polymerization.
- Material Jetting – a process in which droplets of build material are selectively deposited.
- Binder Jetting – a process in which a liquid bonding agent is selectively deposited to join powder materials.
- Powder Bed Fusion – a process in which thermal energy selectively fuses regions of a powder bed.
- Directed Energy Deposition – a process in which focused thermal energy is used to fuse materials by melting as they are being deposited.
- Sheet Lamination – a process in which sheets of material are bonded to form a part.

DDM offers many advantages, such as a high degree of geometric freedom for design, reduction of material waste, reduction of tooling, time-to-market and, therefore, costs, when compared to conventional manufacturing techniques. The high flexibility of these technologies in terms of geometry, by building parts layer by layer, enables the production of complex and light functional parts, with associate weight and material savings. Thus, DDM is gaining recognition as an energy-efficient technology. The reduction or complete elimination in the need for tooling means that the cost of producing a single part through DDM is approximately the same despite the amount of parts needed. This makes DDM suitable for the production of smaller batch sizes when compared to conventional processes like injection moulding, also being an amazing alternative to produce personalized and high-quality products that respond to an individual need (Gibson et al. 2015). For these reasons, DDM is seen by the industry as a disruptive technology that will enable the growth of new products and new business models, with applications in various areas (e.g. aerospace, automotive, biomedical, food industry).

6.2.1 DDM for Biopolymers Production

DDM technologies using biobased and biodegradable polymers have been an emerging field in the last few years, due to the increasing interest in developing sustainable products and solutions, instead of using limited resources such as fuel-based polymers (Jiang and Zhang 2017). However, DDM has not yet achieved its full potential to be used as a regular production technology, due to limitations related with the availability of materials, their sustainability and their lack of processability concerning the DDM processes and equipment requirements (Liu et al. 2019).

In the last few years, the number of articles published regarding biopolymer DDM increased from less than 5 articles in 2013 to more than 1000 in 2020 (Santoni et al. 2022), which shows the effort on exploring natural and renewable biopolymers to achieve a recycling and sustainable economy. Biomass from residuals of renewable resources, such as woody or marine, is seen as a thriving alternative to fossil

resources. By using the biorefinery concept it is possible to apply biomass conversion processes and extract different components (e.g. collagen, chitosan, alginate) and then incorporate them into innovative materials for specific 3D printing techniques (Li et al. 2021).

The most promising DDM technologies for industrial biopolymers production are extrusion-based processes—Fused Filament Fabrication (FFF) and Direct-Ink Writing (DIW), Inkjet 3D printing—Drop on Demand (DOD) and Laser-assisted 3D printing—Stereolithography (SLA).

6.2.1.1 Fused Filament Fabrication

Fused Filament Fabrication (FFF) is a material extrusion AM process in which a thermoplastic filament is melted and forced through a nozzle to the build platform, layer by layer (Fig. 6.9). Scott Crump, founder of Stratasys Inc., developed this technology in 1992 under the term of Fused Deposition Modelling (FDM™) (Scott Crump 1992). Since FDM™ is the term trademarked by Stratasys Inc., FFF is commonly used to refer to the same process. FFF is the most used DDM technology for polymer AM due to the simplicity and low cost of the process when compared to other techniques. FFF is commonly used for printing polymeric materials such as polylactic acid (PLA), polyethylene terephthalate (PET), acrylic butadiene styrene (ABS), among others. The polymeric filament is heated above the melting temperature and rapidly hardens after being deposited in the build platform, which allows

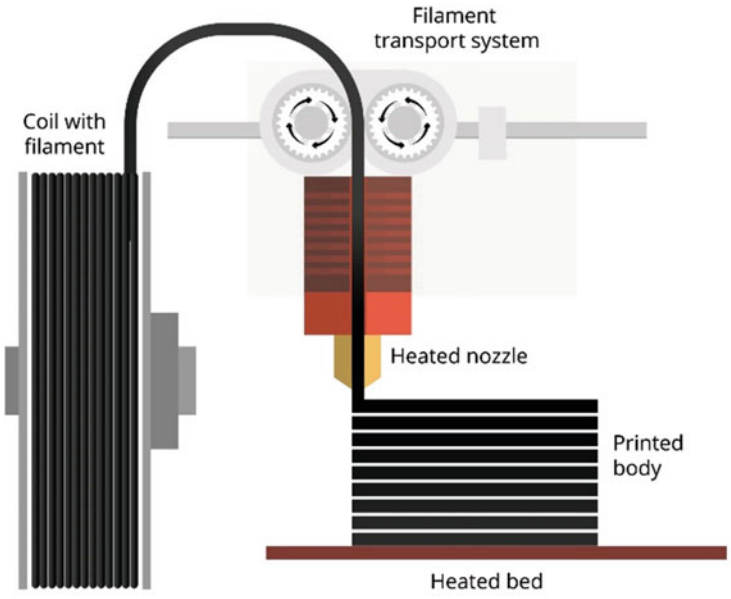

Fig. 6.9 Schematic diagram of a typical Fused Filament Fabrication (FFF) printer

the adhesion of the layer immediately deposited after on it. In addition, it is also required to make sure that the filaments produced have the appropriate modulus and flexural strength so they can be spooled and pushed by the drive gear and the idle pulley (Ngo et al. 2018).

FFF was first developed to produce prototypes from plastic material. However, nowadays it is also used to fabricate 3D parts with biopolymers with applications in biomedical fields. A few studies reported that FFF is a capable technology in the production of bioproducts and scaffolds for tissue engineering using polyhydroxyalkanoates (PHAs) (Ausejo et al. 2018; Wu et al. 2017; Wu 2018; Rydz et al. 2020) and polyhydroxybutyrate (PHB) biopolymers (Kontárová et al. 2020; Duan et al. 2011; Giubilini et al. 2020). PHA and PHB are biodegradable and environmentally friendly polymers that can be extracted from marine microbes and organisms.

The main challenges regarding the application of biopolymers in FFF are related with the clogging of the printing nozzle, associated with the fragility of the filaments or with the changes of viscosity caused by the filler content (Mazzanti et al. 2019). One of the solutions is to combine the principles of FFF technology and use a different mechanism to push the material through the heated nozzle, through a material extrusion principle but without the need of preparing filaments.

6.2.1.2 Direct-Ink Writing

Direct-Ink Writing (DIW), like FFF, is a material extrusion AM process. In DIW, a viscoelastic ink is forced through the printing nozzle onto a build platform under controlled flow rates (Fig. 6.10), in order to form 3D fibre structures at ambient temperature with a predefined pattern, layer by layer (Saadi et al. 2022). The extrusion of the ink can be done by different extrusion mechanism, such as pneumatic-based, piston-based or screw-based extrusion, which make this process suitable for working with a huge range of fluids with different viscosity values (Murphy and Atala 2014).

Typically, the inks used in DIW are in the form of pastes or gels, which normally includes organic, colloidal, nanoparticle-filled and sol-gel inks. Since DIW allows the production of 3D parts directly from pastes and gels, instead of using heat in the extrusion process like in FFF, the inks need to go through a solidification process, such as evaporation, gelation or solvent-driven steps. Therefore, it is important that DIW materials match rigorous requirements in terms of rheology in order to flow with ease and recover after the deposition. Their formulations should behave as non-Newtonian fluids, usually referred to as yield stress fluids, which means that they are able to flow or deform indefinitely when submitted to a specific stress (Gibson et al. 2015; Rocha et al. 2020).

DIW is a promising technology in the production of biopolymers for biomedical areas, for example in tissue engineering. Several studies have been performed using alginate, a polysaccharide derived from seaweed with biocompatibility and non-toxic properties, to produce lattice 3D structures successfully (Bendtsen et al.

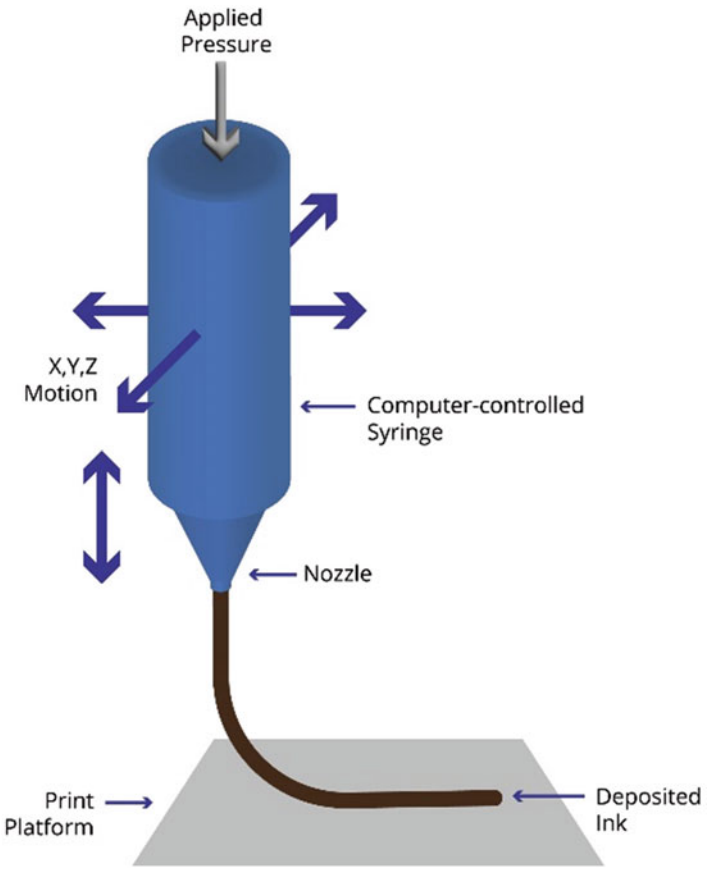

Fig. 6.10 Schematic diagram of Direct-Ink Writing (DIW) process

2017; Möller et al. 2017; Wu et al. 2016; Chawla et al. 2020; Choe et al. 2022). Other study evaluated the printability of biopolymers with collagen, an important protein that can be extracted from fish bones, with applications in biomedical or drug discovery (Zhao et al. 2015), and also the production of scaffolds for tissue regeneration using chitosan, a biocompatible polysaccharide with wound healing and antifungal effects that comes from the skeleton of shellfish (Demirtaş et al. 2017; Elviri et al. 2017).

6.2.1.3 Inkjet 3D Printing

Inkjet 3D Printing is a low-pressure and low-temperature process that involves the deposition of ink droplets on a platform, layer by layer (Fig. 6.11). The printing material is extruded through a small nozzle within a print head, with each individual layer cured between consecutive depositions (using an infrared or ultraviolet light)

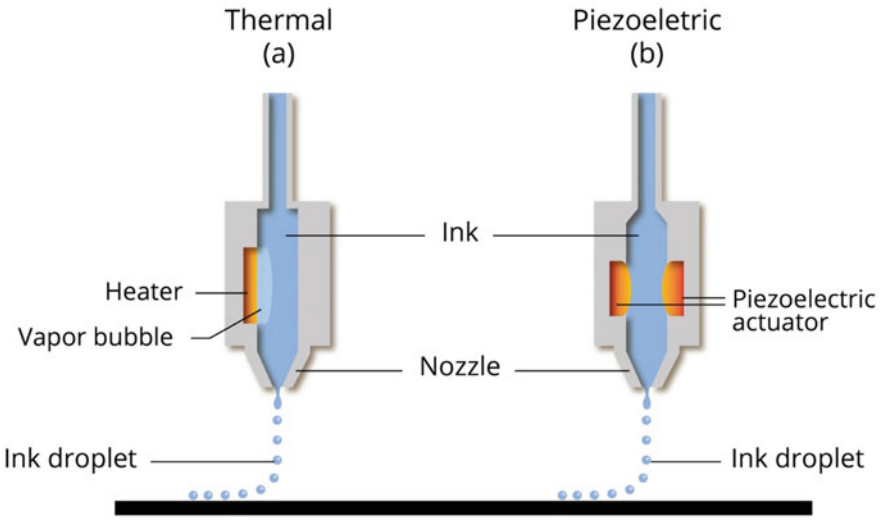

Fig. 6.11 Schematic diagram of a Drop on Demand (DOD) inkjet process

(Gibson et al. 2015). Inkjet 3D Printing is able to produce a wide range of materials, such as polymers, dielectric and nano-conductive nanoparticles. This technology can be performed using two different mechanisms, namely Drop on Demand (DOD) and Continuous Inkjet (CIJ) printing. In the CIJ process, a continuous liquid ink flows through the nozzle into a droplet format. In DOD inkjet printing, the most common and more cost-effective when compared to CIJ, tiny droplets are deposited in the build substrate due to the action generated by thermal or piezoelectric actuators (Guo et al. 2017). In thermal DOD printing, heat is produced to create vapour bubbles that force the ejection of the material droplets. On the other hand, in piezoelectric DOD printing, an electric signal is applied to a piezoelectric material that generates acoustic pulses, which forces the ejection of the ink droplets (Derby 2010).

Besides the high printing speed and cost-effectiveness, this process provides higher precision and quality of production because the actuator mechanism allows to control the droplet uniformity and size. Therefore, DOD inkjet printing is gathering a lot of attention as a disruptive process in biomaterials production (Guo et al. 2017).

Köpf et al. reported that the use of agarose, a polysaccharide commonly extracted from red seaweed that is used in molecular biology for the separation of large molecules, combined with collagen allows to successfully print 3D complex structures that can be used in disease and drug discovery (Köpf et al. 2016). Another study showed that is possible to 3D print pre-vascularised tissue replacements by DOD inkjet printing using Gelatin-methacryloyl (GelMA) and collagen hydrogel solutions (Stratesteffen et al. 2017).

6.2.1.4 Stereolithography

Stereolithography Apparatus (SLA) or usually Stereolithography (SL) was the first patented (1986) and commercialized (1988) AM process, developed by Charles W. Hull, founder of Stratasys Inc. (Hull 1986). In SL, a UV laser is used to selectively polymerize the photocurable resin contained in a vat (Fig. 6.12). This process starts with the scanning of the photosensitivity resin surface and after the first layer is complete, the build platform shifts down and another laser scanning is performed to the next layer, and so on until the 3D solid part is finished. After the print is completed, the part is submersed in a solvent, such as isopropyl alcohol, to remove any excess of resin and the support structures, which are usually necessary to ensure the success of the print and avoid deformations, can be removed manually. After that, and depending on the desired application, the final part can be submitted to a post-curing step to enhance material properties, where temperature and UV light are combined (Gibson et al. 2015).

The materials used in SLA are usually thermosetting photopolymer resins. This means that the photopolymerization process is irreversible and it is not possible to

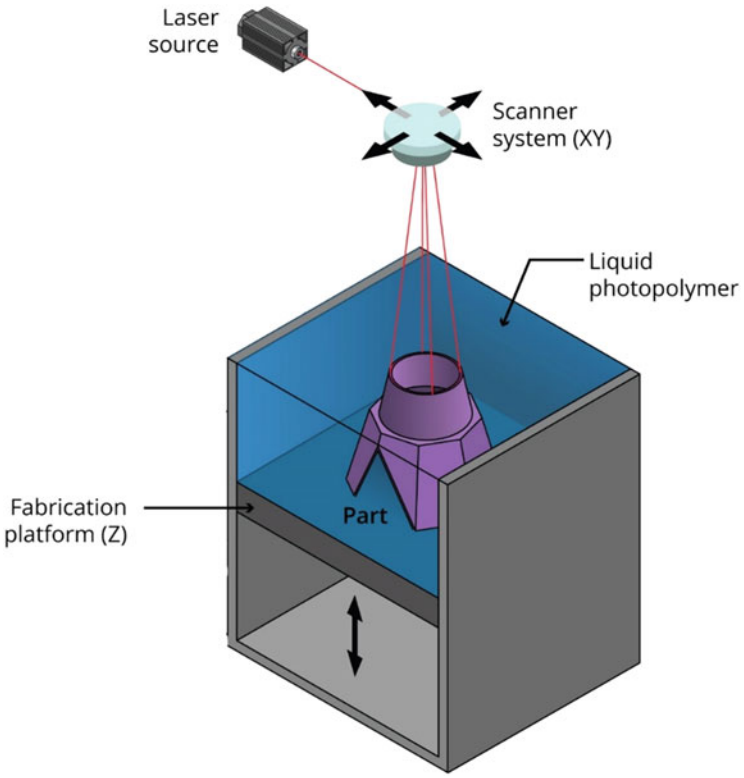

Fig. 6.12 Schematic diagram of a stereolithography (SLA) process

heat and convert the printed SLA parts back to their original liquid form. SLA technique is capable of produce fine and complex structures with high dimensional accuracy and good surface properties with minimal stepping effect, when compared to the material extrusion AM processes. Due to the characteristics of SLA process, especially attention is being paid to the bioprinting area (Grigoryan et al. 2021).

The development of calcium phosphate suspensions for SLA was studied and reported as a viable solution for producing 3D components (Goutagny et al. 2021). Calcium phosphate is a mineral extracted from marine fish bones with good biocompatibility and osteoconductivity. Gyroid scaffolds were successfully produced by SLA using calcium phosphate nanoparticles, with great potential for bone repair applications (Ullah et al. 2021). 3D hybrid scaffolds with ear shape were also produced by SLA using a hybrid biocompatible resin with chitosan and polyethylene glycol diacrylate (PEGDA), a synthetic polymer. Long-term cell viability and spreading were observed, confirming the possibility of printing chitosan by SLA and providing cell-adhesive properties to the scaffolds, suitable for repairing complex geometries (Morris et al. 2016).

6.3 Current Challenges and Future Perspectives

In the last few years was made incredible progress in DDM of biopolymers, mainly in biomedical areas, with many process improvements and advances in feedstock materials. As a raw material, biopolymers represent an ideal option to be used in AM processes, combining their biodegradable, non-toxic and environment-friendly properties with the possibility of producing complex 3D porous structures.

However, the feedstock formulation is still the major challenge to overcome for the large adoption and large-scale production by the industry using DDM. Questions related to feedstock printability, sustainability and functionality need further attention to match and achieve the cost and eco-effectiveness inherent to DDM technologies. Properties such as material degradation, compatibility with living cells and mechanical strength and stiffness are the main barriers in today's biopolymer 3D printing. The development of biobased materials for DDM processes will be a growing trend, with an increasing focus on discovering sustainable and environment-friendly feedstocks.

Therefore, DDM shows a great potential for the development of natural-delivered biopolymer 3D structures, with many biocomponents available in marine environments or residues, the largest renewable resource on the planet.

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Chapter 7
Exploiting Marine Fungi in the Removal of Hazardous Pollutants and Biomass Valorisation

Dushyant R. Dudhagara, Bhumi M. Javia, and Anjana K. Vala

Abstract The environment and human health are now seriously threatened by pollution. Organic contaminants have a long half-life in the environment and possess hydrophobic, mutagenic, and cytotoxic properties. Hence, they are a big challenge to the health of environment. Due to their hydrophobic and persistent character, non-biodegradable and recalcitrant chemicals have an adverse effect on terrestrial and aquatic ecosystems. According to current pollution management methods, biodegradation and bioremediation using various microorganisms is an efficient, reliable, and eco-friendly approach to combat the pollutants. Marine-derived fungi play a significant role in the remediation of organic pollutants because their unique morphological and physiological properties including survival in the extreme conditions and a diverse metabolic capability. This chapter highlights the role and mechanisms of marine-derived fungi in removal of various pollutants as polyaromatic hydrocarbons (PAHs), heavy metals, dyes, and the biomass valorization. Fungi can oxidize PAHs, alkanes, and other complex hydrocarbons using various intracellular and extracellular enzymatic machineries including monooxygenases and lignin-modifying enzymes. Marine-derived fungi act as biosorbents to remove heavy metal contaminants through active and passive mechanisms. Both living and non-living fungal biomass can be used to detoxify and degrade dyes from the contaminated environment. Furthermore, fungal biomass valorization facilitates the sustainable development of value-added products such as biofuels, enzymes, amino acids, organic acids, alcohol, pigments etc. Thus, the chapter emphasizes to understand the mechanisms of marine-derived fungi in degradation of organic pollutant, metabolic pathways, and enzymes responsible for degradation of organic compounds which would help to develop the future

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mycoremediation policies and production of value-added products for sustainable future development.

Keywords Organic pollutants · Bioremediation · Biomass valorization · Value-added products · Sustainable development

7.1 Introduction

Pollution is a matter of grave concern in this age. The condition of environment directly influences the quality of life in the ecosystem on earth. Industrialization and growing affluence in the developed world along with population explosion and rapid development in the developing countries has resulted in accelerated environmental degradation on a large-scale. Major sources of pollutants include industrial effluents, injudicious use of fertilizers, insecticides, pesticides, mining activities, sewage sludge, etc. Pollutants can be divided into two major types: biodegradable and non-biodegradable pollutants. Non-biodegradable pollutants cause hazardous effect on environment. Non-biodegradable pollutant includes heavy metals, pesticides, polyaromatic compounds and radionuclear material etc. (Peng et al. 2008). Many conventional physico-chemical methods of treatment/removal of these compounds, though effective, are not feasible for application on a large scale. Hence, there is need to develop treatments that can minimize or even eliminate such pollutants from environment. In recent years, the application of microorganisms which degrade or convert hazardous pollutants to less toxic compounds have become popular. Fungi proved to have high potential in the degradation of high molecular weight compounds and therefore are used widely to remediate environmental pollution (Akcil et al. 2015; Deshmukh et al. 2016; Varjani 2017).

Fungi are eukaryotic, chemoheterotrophic, parasitic or saprophytic, unicellular or multicellular filamentous organisms that include molds, yeasts, and mushrooms. The kingdom Fungi includes eight phyla. Fungi are found in variety of habitats like soil, fresh, and marine waters (Anastasi et al. 2013). Fungi produce secondary metabolites, enzymes, biosurfactants, and polysaccharides and applied in bioremediation of pollutants. Fungi have been efficiently used to treat water samples contaminated with micropollutants (Badia-Fabregat et al. 2015)**.** Marine fungi are able to sustain high saline conditions and extreme pH this trait provides biological advantage over terrestrial fungi (Thatoi et al. 2013; Singh et al. 2019).

Marine fungi are found in oceans and colonize different niches. They act as parasites, saprobes, or symbionts and associated with organisms (Wang et al. 2012). Marine fungi can be isolated from different samples such as sediment, seawater, mangrove detritus, decaying wood, seaweeds, and invertebrates (Pang et al. 2016). Marine fungi have capacity to produce different pharmacological metabolites (Imhoff 2016). Marine fungi are source of novel antibacterial, antiviral, anticancer, antiplasmodial, and anti-inflammatory compounds (Bovio et al. 2019), enzymes, biosurfactant (Cicatiello et al. 2016; Nicoletti and Andolfi 2018) and are also useful in the bioremediation of pollutants (Bovio et al. 2017). Marine fungi tolerate high

concentrations of heavy metals such as copper and lead (Gazem and Nazareth 2013). Role of marine fungi in heavy metals, dyes, and hydrocarbons degradation has been well documented. Furthermore, enzymes from marine fungi can be used for paper, pulp, textile, leather, biofuel industries, food and beverages, for animal feed, for pharmaceutical, cosmetic, and environmental applications (Damare et al. 2012; Bonugli-Santos et al. 2015; Deshmukh et al. 2016).

It has been reported that marine fungi have capacity to bioremediate highly recalcitrant pollutants. Bioremediation is a process in which living organisms degrade or convert harmful organic contaminants to less toxic compounds. Mycoremediation is the process in which fungi are used for bioremediation. Mycoremediation is eco-friendly and effective method to combat increasing pollution of soil and water (Arun et al. 2008). Fungi are ideal candidates for remediation of pollutants due to their unique traits including ability to withstand fluctuation in temperature and pH, heavy metal resistance, high surface area to volume ratio, mycelial growth, hyphal network, and extracellular ligninolytic enzymes (Khan et al. 2019). Fungi produce variety of intracellular as well as extracellular enzymes including peroxidase and cytochrome P450, respectively, for detoxification and biodegradation of pollutants (Durairaj et al. 2015). The diversity of habitats and ability for secreting multitude of enzymes makes fungi potential candidates for bioremediation at various locations (Divya et al. 2014).

In this chapter, the role of marine fungi in degrading various recalcitrant, persistent, and harmful pollutants like polycyclicaromatic hydrocarbons (PAHs), heavy metals, dyes, and mechanisms behind the mycoremediation of these pollutants are summarized with process of biomass valorization. An attempt is made to understand how the process of degradation can be accelerated and the future strategy to overcome the existing limitations is discussed.

7.2 Hydrocarbon Degradation by Marine Fungi

Hydrocarbon contamination from petrochemical industry is the major environmental problem faced by humanity. Leakage and accidental spillage of petroleum products are to the tune of 2,00,000–6,00,000 metric tons per year (Kvenvolden and Cooper 2003; Das and Chandran 2011). These organic pollutants are carcinogenic and neurotoxic which cause the harmful effects on animal and plant.

Bioremediation or biodegradation by application of natural populations of microorganisms is one of the mechanisms by which hydrocarbon and other pollutants can be removed from the environment (Ulrici 2000). Biodegradation of petroleum hydrocarbons is a complex process which depends on the environment and the amount of the pollutants present in the site. Petroleum hydrocarbons are divided into four classes: saturates, aromatics, the asphaltenes (ketones, phenols, porphyrins, esters and fatty acids), and the resins (sulfoxides, amides, pyridines, carbazole, and quinolones). Hydrocarbons differ in their degradation susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation is linear alkanes

> branched alkanes > small aromatics > cyclic alkanes (Ulrici 2000). Some compounds such as high molecular weight containing polycyclic aromatic hydrocarbon may be difficult to be degraded (Atlas 1995) but many fungal species are capable to degrade this recalcitrant hydrocarbon-containing pollutants. It has been observed that fungi present in polluted environment have developed adaptive mechanisms by which they are able to utilize hydrocarbons as the sole source of carbon (Dacco et al. 2020).

Chaillan et al. (2004) isolated fungi *Amorphotheca*, *Talaromyces, Neosartorya*, and *Graphium* from the soil contaminated by petroleum and have higher efficiency in the degradation of petroleum hydrocarbons. Some terrestrial fungi *Aspergillus*, *Penicillium,* and *Cephalosporium* are reported as idea candidate for the degradation of crude oil (Singh 2006; Das and Chandran 2011). The different hydrocarbon degrading pathways and their mechanisms operated by fungi are mentioned below.

7.2.1 Degradation Process of Alkane

Alkane is a saturated hydrocarbon with all single bonds in its structure and cycloalkane is a saturated hydrocarbon with several carbon rings in its structure. Alkane degradation can be catalyzed by some enzymes such as oxidase, dehydrogenase and converted into fatty acids, and followed by acetyl coA and which can be further mineralized as CO_2 and H_2O (Singh 2006). It has been observed that in the process of Alkane degradation many enzymes played an important role such as alkane monooxygenase, fatty alcohol dehydrogenase, fatty aldehyde dehydrogenase, etc. Biological degradation of alkane is a subterminal oxidation process. In the primary step, cycloalkane is oxidized into alcohols by n-alkane monooxygenase type of oxidizing enzymes. Then, alcohols are converted into ketone by alcohol and aldehyde dehydrogenase. Furthermore, the ketone is oxidized into fatty acid. Cyclohexane is converted into corresponding compound, followed by cyclohexanol, cyclohexanone, and fatty acids. At last, the compound is mineralized and form CO_2 and H_2O along with fungal biomass production as the end product (Fig. 7.1) (Dacco et al. 2020).

7.2.2 Degradation Process of Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAH) are widely distributed in the environment and may persist for extended period of time. PAHs are composed of two or more fused benzene rings and are formed during combustion of organic molecules (Haritash and Kaushik 2009). Polycyclic aromatic hydrocarbons have carcinogenic, mutagenic, and teratogenic properties. Forest, oil seep, volcanic eruption and

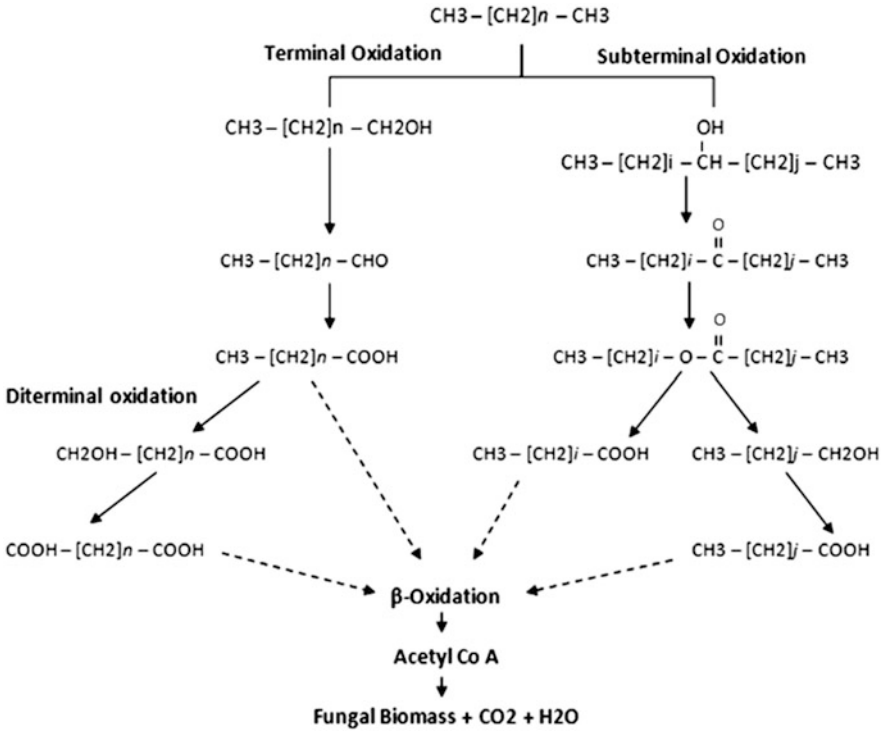


Fig. 7.1 Degradation shows the terminal, diterminal, and subterminal pathways for the n-alkane (modified from Dacco et al. 2020)

exudates from trees are natural sources of PAHs. Anthropogenic sources include fossil fuel burning, coal tar, wood, garbage, lubricating oil, municipal waste incineration and petroleum spills, etc., are the major source of pollutants (Kaushik and Haritash 2006). Most important and ecologically damaging components of pollution are the PAHs and cause depletion of the ozone layer and affect Earth's heat balance, adding acidic air pollutants to atmosphere and reduce visibility (Chauhan et al. 2000).

According to Yumoto et al. (2002), PAHs can be degraded by enzymes and catalyzed into glycol or catechol, then decomposed into succinic acid or acetyl coA. In degradation pathway, PAHs are gradually degraded into epoxide, trans diol, phenol, and trans dihydro 2 phenol by monooxygenase enzyme of yeast. In another study of Mills et al. (2004), PAHs can be degraded by dioxygenase into cis dihydro 2 phenol, epoxide, cis diol etc. The final metabolites in both pathways are $co_2$and water. The ligninolytic and monooxygenase system of cytochrome P-450 may be involved in polycyclic aromatic hydrocarbon degradation by filamentous fungi. Induction of the monooxygenase before application in degradation of hydrocarbon could result in enhanced removal of PAHs (Haritash and Kaushik 2009). Fungal cytochrome P-450 Monooxygenase from white rot fungi *Phenerochaete*

chrysosporium have capacity to oxidize pollutants like aliphatic hydrocarbons, crude oil, n-alkane, polyaromatic hydrocarbons alkylphenol, etc.

PAHs degradation depends on solubility, number of benzene rings, the species and number of substituent species, the properties of heterocyclic atom of PAHs. Asphalt is very difficult to be degraded by biodegradation due to its most complicated structure. Many researches indicated that the PAH can be degraded only in aerobic conditions. While it has been observed that PAH can be degraded in anaerobic condition as well like sulfate reduction, denitrification, or methanogenic fermentation. Though rate of aerobic degradation of PAHs is higher than the rate of anaerobic degradation. (Meckenstock Rainer 2004).

Ligninolytic fungi are capable of oxidizing PAH by non-specific extracellular enzymatic complexes, normally used to depolymerize lignin. These lignin-degrading enzymes include laccase, lignin peroxidase, manganese peroxidase, etc. (Peng et al. 2008). A novel PAH metabolic pathway in fungi involves hydroxylation by cytochrome P-450 monooxygenase enzyme through a sequence of reactions similar to mammalian metabolism (Capotorti et al. 2004). In many non-ligninolytic fungi, this pathway occurs to effectively degrade hydrocarbons (Ravelet et al. 2000). Many researchers have studied that purified fungal laccase enzyme can be used for the oxidation of PAH. Laccase enzyme of *T. versicolor*, *C. hirsutus, P. ostreatus*, and *Coriolopsis gallica* was the most studied in the fungi. It has been reported that activity of enzyme in *T. versicolor* fungi is 29 times higher than other microorganisms (Margot et al. 2013). For example, *T. versicolor* laccase, in combination with 1-hyrdoxybenzotriazole (HBT), was capable to oxidize two PAHs, acenaphthene and acenaphthylene; Laccasewithout mediator oxidized about 35% of the acenaphthene and only 3% of acenaphthylene. The end products obtained after incubation were 1,2-acenaphthenedione and 1,8-naphthalic acid anhydride (Johannes et al. 1998).

Kirk and Gordon (1988) explained that 14 strains of obligate marine fungi belonging to genus, *Varicosporina, Dendryphiella*, *Lulworthia*, and *Corollospora* species can grow using alkenes and alkanes as a sole source of carbon and mineralized into n-hexadecane. The study has shown that the 14 lignicolous and arenicolous strains utilized pristine, 1-hexadecene and some degree of tetradecane as a sole source of carbon. Raikar et al. (2001) isolated*Thraustochytrids* fungi from several oil spills polluted sites in Goa and they were capable of degrading tar-balls added to peptone broth and degradation was observed up to 30% in 7 days as estimated by gas chromatography and gravimetry**.** *A.sclerotiorum* showed 99.7% pyrene 2 mg in 30 mL and 76.6% benzo pyrene 1 mg in 30 mL degradation after 8 and 16 days, respectively (Passarini et al. 2011). Two non- identified marine-derived fungi were able to remove phenanthrene from a media by adsorption through fungal mycelium. Fungus *Aspergillus* sp. BAP14 isolated from marine sediment showed degradation of benzopyrene and removed approx 30% BaP after 3 days (Raghukumar et al. 2006; Damare et al. 2012).

In the degradation process of aromatic hydrocarbons, aromatic hydrocarbon is oxidized by oxidase into dihydrodiol. Then, the dihydrodiol is degraded into o-dihydroxybenzene. Dihydroxybenzene is degraded by following two processes,

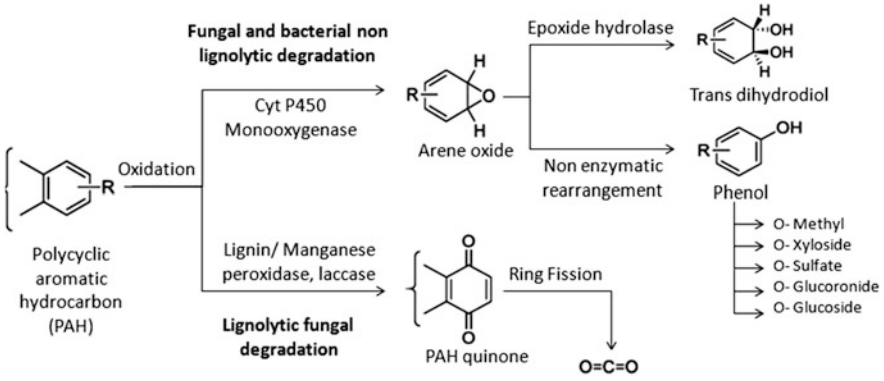


Fig. 7.2 Degradation of polycyclic aromatic hydrocarbons by fungi

which are ortho position ring opening and meta position ring opening reaction. Then, these compounds are oxidized into long chain compounds and gradually metabolized into acetyl coA. Degradation process by microorganisms such as bacteria and fungi are comparatively different from each other. In bacteria, aromatic hydrocarbon is oxidized by two oxygen atoms and converted into cis-dihydrodiol. While in fungi, aromatic hydrocarbon is oxidized and converted into trans-dihydrodiol (Fig. 7.2) (Xue et al. 2015; Kadri et al. 2017).

7.3 Heavy Metal Removal by Marine Fungi

Heavy metals are considered as one of the most hazardous pollutants having a specific density of more than 5 gm/cm^3. Heavy toxic metals are directly or indirectly released into environment and as a result, annual worldwide release of heavy metals reached 13,50,000 tons of zinc, 9,39,000 tons of copper, 22,000 tons of cadmium and 7,83,000 tons of lead (Singh et al. 2003). Metals like iron, zinc, copper, and manganese are essential for biological process. While mercury, cadmium, and nickel have no physicological role but these metals can cause harmful disorders at high concentration (Lenin et al. 2014). High concentration of heavy metals can cause serious environmental as well as health problems. Unlike organic contaminants, these pollutants from heavy metals are non-biodegradable and cause bioaccumulation in food chain. Conventional physico-chemical treatment technologies become less effective and more expensive when metal concentrations are in the higher range (1–100 ppm) (Dermont et al. 2008).

Marine fungi can tolerate high concentration of heavy metals and its their interaction to metal ions can be used to remove heavy metal pollutants from environment (Lopez Errasquín and Vázquez 2003). Marine fungi can remove toxic metals from the environment by adsorption as well as their metabolic activities (Davis et al. 2003). Living as well as dead fungal biomass has been recognized for the removal of

heavy metals through absorption (Bishnoi and Garima 2005). Fungi can be used as a biosorbent for the removal of heavy metals with excellent metal uptake and recovery (Fu et al. 2012). Rehman et al. (2008) isolated yeast *Lodderomyces elongisporus* from metal-contaminated site and found to tolerate various heavy metals. Damare et al. 2012 stated that the fungus *Thraustochytrids* from shallow water hydrothermal vents have efficiency to withstand high concentration of heavy metals. Majeau et al. (2010) reported that psychrophilic fungi, *Cryptococcus* sp. found in deep-sea sediments have capability to tolerate high concentration of heavy metals such as $ZnSO_4$, $CuSO_4$, Pb $(CH_3COO)_2$ and $CdCl_2$ up to 100 mg/L.

Vala et al. (2004) while searching for new sources of marine fungi for the removal and tolerance of heavy metal confirmed two seaweeds associated fungi *Aspergillus flavus* and *A. niger* for their tolerance potential against hexavalent chromium. Both the confirmed fungi had remarkable chromium tolerance and removal capability. Chromium removal (mg/g dry wt) was noticed to increase with increasing chromium concentrations.

Khambhaty et al. (2009) isolated three marine-derived aspergilli viz. *Aspergillus niger*, *A. wentii* and *A. terreus* from Gujarat coastal area and were examined for their hexavalent chromium (Cr(VI))removal capacity. Out of the three, *A. niger* was monitored as the most potential candidate for Chromium removal. Complete analyses of biosorption and sorption capability discovered 117.33 mg/g adsorption by *A. niger* under optimized conditions and sorption efficiency was noticed to be 100%. Biosorption process was observed to be endothermic. On the basis of FTIR analysis, amino, methylene, hydroxyl, and phosphorous groups were involved in binding of chromium to fungal biomass.

Taboski et al. (2005) evaluated the toxicity level of Cadmium (Cd) and lead (Pb) to two fungal species *Corollospora lacera* and *Monodictys pelagica* isolated from the marine environment by exploring their growth rate and biomass. Biosorption of metals was also checked. Growth rate of fungi was not affected by lead, though, high cadmium concentration reduced the growth rate of fungi, particularly, *M. pelagica*. About 93% of extracellular lead segregation by *C. lacera* was observed. *M. Pelagica* accumulated about 60 mg/g Cd and about 6 mg/gPb. About 7 mg/g Cd and up to 250 mg/g Pb was accumulated by *C. lacera*.

Khambhaty et al. (2009) studied dead fungal biomass of four marine *Aspergillus* species for Hg(II) biosorption and noticed *Aspergillus niger* as the most efficient Hg (II) biosorbent. Dead biomass of *A. niger* showed 40.53 mg/g Hg(II) removal under optimized conditions. Assessment of possible cell-metal ion interaction disclosed involvement of hydroxyl(–OH) and amino (NH_2) groups present on the cell surface in Hg(II) biosorption.

El-Kassas and El-Taher (2009) isolated hexavalent chromium (Cr(VI)) tolerant strain of *Trichoderma viride* from water samples of the Mediterranean Sea. The fungus could remove 4.66 mg/g Cr(VI). On the basis of transmission electron microscopic (TEM) analysis, it was observed that accumulation of chromium by the fungus did not affect its mycelial and conidial structures. Mendoza et al.(2010) studied that two marine fungal strains of *Dendryphiella salina* were observed to absorb 80–92% Hg^{2+} from the liquid media. Strain Den32 had higher absorption

Table 7.1 Metal biosorption by marine fungal biosorbents (Ayangbenro and Babalola 2017)

Fungal Biosorbent	Metal	Initial Metal Ion Concentration (mg/L)	Sorption capacity (mg/g)	References
Aspergillus Niger	Cu Pb Cr (VI)	100 100 50	15.6 34.6 6.6	Dursun et al. (2003)
Botrytis cinerea	Pb	350	107.1	Akar and Tunali (2005)
Phanerochaete chrysosporium	Cu Pb Zn	100 100 100	88.16 68.73 39.62	Iqbal and Edyvean (2004)
Pleurotus platypus	Ag	200	46.7	Das et al. (2010)
Rhizopus oryzae	Cu	100	34	Fu et al. (2012)

efficiency than strain Den35. The study disclosed the potential application of both the strains for bioremediation of mercury, mainly through biosorption.

Vala and Upadhyay (2008) isolated arsenic tolerating *Aspergillus* sp. from coastal waters of Bhavnagar, Gulf of Khambhat, West coast of India, disclosed the fungus to tolerate supplied 100mg/L As (III) or As(V). Hydride generation atomic absorption spectrometric (HGAAS) analysis revealed higher removal of As (V) than As(III). Energy Dispersive X-ray spectroscopic (EDX) data further confirmed the presence of arsenic in fungal biomass.

Vala (2010) explored removal and tolerance of arsenic by *Aspergillus candidus* isolated from coastal waters of Bhavnagar, Gulf of Khambhat, West coast of India. The fungus showed tolerance for the trivalent and pentavalent forms of arsenic (25 and 50 mg/L). Maximum arsenic removal (mg/g) by the fungus was observed on third day. Vala (2010) also suggested that facultative marine fungus *A. candidus* was one of the most promising fungus for bioremediation of arsenic.

Vala (2010) and Vala et al. (2011) reported that *Aspergillus flavus* and *A. niger,* facultative marine fungi, have tolerance and removal capability for arsenic. Vala (2010) has reviewed *A. niger* as potential biosorbent. This perception was supported by marine-derived fungus *A. niger*. Vala and Patel (2011) explained that heat-killed biomass of marine-derived *A. niger was* studied for its As(III) biosorption capacity, it was noted to remove more than 90% of provided As(III) concentrations. Highest biosorption was found108.083 mg/g at the concentration 600 mg/L. (Table 7.1 depicts the sorption capacity of fungi for various metal ions).

Vala and Sutariya (2012) explored the amount of arsenic tolerance and removal efficacy of two facultative marine fungi *A. flavus* and *Rhizopus* sp. Upon exposure to 25 mg/L and 50 mg/L sodium arsenite (As (III)), both the fungi showed arsenic tolerance and accumulation. A little better accumulation was observed by *Rhizopus* sp. Increase in accumulation was observed with increasing concentration representing higher complexation rates between arsenic and arsenic complexing group on the fungal biomass.

Yeasts from the marine environment have been less studied for heavy metal removal. Strains of *Yarrowia lipolytica* have been reported as potential hexavalent chromium remediators by several workers (Rao et al. 2013; Imandi et al. 2014). Likewise, marine yeast *Rhodotorula rubra* has been exploited for arsenic metabolism (Cullen and Reimer 1989; Maher and Butler 1988). Though, arsenic remediation by marine yeasts has not been attended much attention in the recent past (Vala and Dave 2017). Abe et al. (2001) isolated thirteen yeast strains from deep-sea sediment samples of Japan Trench. Among them, *Cryptococcus* sp. was observed to have the maximum tolerance for Cu^{2+}. The authors also suggested the importance of enzyme superoxide dismutase (SOD) to resist high Cu^{2+} stress.

Deep-sea psychrotolerant yeast isolates *Cryptococcus* sp., when grown in presence of various concentrations of heavy metal salts viz. $CdCl_2$, $CuSO_4$, Pb $(CH_3COO)_2$, and $ZnSO_4$, demonstrated remarkable growth in the presence of 100 mg/lmetal concentrations. Tolerance to these metals showed by the isolate was comparatively higher than other deep-sea and terrestrial yeasts. Modification in the cell morphology was observed in presence of heavy metals. The yeast can remove 30–90% of the provided heavy metals. The authors recommended the *Cryptococcus* sp. as a potential candidate for bioremediation of heavy metal-contaminated sites. The authors postulated the metal-tolerant property and characteristics of the yeast for the contribution to its ecological role and adaptations in extreme environments (Singh et al. 2013).

Oyetibo et al. (2015) isolated both resting and growing cells of mercury-resistant *Yarrowia* spp. from estuarine sediments polluted with mercury. The resting cells of yeast strain were recommended to be applicable as a reusable bioadsorbent, whereas the growing cells were recommended to be more suitable as efficient mercury bioreduction and volatilization agent.

Srivastava and Thakur (2006) reported efficiency of *Aspergillus* sp. for the removal of chromium in tannery wastewater. 85% chromium can be removed at 6 pH in a bioreactor by using synthetic medium, compared to 65% removal from the effluent. Lakkireddy and Kues (2017) studied*Coprinopsis atramentaria* for its ability to accumulate 76% cadmium at concentration 1 mg/L and 94.7% of lead at concentration 800 mg/L. Study of Park et al. (2005) suggested that dead fungal biomass of *A. niger, Rhizopus oryzae, Saccharomyces cerevisiae*, and *Penicilliumchrysogenum* can be used to convert toxic metal Cr (VI) into less toxic or non-toxic Cr (III). It has been reported that catalase enzyme provides heavy metal tolerance capacity to fungi such as lead, copper, zinc, cadmium, etc. It has been observed *A. niger*, *Rhizopus,* and *Penicillium* fungi produce high amount catalase in the presence of heavy metals such as Pb^{2+}, Cu^{2+} (Thippeswamy et al. 2014). *Aspergillus foetidus* has capacity to tolerate lead (Pb) concentration up to 200 mg/L and can produce antioxidative enzymes including catalase for detoxifying H_2O_2 and malondialdehyde. *Aspergillus* spp. Have oxidative stress tolerance for heavy metals like zinc and Copper. (Chakraborty et al. 2013; Mitra et al. 2014; Deshmukh et al. 2016).

Luna et al. (2016) stated that *Candida sphaerica* can produce biosurfactants with the removal of 95%, 90%, and 79% for Fe, Zn, and Pb, respectively. According to

Mulligan et al. (2001) biosurfactants have been widely used in recent years because of their low toxicity, biodegradability, and diversity. Surfactin, rhamnolipid, and sophorolipid can be used for the removal of copper and zinc. A single wash of 0.5% rhamnolipid can remove 65% copper and 18% zinc, while 4% sophorolipid can remove 25% copper and 60% zinc. Chatterjee et al. (2012) reported that *Hansenula polymorpha, S. cerevisiae, Yarrowia lipolytica, Rhodotorula pilimanae, Pichia guilliermondii,* and *Rhodotorula mucilage* can be used to convert toxic Cr (VI) to less toxic Cr (III).

Over the past few years, many bioremediation technologies have been applied all over the world to solve the problem of contaminated environment. Many research and review articles on these technologies for bioremediating heavy metals are available (Khan et al. 2004). Though, there are several gaps in the understanding of heavy metals bioremediation specifically because of the great complexity of soil chemistry. Therefore, expansive and site-specific research is still required to bring out the optimum performance from the technologies of fungal remediation.

7.3.1 Mechanisms of Mycoremediation for the Removal of Heavy Metals

Mycoremediation of heavy metals involves interaction between fungi and metal. Bioremediation process includes adsorption, precipitation, oxidation, reductions, and complexation reactions. The chemical reactions between microorganisms and metals can be categorized into six different processes: intracellular accumulation, cell wall-associated metals, extracellular mobilization or immobilization of metals, metal siderophore interactions, extracellular polymer-metals interaction with transformation and volatilization of metals (Davies and Bennett 1983; Siddiquee et al. 2015).

Gadd (2007) demonstrated that Fungi can use three possible strategies for toxic metal removal: (1) Active metal bioaccumulation in fungal cell and storage in vacuoles and/or passive metals bioabsorption on fungal wall; (2) Metalmobilization/transformation/immobilization in the external environments, due to metabolites and secondary organic acids production; (3) metal exclusion.

Gadd (2007) also mentioned that fungi can restrict entry of toxic metal into cells by these three mechanisms: (1) reduced metal uptake and/or increased metal efflux; (2) metal immobilization, e.g., cell wall adsorption, extracellular precipitation of secondary neoformed minerals (e.g., oxalates); (3) extracellular metal sequestration by, e.g., exopolysaccharides and other extracellular metabolites. Five different mechanisms for the heavy metal removal in fungi are mentioned below; (Fig. 7.3 depicts the mechanisms for the removal of heavy metals).

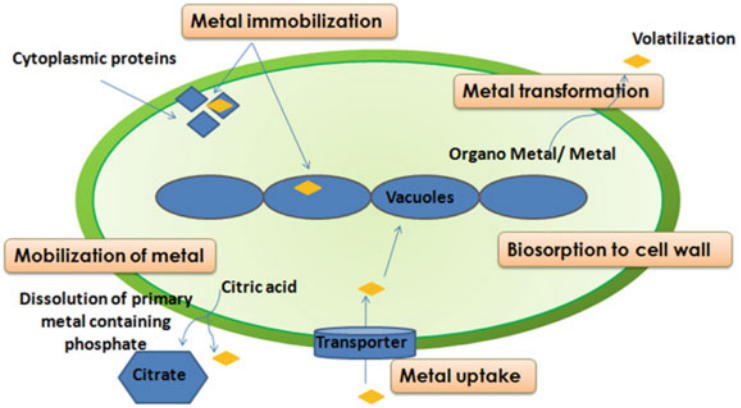


Fig. 7.3 Mechanisms of mycoremediation for the removal of heavy metals (modified from Siddiquee et al. 2015)

7.3.1.1 Mobilization of Metals

Mobilization of metals by fungi takes place due to the production and secretion of fungal products like citric acid, a metal ions chelator and oxalic acid that can interact with metal ions to form insoluble oxalate, which can be resulted from the dissolution of primary metals containing phosphate. These organic acids produced by fungi can increase the metal solubility by acidification and formation of metal-complex structure (Siddiquee et al. 2015).

7.3.1.2 Biosorption to Cell Wall

Fungal cell wall is the protective layer and barrier that controls uptake of toxic metals into the cell and the first cellular components that interact with metal. Heavy metal affects fungal growth and metabolism results in uptake of metals through chemisorption processes (Kapoor and Viraraghavan 1997), which includes adsorption coupled reduction process, ion exchange, precipitation, crystallization, and electrostatic interaction (Pundir et al. 2016). *Aspergillus niger* strains have been found better in biosorption capabilities of heavy metals such as Cu^{2+}, Zn^{2+} and Ni^{+2} at 4 to 6 pH (Siddiquee et al. 2015).

7.3.1.3 Metal Uptake and Translocation through Cell Membrane

Transporters are located on the cell wall of fungi and they are responsible to uptake essential metals. Carriers may consist of all the metabolically-coupled and H^+ gradient-driven transporter system (Siddiquee et al. 2015). Transportation of heavy metals into the cell from the extracellular environment using active or passive

transport mechanism through the cell membrane requires for the removal of heavy metal ions from the environment. Transportation system requires energy for the removal of heavy metals from the aqueous solution (Veglio and Beolchini 1997).

7.3.1.4 Intracellular Metal Immobilization

Intracellular metal immobilization includes two processes that are vacuoles compartmentation and complexation by cytoplasmic protein, called metallothioneins and phytochelatins (Siddiquee et al. 2015). Fungal vacuole plays important roles in molecular degradation, storage of metabolites, regulation of cytosolic concentrations of metal ions and detoxifies potentially toxic metal ions. Metal-tolerant fungi can survive due to their abilities of intracellular chelation, for example, metallothioneins, phytochelatins, and metal sequestration within vacuoles (Liu and Culotta 1999). Metallothioneins is a metal-binding protein that can modulate the intracellular concentrations and bind both the essential metals such as Cu and Zn and inessential metals such as Cd.

7.3.1.5 Metal Transformations

In fungi, biotransformation of metal occurs through chemical reactions such as oxidation, reduction, methylation, and dealkylation. These reactions convert metal ion into non or less toxic form. Chemisorption involves bond formation in chemical group (hydroxyl, amine, phosphoryl, thiol, etc.) present on fungal cell wall or on the surface and substrate to be adsorbed. Chemisorption excludes dependency on metabolic reaction within fungal cell (Bhainsa and D'Souza 2009) and forms strong bonding than ion exchange (Sheoran and Sheoran 2006). Metals may transfer to other parts of the fungi mycelium and plant symbionts by cytosplasmic vesicles and vacuoles. Condensation of heavy metal concentration in the absence of water molecules known as precipitation that helps in removal of contaminants in bulk amount (Gibert et al. 2005; Siddiquee et al. 2015).

7.4 Dye Degradation

Dyes are synthetic chemicals and recalcitrant in nature. More than 1,00,000 commercial dyes including acidic, basic, reactive, azo, and anthraquinone-based dyes are produced every year (Campos et al. 2001). Synthetic dyes are widely used in textile dyeing, color photography, paper printing, food, pharmaceutical, cosmetic, and leather industries. Among various industries, the textile dying industries discharge large amount of wastewater effluent after dyeing process. More than 7×10^5 metric tons of dyes are produced worldwide yearly (Supaka et al. 2004). The amount of dyes that does not bind to the fibers, enters into wastewater during textile processing

(Rai et al. 2005). It has been estimated that 2,80,000 tons of textile dyes are discharged in textile industrial effluents every year worldwide (Jin et al. 2007). Many dyes are visible in water at concentration as low as 1 mg/L (Sandhya 2010). Synthetic dyes can cause environmental pollution and serious health-risk factors due to large-scale production and extensive application (Forgacs et al. 2004).

Based on the chemical structure of the chromophoric group, dyes are classified as azo, triphenylmethane, anthraquinone, polymeric, and heterocyclic dyes. The versatile triphenylmethane and azo dyes account for most textile dyes (Yang et al. 2009). Azo dyes are characterized by the presence of one or more azo bonds [–N=N–] with aromatic ring. Different substitutions on aromatic nucleus give structurally different and versatile group of compounds which makes them recalcitrant and xenobiotic compound (Khan et al. 2013; Jain et al. 2012). Many dyes contain known carcinogens such as benzidine and other aromatic carcinogens (Singh 2006b).

In aquatic environment, dyes can interfere with photosynthetic activities of aquatic flora, diffusion of gases and badly affect food source of aquatic organisms and are of human health concern also. Dye forms thin layer over the surface of a water and thus decreases the amount of dissolved oxygen in the water, therefore adversely affects the aquatic flora and fauna. Dye-containing effluent increases biochemical oxygen demand of the contaminated water (Ciullini et al. 2008; Annuar et al. 2009; Ali 2010). Thus, nowadays degradation of dye is major point of concern.

Due to complex chemical nature, most of the synthetic dyes are highly resistant to degradation (Lin et al. 2010). Physical and chemical treatment methods such as precipitation, coagulation, adsorption, flocculation, flotation, electrochemical destruction, and mineralization and decolorization process have some disadvantages such as cost, time, and release of residues. All these techniques are minimizing the toxicity level but not neutralizing the toxicity. To replace these techniques, biodegradation can be used to completely degrade the dyes (Pandey et al. 2007).

Dye-contaminated industrial effluent is usually treated by physico-chemical processes include membrane filtration, precipitation, ion exchange, flocculation, flotation, ozonation, electro flotation, irradiation, and adsorption using activated carbon or by using bacteria, fungi, algae, plant biomass or other biological material (Robinson et al. 2001). Both living as well as dead cells are used for biosorption. Biodegradation is the most efficient method to remove dyes from industrial effluent and is energy-dependent process that involves the breakdown of dye into various byproducts by action of various enzymes such as laccase, azo reductase, peroxidase, and hydrogenase (Fu and Viraraghavan 2001).

Decolorization of the dye occurs when the chromophoric center of the dye is cleaved (Kaushik and Malik 2009). In biosorption process, the original structure of the dye remains intact and not degraded into fragments. Biosorption plays important role in the decolorization of dye by living fungi (Fu and Viraraghavan 2001). Several microorganisms, including bacteria, fungi, yeasts, and algae, can decolorize and completely mineralize many azo dyes under certain environmental conditions (Pandey et al. 2007). Both live as well as dead fungal biomasses can be utilized to remove dyes from the contaminated ecosystem.

7.4.1 Types of Toxic Dyes

During the manufacture and processing of textiles many different chemical reagents, such as acids, bases, water softeners, salts, and organic solvents dyes are utilized. From the 12 classes of chromogenic groups, azo dyes are largest group of synthetic colorants and the most common synthetic dyes released into environment (Zhao and Hardin 2007) followed by the anthraquinone type. They are widely used in the textile, food, pharmaceutical, cosmetics, plastics, paint, ink, photographic, and paper industries. Different types of dyes include azo, direct, acidic, basic and anthraquinone etc., are mentioned below with its structure and molecular weight in Table 7.2. (Pande et al. 2019).

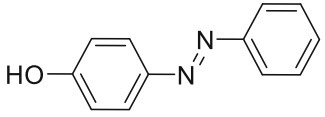
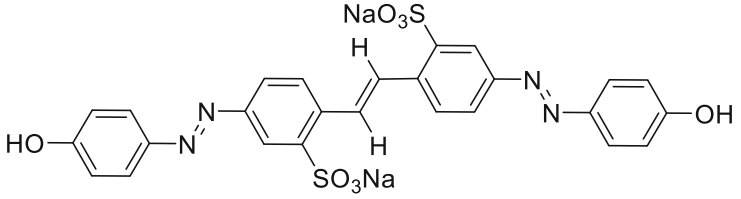
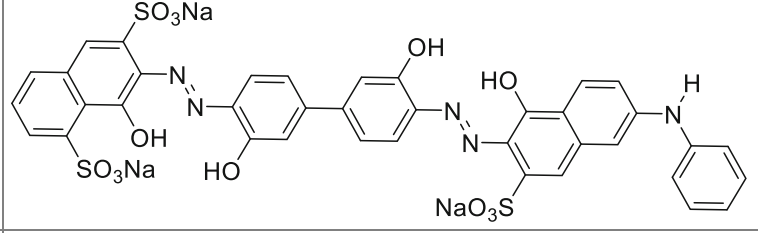
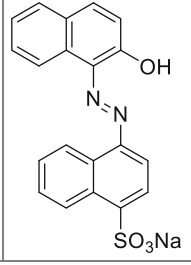
7.4.2 Dye Decolorization and Degradation by Marine Fungi

Marine-derived fungi, due to their adaptability to extreme conditions are better suitable in treatment of colored effluents than their counterparts in the terrestrial environment (Raghukumar 2004; Bonugli-Santos et al. 2015; Vala and Dave 2017). Fungi growing under marine conditions have adapted to grow under saline and alkaline conditions since the pH of seawater ranges from 7.5 to 8.2. *Alternaria tenuissima* (El Aty et al. 2017), *Cerrena unicolor* (D'Souza-Ticlo et al. 2009), *Aspergillus niger* (Lu et al. 2016; Joshi et al. 2012), *Flavodon flavus*, *Penicillium janthinellum* (Wang et al. 2015), *Peniophora* sp. (Bonugli-Santos et al. 2015), *Tinctoporellus* sp. (CBMAI 1061), *Marasmiellus* sp. (CBMAI 1062), and *Peniophora* sp. (CBMAI 1063) (Bonugli-Santos et al. 2012) *Phialophora* sp. (MF 6), *Penicillium* sp. (MF 49), and *Cladosporium* sp. (Torres et al. 2011) are some examples of potential dye degrading fungi.

It has been reported that marine-derived fungi have potential for the decolorization of textile effluents and synthetic dyes such as Congo red, Brilliant green and RBBR (Raghukumar 2004, Raghukumar et al. 2008; D'Souza et al. 2006). According to Arun et al. (2008) the lignolytic extracellular enzymes produced by filamentous fungi have great relevance in bioremediation of toxic dyes. Bartlett (1971) illustrated that Some dyes are used as indicators for production of lignolytic enzymes and also play a very important role in bioremediation of lignin-based derivatives in colored industrial pollutants such as paper and pulp mills, textile mills, tanneries, and molasses-based distilleries.

Baccar et al. (2011) explained that White rot fungi such as *Trametes versicolor*, *Ganoderma lucidum*, and *Irpex lacteus* were evaluated for decolorization of Tannery Dye Black Dycem TTO and suggested adsorption and biodegradation as a key mechanism for removal of dye. Aksu et al. (2007) analyzed that *Trichoderma versicolor* biomass can be utilized for biosorption of Remazol Black B reactive dye. Laccase produced from Marine fungi can decolourize and mineralize high concentrations of pollutants (Vishwanath et al. 2014). *Myceliopthora thermophila*

Table 7.2 Chemical structures of toxic dyes used in the textile industry (modified from Pande et al. 2019)

Types of dyes	Structure	M.Wt.
Azo dye		248.28
Direct yellow 4		624.55
Direct blue 98		928.79
Acid dye		400.38

Basic dye		365.89
Anthraquinone dye		208.21

(*Ascomycetes*) have capacity for decolourization of flexographic inks in presence of mediators (Fillat et al. 2012; Deshmukh et al. 2016).

Young and Yu (1997) stated that Binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation by extracellular and intracellular enzymes are major mechanisms for the dye degradation (Young and Yu 1997). White rot fungi produce lignin peroxidase, manganese peroxidase and laccase that degrades many aromatic compounds due to their non-specific enzyme systems (Robinson et al. 2001; Madhavi et al. 2007). The predominantly reported enzymes for dye degradation are azoreductase, laccases, lignin peroxidase, manganese peroxidase, and hydroxylases. Azoreductase and Laccase have been shown to degenerate azo dyes (Rodrigue et al. 1999).

Chivukula and Renganathan (1995) stated that laccase enzymes can degrade the azo dye through a non-specific free radical mechanism to form phenolic compounds and prevent the formation of toxic aromatic amines. Manganese peroxidase has been reported as the main enzyme involved in dye decolorization by fungus *Phanerochaete chrysosporium* (Chagas and Durrant 2001). In fungus *Bjerkandera adusta,* lignin peroxidase has been reported as important enzyme in dye degradation (Robinson et al. 2001).

Dwivedi and Singh Tomar (2018) explained that *A.allhabadii* and *A. sulphureus* have higher decolorization capacity up to 95.13% and 93.01%, while *A. niger* has little lesser 83% decolorization capacity. Namdhari et al. (2012) explained that decolorization of azo and anthraquinonic dyes can be achieved by brown rot fungi such as *Coprinus micaceus, Fomtopsispinicola*, and *Gloeophyllum odoratum*. Saranraj et al. (2010) isolated *Aserpgillus niger, Aspergillus flavus, Aspergillus fumigatus, Trichoderma viride, Fusarium oxysporum, Penicillium chrysogenum* and Mucor sp. that are responsible for the degradation of a wide range of textile dyes.

Huanga et al. (2016) modified *Aspergillus versicolor* by using cetyl trimethyl ammonium bromide (CTAB) to enhance the fungal biosorption of Reactive Black 5 at various physiochemical conditions. Basidiomycetes fungi have been reported to decolourize dye by adsorption to the mycelial surface and further metabolic breakdown by both batch mode and continuous mode. *Schizophyllum commune* has found to be more efficient than *Lenzites eximia* for the treatment of azo dyes and textile dye industry effluent, (Selvam and Shanmuga Priya 2012). Yesilada (1995) analyzed that *Coriolus versicolour* and *Funalia trogii*are responsible for decolorization of crystal violet dye. Versatile peroxidase and lignin peroxidase have ability to oxidize non-phenolic aromatic compounds, reactive Black B dye and low redox potential was observed as seen from oxidation of phenolic substrates (Karigar and Rao 2011; Deshmukh et al. 2016).

Ollikka et al. (1993) described that isozymes of lignin peroxidase found in*Phanerochaete chrysosporium*has capability for decolourization of synthetic dye. *Phanerochaetechrysosporium* has been observed to degrade many dyes such as polymeric dyes, azo dyes, heterocyclic dyes, and crystal violet dyes. The fungus *P. Chrysosporium* can aerobically degrade three azo dyes includes congo red, orange II, and tropaeolin (Cripps et al. 1990).

Bonugli-Santos et al. (2015) studied that the ascomycetes and basidiomycetes can decolorize 30 to 60% of azo dye-containing effluent and 33 to 80% decolorization of mixture of 8 reactive dyes under saline conditions. Bucher et al. (2004) illustrated that decolorization of azure B is possible due to the production of ligninolytic peroxidase by fungi *Rhizophila marina, Bathyascus grandisporus*, *Verruculina enalia,* and *Cryptovalsa halosarceicola*. It has been found that *Penicillium citrinum* CBMAI 853 is the most efficient fungus that decolorizes RBBR (100%) after 12 days, *A. sulphurous* CBMAI 849 (95%), *Cladosporium cladosporioides* CBMAI 857 (93%), and *Trichoderma* sp. CBMAI 852 (89%) (Da Silva et al. 2008). Molitoris et al. (2000) isolated filamentous halophilic fungi *Gymnoscella marismortui* from the Dead Sea which is responsible for the decolorization of synthetic dye belonging to 4 different groups. Lalitha et al. (2011) explained that marine *Aspergillus flavus* has capacity for the bioremediation of synthetic, color photographic and paper mill dyes and can remove 80% and 90% synthetic dyes and 100% of color. It has been observed that sponge-derived basidiomycetes fungi have ability to decolorize textile dyes in solid medium under both saline as well as non-saline condition (Bonugli-Santos et al. 2012; Vala et al. 2018).

Raghukumar et al. (2008) explained that whole cell immobilization of marine-derived fungi *Penicillium janthinellum* P1 and *Pestalotiopsis* sp. J63 showed the decolorization of Azure B dye. 70% decolorization of MSW in five days has been observed when marine-derived fungus NIOCC #312 is Immobilized on polyurethane foam (PUF) cubes. It has been seen that *Basidiomycetes* fungus, *Flavodon flavus* can decolorize synthetic dyes such as Congo red, Remazol brilliant blue R, Poly-B, and Poly-R. This fungus efficiently decolorized pigments in the molasses spent wash and could also reduce the total phenolic and COD up to 50% and toxicity completely. Verma et al. (2012) described that rapid decolorization and detoxification of anthraquinone dye Reactive Blue 4 can be achieved by enzymatic oxidation and sorption of degraded products on marine-derived fungal biomass. *Saagaromyces ratnagiriensis*, a non-white rot obligate marine ascomycete fungus has capability to decolorize effluent of paper mill (Sarma 2018).

7.5 Biomass Valorization

Rapid exploration of Earth's resources has been steadily increased, giving rise to depletion of resources and rapid generation of waste product (Ferreira 2015). Nowadays, the best way to reduce the waste generation in large amount is their application for the production of value-added products. Valorization is the approach that facilitates sustainable development by value-added products from the waste (Kumari et al. 2018). Waste valorization is the process of converting waste materials into more useful products such as chemicals, reusable materials and fuels (Arancon et al. 2013).

Fungi are heterotrophic and obtain sustenance by hydrolyzing complex material and convert into simple form by taking up and utilizing substance for their

biosynthesis and energy production (Hanson 2008). Filamentous fungi exist in a myriad of environments and have capacity to process complex and diverse substrates include starch or lignocellulosic polymers such as cellulose, hemicellulose, and lignin (Maity 2015). The biomass and byproducts produced by fungi during waste treatment are better valorized than bacteria. Fungi provide valuable enzymes as well as proteins. Several value-added products such as biofuels and biochemicals are produced by industrial cultivation of fungi using industrial waste as substrates.

It has been reported that processing of waste from dairy, sugarcane, tanning, oil, cotton, bioethanol, agro, marine, and poultry industries can be utilized as an attractive alternative source of low-cost organics and nutrients to valorize waste into fungal products with associated waste treatment. (Ali et al. 2020; Mahari et al. 2020; Gaur et al. 2020; Patel et al. 2017; Koutra et al. 2018). Compared to conventional physico-chemical processes, biological processes by using microorganisms including bacteria, fungi, and yeasts are offers a promising technique to produce biofuel while decolorizing recalcitrant synthetic dyes and lignin (Ali et al. 2019, 2020; Kiayia et al. 2019). Waste produced from the agro-industrial could be used as a sugar-based carbon source that can be either used alone or supplemented with various expensive nutrients like yeast extract for the production value-added products such as biodiesel, bioplastic, and exopolysaccharides at laboratory scale and pilot scale. Several value-added products that have been produced from wastes include biofuels like bioethanol and biohydrogen, short-chain organic acids, building-blocks, including 2, 3-butanediol, 1, 3-propanediol, and succinic acid, polymers like bioplastics, i.e., polyhydroxyalkanoates (Koutinas et al. 2014). The major waste generated in paper pulp industry is cellulose-based fibers that can be further treated for the production of useful products like fabric and paper.

Palmqvist and Hahn-Hägerdal (2000) explained that waste generated in pulp and paper industry is spent sulphite liquor (SSL) that can be used for the fabrication of phenolic compounds mainly aromatics syringic, gallic, and vanillic acids. According to Alexandri et al. (2016) the SSL can also be utilized as a raw material for the production of single-cell protein, bioethanol, bioplastics, bacterial cellulose and other valuable products. Mukherjee et al. (2015) stated that food waste can be utilized for the production of another value-added product called hydroxymethylfurfural (HMF), which could be utilized as the precursor of medicines, polymers, resins, solvents, and biofuels.

Similarly, lignocellulosic waste biomass has been used for the production of phytosterols, polypropylene, acrylic acid and esters (Bardhan et al. 2015). The main enzymes identified for lignin degradation include lignin peroxidase (LiP), manganese peroxidase (MnP), and the copper-containing phenoloxidase known as laccase. These ligninolytic enzymes have already been demonstrated utility in the food industry, pulp and paper industry, textile industry, and as biocatalysts (Jaqueline et al. 2010). Studies on utilization of lignin and/or lignin-like dyes by oleaginous yeasts hold much promise for achieving overall efficiency and sustainable utilization of lignocellulosic biomass and textile azo dyes for biofuel production (Ali et al. 2021).

White rot fungi, especially *Phanerochaete*, *Trametes*, *Bjerkandera*, and *Pleurotus* genera have ability to produce several lignocellulolytic extracellular enzymes. In general, the conversion of lignocellulosic biomass includes a pretreatment step for removal of protective lignin seal surrounding structural polysaccharides, followed by enzymatic hydrolysis and subsequent fermentation of released soluble sugars. Thus, the reducing sugars from hydrolysis of cellulose and hemicellulose fractions could be metabolized by other microorganisms producing value-added compounds such as alcohols, flavonoids, organic acids, and phenolics (Sánchez 2009; Mateo and Maicas 2015).

According to the study of pandey et al. (2015) *Aspergillus* spp. is involved in the production of many value-added products including enzymes such as amylase, protease, lipase, phytase, lactase, and catalase. *Trichoderma* spp. can be used for production of cellulose and xylanase. Moreover, *Aspergillus* spp. are responsible for a major fraction of commercial production of organic acids including citric acid, gluconic acid, and itaconic acid and they are also potential sources of malic and oxalic acid. Chitosan is used for production of superabsorbents can be obtained via hydrolysis of chitin from the cell walls of *Aspergillus* spp. and these ascomycetes can be used for production of keratinase hydrolysates (Pandey et al. 2015; Zamani 2010).

Monascus spp. have been important sources of pigments for the food industry and together with *Aspergillus* spp., *Fusarium* spp. and *Neurospora* spp. have been a source of different human food products (Ferreira 2015). However, unicellular ascomycetes, that is, yeasts such as *Saccharomyces* spp., *Pichia* spp., and *Yarrowia* spp. have also been reported to be potential sources of organic acids (such as a-ketoglutaric acid, lactic acid, malic acid and pyruvic acid), polysaccharides such as glucan, proteins like collagen, polyunsaturated fatty acids, sterols (e.g., squalene) and lipids (e.g., ceramides). Marine fungi can potentially play an important role as a bio catalyst in waste biorefineries due to their ability to produce enzymes that can break down these recalcitrant structures. By using filamentous ascomycetes, their biomass, normally rich in proteins and lipids, can represent another value-added product of the biorefinery (Ferreira 2015; Ferreira et al. 2016).

7.6 Conclusions and Future Prospectives

Fungi are considered as natural decomposers which can significantly reduce and degrade various recalcitrant, persistent, and toxic pollutants like hydrocarbons, heavy metals, and dyes. Most of the studies show the role of various extracellular ligninolytic enzymes and cytochrome 450 in the degradation of these pollutants. However, in most cases, the underlying mechanism of the mycoremediation of these harmful pollutants is elusive and needs further research. Mycoremediation can be augmented by adding carbon sources at polluted sites and providing optimum condition to increase degradation process. Naturally present community of microbes acts in concert with the fungi to decompose the harmful contaminants. White rot

fungi are extremely effective in decomposing toxic aromatic pollutants, heavy metals and dyes etc. Further studies could be helpful in understanding the mechanism and optimizing the process of mycoremediation. Benefit is offered that land that is contaminated and unfit for agriculture could be both restored and made to yield a nutritious food crop. Biomass valorization is the process which can convert waste materials into more useful products such as chemicals, reusable materials and fuels. This chapter will help to expand our understanding for the fungi from marine environment as potential candidates for biomass valorization and mycoremediation of hazardous pollutants that would be important for economical, ecological, and legal reasons as well.

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Chapter 8
Marine Bacteria for Bioremediation

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Abstract Marine pollution has been increasing over the years and can impact directly living organisms. The continued pollution of soil and fresh water by agriculture, industrial and urban activities frequently reaches the rivers and the ocean by run over polluting from these environments. Bioremediation is an eco-friendly technique that can immobilize, reduce damage, inactivate or remove contaminants using living organisms or their structures or products for cleaning up the environment. The technique used for bioremediation depends on the type of contaminant, including structure, oxidation stage, complexation form. Bacteria are recognized as important agents in bioremediation processes, including removal of heavy metals, biodegradation of polyaromatic and halogenated hydrocarbons, petroleum and diesel, and biodegradation of plastics. Marine bacteria present a great diversity of metabolic activities and their potential for bioremediation is still poorly exploited.

Keywords Marine pollution · Biodegradation · Biotechnology · Bacterial metabolism · Green technology

8.1 Introduction

Environmental pollution has becoming gradually a more complex and severe issue, generating great concern worldwide. By definition, environmental pollution refers to changes in physicochemical and biological components to such an extent that adversely affects the environmental process and quality of life. The unbalance

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between use of natural resources and its restoring capacity can lead to damage in different spheres, including water, soil, and air (Krishna et al. 2017; Li and Zhou 2020).

Regarding water pollution, the pollutants reaching aquatic environment can be classified as point and non-point origin, being the point source normally from an identifiable effluent discharge and the non-point source being widely distributed over an area. The release of untreated effluents into the environment as a result of different activities in industry is listed as one of the causes of water contamination, along with domestic sewage discharge (Singh et al. 2020). The contaminants with great concern in water can be both inorganic and organic: heavy metals, persistent organic pollutants (POPs), emerging pollutants, pesticides, herbicides, among others (Jeevanantham et al. 2019).

Amongst the effects of water pollution, the nutrient in excess causes eutrophication of aquatic environment. It can be highlighted by the oxygen reduction and photosynthesis damage due to growth of aquatic weed blocking the light entrance in watercourses (Bougarne et al. 2019). This eutrophic condition affects the natural cycle, being responsible for death of several species. Another important impact is the bioaccumulation of contaminants in trophic chain, leading to toxicity to human health (Ali et al. 2019).

When mentioning air pollution, the source of emission and specific climatic conditions from a location make the air pollution to be a complex combination of several particulates and gases (Leni et al. 2020). Among the pollutants affecting air quality, sulfur dioxide, ozone, carbon monoxide, and volatile organic compounds (VOCs) are emphasized. The effects of long-term exposure to air pollution in human health include cardiovascular diseases/increased mortality, along with pulmonary insufficiency and asthma (Manisalidis et al. 2020).

On the other hand, among the sources of soil pollution from anthropogenic activities, it can be mentioned the release of untreated effluent and solid wastes from industrial sector, along with accidental spill and agricultural application of pesticides, herbicides, and fertilizers. The main consequences of soil pollution are related with security in food production, along with the surface runoff carrying contaminants that end up reaching the aquatic environments (Cachada et al. 2018). This consequence poses a serious threat to population, considering the presence and accumulation of several contaminants in water, such as emerging contaminants, heavy metals, and pathological agents, potentially causing contagious diseases and toxicity effects on human health (Zulkifli et al. 2018).

The study and attempt to recover these affected areas and control the environment pollution is increasingly growing, considering the importance for population health and ecosystem equilibrium. The recovery of degraded areas can be done through several different conventional techniques, including the physicochemical methods. However, the use of eco-friendly alternatives, as the bioremediation, is gaining attention and presents cost benefits, in addition to being a more sustainable approach. In this sense, the bioremediation stands out as a technique that is based on the ability of microorganisms in reducing the pollution level through their metabolism (Verma and Kuila 2019).

In the case of heavy metal remediation, for example, some conventional techniques include the membrane filtration, chemical precipitation, coagulation-filtration, electrochemical treatment, solid-liquid separation, among others (Rahman and Singh 2020). The use of bioremediation instead of the aforementioned methods aims to overcome the disadvantage of operation cost and generation of secondary pollution as sludge formation, also being able to remove concentrations in levels that could not be removed by other practices (Leong and Chang 2020).

The marine environment presents a wide variation in its characteristics, changing the temperature, precipitation regimes, pH, salinity, pressure, currents, and prevailing winds. Considering this great environmental variability, marine organisms present great potential of adaptation, thus resisting to several adverse conditions when applied to bioremediation (Dash et al. 2013; Theerachat et al. 2018).

In addition, the marine environment is well known by possessing an enormous diversity of organisms, as yeasts, algae, archaea, protozoa, fungi, microalgae, eubacteria, cyanobacteria, and actinomycetes (Beygmoradi and Homaei 2017). Besides are being studied for bioremediation purposes, some marine organisms have already important potential uses in industry such as the production of biosurfactants, enzymes, and antibiotics (Tipre et al. 2020).

The importance of eco-friendly solutions to mitigate the environment pollution is in evidence, since they are a less aggressive alternative, considering that can be made in situ and without changing great amounts of soil from one location to another (physical changes in landscape), in addition with the fact that microorganisms or plants are used to reduce the contamination level. This alternative presents a high acceptability by the population, besides the fact that it reduces the costs for decontamination (Azubuike et al. 2020).

The understanding of gene pathways and degradation mechanisms is extremely important in discovering organisms that can enhance even more the bioremediation process of a degraded area. Thus, the genetic investigation allows the screening of bacteria with amplified potential of pollution control, along with the screening of genes and increasing the expression of enzymes responsible for degrading the contaminants. In addition, the genetically engineered bacteria are also being studied considering the advances in biotechnological and microbiological areas, where these organisms can be able to enhance the efficiency of remediation of several contaminants (Liu et al. 2019).

Thus, this chapter aims to elucidate the bioremediation principles and general aspects, along with presenting the related techniques, as bioaugmentation, bioleaching, composting, among others, showing the most recent information available. The chapter also focus on illustrating the importance of bacteria for bioremediation of different contaminants, as the removal of heavy metals, biodegradation of polyaromatic and halogenated hydrocarbons, biodegradation of petroleum and diesel, and biodegradation of plastics. Another important point of this chapter is bringing up the potential of marine organisms in bioremediation, along with the innovative use of engineering bacteria for bioremediation. The focus is also to demonstrate the great advances and benefits of application of this eco-friendly technique to remediate polluted environments.

8.2 Bioremediation

The bioremediation approach is based on the use of metabolic systems of microorganisms or plants to clean up the environment. The microorganisms that are used to perform the breakdown or transformation of the pollutant can be naturally existing—thus named indigenous microorganisms—or from other location, intentionally added to the remediation area. The bioremediating may involve several mechanisms executed by the decontaminating agents (bacteria, fungi, or plants), such as degradation, immobilization, and alteration of contaminant, along with its entire removal from the environment (Abatenh et al. 2017).

The bioremediation can be classified into two different types: ex situ and in situ. The in situ treatment is expected to be less expensive and more easily applicable, since there is no need for moving large amounts of soil from one location to another. However, the design and installation of equipment to improve the microbial activities should be considered, which can increase the operation costs (Azubuike et al. 2016). In this technique, the microorganisms are placed in the area, along with oxygen adjustment through aeration and nutrient supply, permitting the surveillance and efficient removal of the target contaminant. It is widely used to remediate petroleum contaminated areas. On the other hand, the ex situ application is based on the removal of soil or water from the contaminated area in order to decontaminate and thereafter, the material is bring back to the original location. The ex situ is known to be more efficient and faster than the in situ method and can be applied in two most common ways, the slurry-phase in bioreactors, and the solid-phase, which includes landfarming, biopiles, and composting techniques (Kumar et al. 2018a, b, c).

The efficiency of bioremediation is directly related with environmental parameters as the pH, moisture content, and temperature. These aspects have direct influence on microbial growth and decontamination rate in such a way that studies in this area aims to enhance the efficiency of the technique through the understanding of the influence of these parameters on bioremediation mechanisms. The researchers intend to make the microorganisms capable to remove contaminants in field conditions, in addition to controlled simulations as laboratory conditions (Sharma et al. 2018a, b).

One of the factors most influencing the bioremediation is the availability of nutrients, since it impacts the growth of microorganisms, reflecting directly in the biodegradation rate and effectiveness. Mainly N, C, and P are essential nutrients for bacterial activities. The adequate C:N ratio is essential in order to guarantee the complete degradation of the target compounds, increasing the metabolic activity of microorganisms (Huang et al. 2018).

The temperature is another parameter widely affecting the bioremediation process, since it determines the survival of organisms and the inactivation/activation of their metabolism. The enzyme activities that participate in degradation of contaminants present a specific optimum temperature, where it reaches the maximum, thus declining when this value is changed. Moreover, this physical parameter is

responsible for directly influencing physiological proprieties of microorganisms (Hong et al. 2020).

Regarding the concentration of oxygen, it influences the degradation condition of contaminants, promoting the aerobic or anaerobic condition. The necessity of aeration aims to increase the oxygen level, since the element is the main electron acceptor for aerobic bioremediation. The pH of environment widely affects the bioremediation process since there are values for optimum growth and removal of contaminants by the microbial metabolism. Any slight alteration from this value can change metabolic processes and affect the removal. The moisture content also affects directly the bioremediation, since each microorganism presents a requirement for its growth and development, and it can change the availability and solubility of contaminants (Kumar et al. 2018a, b, c).

Some additional factors may be considered when the bioremediation is intended to be applied to an area, according to Das and Dash et al. (2014). Firstly, it is related with levels, toxicity, mobility, and degradation property of the contaminants. Thus, it is highly recommended that the location should be scanned and properly characterized, aiming the determination of the real extent of the contamination and probable transport to other areas. Regarding the degradability of the contaminants, this property is essential in determining the type of microorganism able to perform the environmental removal and the time for remediating the area. Some compounds present slow degradation rate due to its high molecular weight, presence of complex ring structures or chlorine in its composition, affecting directly the time for achieving the expected results.

Secondly, it is necessary to verify the vicinity areas in order to prevent the contamination in achieving population and/or environmental receptors. In addition, in order to select the best bioremediation technique for the location, the future use of the bioremediation site might be considered, along with planning the most adequate monitoring of physicochemical and biological parameters (Das and Dash 2014).

Among the inorganic contaminants to which bioremediation can be applied for removal, heavy metals stand out. The bioremediation of heavy metals has widely been studied, as showed by the research of Talukdar et al. (2020), where a consortium using two fungal isolates, namely *Aspergillus fumigatus* and *Aspergillus flavus* was reported as presenting bioremediation potential of highly toxic heavy metals Cr (VI) and Cd (II). This high affinity of fungal consortium reveals great ability for uses in environments contaminated with heavy metals.

The microorganisms that can perform efficient bioremediation are widely listed in the literature, with different contaminants being removed from environment with this technique. Some bacterial genera such as *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Achromobacter* have been extensively used in bioremediation since they present ability of converting pollutants to non-toxic compounds (Liu et al. 2019).

Organic contaminants are also widely studied in research area. The petroleum hydrocarbons have successfully been bioremediated, for example, in a study using vermicomposting process bioaugmentating with indigenous bacterial consortium

isolated from petroleum oily sludge, composed of *Acinetobacter radioresistens* and *Enterobacter hormaechei* (Koolivand et al. 2020).

In addition, among these several organisms that are able to remove several contaminants from environment, the marine organisms are extremely important and are receiving attention of studies, considering the great potential for uses in bioremediation technique (Mohanrasu et al. 2020). The bioremediation of plastics, more specifically low-density polyethylene (LDPE), was reported as efficient by the marine bacteria *Microbulbifer hydrolyticus.* The degradation of LDPE by the strain was evidenced by the modification on material surface seen with scanning electron microscopy and the changes in functional groups identified by infrared spectroscopy analysis. The discovery of a novel microorganism able to degrade polyethylene highlights the relevance for application of this bacteria in bioremediation of other contaminants with further studies (Li et al. 2020).

The advantages of application of bioremediation are related with the possibility of remediation on site, thus reducing the physical disruption of the contaminated area and need for transportation of large amounts of contaminated soil from one location to another. Besides that, the residues generated in treatment are harmless compared to other techniques, being mainly water, carbon dioxide, and cell biomass. Additional benefits that can be cited are the cost effectiveness and popular acceptance, since it is a sustainable and eco-friendly approach not generating chemical hazardous waste and thus not demanding disposal of products. The transformation of contaminants into harmless products allows the technique to avoid the future liability related with treatment and disposal of material (Kumar et al. 2018a, b, c).

On the other hand, the disadvantages of bioremediation are related with limitations regarding the biodegradation of some pollutants, that can be incomplete or even undegradable. The use of microorganisms requires the adequate selection of species with affinity with the target pollutant, besides the environment conditions and nutrient supply that must be satisfactory for the efficient bioremoval. Another important point is the time demanded for the organisms to perform the bioremediation, which can be longer than other techniques (Morillo et al. 2020).

The combination of several contaminants also represents a challenge to microorganisms to overcome and perform bioremediation. In addition to type of contaminants, they can occur in distinct phases, as liquid, solid, or gas. The studies in this area aim to select the most suitable organism for performing the contaminant removal; and another difficulty is going from pilot-scale to real conditions, with different factors affecting the microbial metabolism (Abatenh et al. 2017).

Another important type of bioremediation process is through enzyme activity. Enzymes are biological macromolecules that can enhance several chemical reactions, enhancing the reaction rate by lowering the activation energy of specific substrate molecules. There are several enzymes that can act in biodegradation, being the oxygenases that catalyze the oxidation of aromatic compounds, an important example (Gao et al. 2021). Another example are the carboxylesterases that catalyze the hydrolysis of carboxyl ester bonds, a functional group widely present in synthetic pesticides like organophosphates (Zhang et al. 2020). Finally, the genetic engineering applied to enzymatic bioremediation aims to increase the half-life,

activities, and stability of enzymes, along with production of recombinant types (Sharma et al. 2018a, b).

The future prospects in bioremediation consists in overcome the costs and the need of isolation and characterization of microorganism and enzymes for bioprospecting new organisms. In this sense, the techniques of metaproteomics, metagenomics, and biomolecular engineering can be applied for identification and characterization of organisms naturally found in different environments, besides enhancing the biodegradation ability (Gu et al. 2021). More specifically, metagenomics involves the understanding of genetic material from environmental samples. The technique aims to identify new bacteria or genes encoding for specific enzymes able to biodegrade contaminants (Datta et al. 2020). On the other hand, metaproteomics studies the proteins involved in physiological responses caused by pollutants in the organisms, aiming the identification of new metabolic pathways (Zakaria et al. 2021). Lastly, the biomolecular engineering includes the use of engineering biomolecules as nuclei acids and proteins, with biomolecular processes aiming improvement of bioremediation process (Pandey et al. 2018).

8.3 Bioremediation Techniques

Different bioremediation techniques have been used as management tools for recovering and remediating contaminated environments. In the process of removing environmental contaminants, bioremediation uses biological agents, mainly microorganisms, to eliminate, reduce, contain, or transform dangerous substances into less toxic or non-toxic ones (EPA 2001, 2002; Saxena and Bharagava 2016; Bharagava et al. 2019). Bioremediation is classified into two main types, in situ bioremediation and ex situ bioremediation. Different in situ and ex situ bioremediation methods are discussed in the following sections.

8.3.1 In Situ Bioremediation

In situ bioremediation involves the treatment of harmful substances at the contamination site (Kumar et al. 2018a), with no need to excavate, remove, or transport toxic waste from its original position (Enerijiofi 2021). Pollution is eliminated or mitigated directly where it occurs or at the site of contamination. It is an ecologically correct and low-cost technique, with the possibility of treating large volumes of contaminated material (Mishra et al. 2020).

Different techniques can be employed for in situ bioremediation, such as bioattenuation, bioventing, bioaugmentation, biostimulation, biosparging, bioslurping, biofilters, bioleaching and biomining, phytoremediation and mycoremediation.

8.3.1.1 Bioattenuation

This bioremediation technique is also known as natural attenuation, which involves the passive and unattended remediation of a contaminated area without the need for human interference. Although the bioattenuation process involves the absence of any external forces, regular monitoring is necessary for the process to be sustainable and successful (Azubuike et al. 2016; Vásquez-Murrieta et al. 2016; Enerijiofi 2021).

Bioattenuation includes physical, chemical, and biological processes, which cause a reduction in the concentration of pollutants, mass, or toxicity (Vásquez-Murrietaet al. 2016). Anaerobic and aerobic microbial processes in the treatment of recalcitrant and biodegradable pollutants are also involved (Enerijiofi 2021). This technique is applicable to contaminated sites with a low concentration of contaminants, where other remediation techniques do not apply (Vásquez-Murrieta et al. 2016; Ossai et al. 2020).

8.3.1.2 Bioventing

In the process of degradation using the bioventing technique, nutrients and air (oxygen) are supplied in a controlled manner through wells to the contaminated site (i.e., soil) to stimulate microbial growth and proliferation (Enerijiofi 2021). This technique uses reduced airflow rates, providing only the amount of oxygen needed for biodegradative processes, reducing volatilization and release of pollutants into the atmosphere to lower levels (Atlas and Philp 2005).

Biodegradation involves the supply of oxygen and nutrients through aqueous solutions in contaminated soils, allowing the stimulation of native bacteria, thus facilitating the decomposition of biogenic pollutants (Tyagi and Kumar 2021). This technique is applied for removing pollutants in the depths of the surface (vadose zone), biodegradation of petroleum hydrocarbons, reducing volatile compounds (VOCs) rising to the surface, spilled light oil, absorbed fuel residues, non-chlorinated solvents and pesticides (Höhener and Ponsin 2014; Kumar and Gunasundari 2018; Olu-arotiowa et al. 2019).

Some factors must be considered before applying the bioventing technique, such as adequate concentration of pre-existing microorganisms; airflow conditions; the soil pH (6 to 8); hot temperature and water table height—when the water table is close to the surface, then the bioventing is not effective. Other conditions for the ineffectiveness of bioventing are low temperatures and moisture content in extremes levels, low or high (Kumar and Gunasundari 2018).

8.3.1.3 Bioaugmentation

Bioaugmentation involves adding native microbial and exogenous microbial cultures, microbes or genetically modified communities with specific catabolic activity,

at the contamination site, to increase the rate of biodegradation (Andreolli et al. 2015; Nwankwegu and Onwosi 2017; Poi et al. 2017; Enerijiofi 2021).

At bioaugmentation, active agents are characterized by the ability to degrade the contaminant and their resilience to the prevailing environmental conditions, as well as its genetic stability and ability to survive under competition (Gentry et al. 2004).

Bioaugmentation can be applied for treating wastewater and contaminated soil. It is used in the degradation process of substances such as polyvinyl compounds, PAHs (pyrene and benzo[a]pyrene, pesticides, insecticides, trinitrotoluene (TNT), aromatic and chlorinated hydrocarbons) (Kumar et al. 2018b; Olu-arotiowa et al. 2019; Sarkar et al. 2020). It has also been widely used in the treatment of medium and long chain alkanes, xenobiotic compounds, water and soils contaminated with polycyclic and monocyclic aromatic hydrocarbons and others (Akinde et al. 2012; Pal et al. 2017; Varjani 2017).

8.3.1.4 Biostimulation

Biostimulation is applied in the treatment of pollutants such as metals and hydrocarbons (Ossai et al. 2020). It consists of the addition of materials (biosurfactants, bulking agents, biopolymers, nutrient correctives, and slow-release fertilizers) to stimulate the growth of indigenous microorganisms capable of degrading various compounds (Adams et al. 2015; Agarry and Latinwo 2015; Lim et al. 2016; Wu et al. 2016).

Substrates containing micronutrients (copper, chlorine, iron, magnesium, manganese, silicon, sodium, and zinc), macronutrients (phosphorus, potassium, and nitrogen), and organic nutrients are essential to improve the degradative capacity of foreign or indigenous microorganisms, and as an example, the concentration and the types of nutrients interfere with the biodegradation of polycyclic aromatic hydrocarbons (PAHs). The ability to deliver nutrients, C:N:P ratio 30:5:1 for equilibrium growth, presence of target microorganisms, ability to stimulate target microorganisms, are requirements for biostimulation (Hazen 2010; Ossai et al. 2020).

8.3.1.5 Biosparging

In the biosparging technique, air (oxygen) and nutrients are injected into the saturated zone under pressure to stimulate the biological activities of indigenous microorganisms to degrade contaminants, thus increasing the concentration of oxygen in ground water (Azubuike et al. 2016; Enerijiofi 2021). This technique is similar to bioventing (Kumar et al. 2018b), being used to reduce the concentration of the contaminant dissolved in ground water or adsorbed to the soil (within the capillary fringe above the water table).

Factors such as pollutant degradability and soil permeability interfere with the effectiveness of the technique (Philp and Atlas 2005; Kumar et al. 2018a), as well as

predicting the direction of the injected airflow can limit its application, along with a high rate of airflow to achieve the volatilization of the pollutant and promote degradation (Ossai et al. 2020).

Biosparging is widely used in the treatment of aquifers contaminated with medium-weight petroleum hydrocarbons (jet and diesel fuels) and other compounds (xylene, benzene, ethylbenzene, and toluene) (Kumar and Gunasundari 2018; Kumar et al. 2018a).

8.3.1.6 Bioslurping

Bioslurping or multiphase extraction is used to remove the free product that is floating on the water table. It combines vacuum-enhanced free product recovery with bioventing. Thus, vacuum-enhanced free product recovery extracts Light Non-Aqueous Phase Liquid (LNAPLs) from the capillary fringe and groundwater (Kumar and Gunasundari 2018; Mishra et al. 2020).

Bioslurping combines several processes, extracting steam from the soil, vacuum pumping, bioventilation, to remove contaminants from groundwater and soil, using an indirect supply of oxygen and stimulating microbial biodegradation (Vidali 2001). The low permeability of the soil reduces the rate of oxygen transfer by limiting microbial activity, which limits the use of the technique.

As bioventing is used in the bioremediation of contaminated soil/water, a combination of techniques (bioventing and bioslurping) can be designed, mainly to control the release of gas from soil and groundwater. Petroleum hydrocarbon compounds and LNAPLs are treated using bioslurping and bioventing. This technique is economical, and applicable to places with deep water table (Kumar and Gunasundari 2018; Mishra et al. 2020).

8.3.1.7 Biofilters

Biofilters are used to degrade pollutants (gaseous) in industrial air emissions. Columns embedded with microbes are applied to eliminate gaseous pollutants (Boopathy 2000), which means that the community of microorganisms that grow on a solid surface is able to immobilize or degrade the pollutant. Thus, the air containing toxic pollutants when passing through the biofilter is adsorbed on biofilms, incorporated into microbes, degrading the pollutant. Several materials are applied as biofilters, among them, peat, soil, agricultural residues (husks), and others. Biofilters are used to remove terpenes, mercaptans, dimethyl sulfides, hydrogen sulfide, ethylbenzene, sulfur gases, and other compounds from the air (Boopathy 2000; Kumar et al. 2018b).

8.3.1.8 Bioleaching and Biomining

The solubilization of sulfide ore metals or solid residues in aqueous solutions using live microorganisms is known as bioleaching or biomining (Shukla et al. 2017). Bioleaching occurs at the mineral–microorganism interface. Most of the microorganisms that participate in bioleaching grow attached to the surfaces of sulfide ores, as natural biofilms (Senel and Hanay 2017; Shukla et al. 2017). Bioleaching can be applied to recover metals. As it involves the use of microorganisms, the following factors must be considered in its application: (a) the microbial ability to resist toxicity and the heterogeneity of the waste; (b) type of microorganism and its growth rate; (c) inoculum concentration and cell genotype; (d) physical–chemical conditions of the pollutant and microorganisms; (e) nutrients required by microorganisms (Priya and Hait 2017; Minimol et al. 2020).

On an industrial scale, the process of bioleaching can be carried out through bioreactors, considering the behavior and principles of bioleaching of a specific microbe. In this sense, the bioleaching process can be employed by continuous or batch operation mode.

When the microorganism and the material containing the metal are simultaneously added and incubated for solubilization, the technique is named one-step bioleaching (Yang et al. 2008; Rasoulnia and Mousavi 2016). Thus, when using this technique, microbial growth can be inhibited by the presence of metals leached in solution, which reduces the leaching rate. Microbes can take longer to acclimatize in the medium containing leachate metals, so the latency phase would be longer; leachate metals can be adsorbed, accumulated, or used in cells, thus reducing metal recovery (Jagannath et al. 2017).

When the addition of metal-containing solids after the pre-cultured microorganism reaches the late exponential growth phase, it is named two-stage bioleaching (Pradhan and Kumar 2012; Işıldar et al. 2016). In this process, the inhibitory effect of metals on growth is reduced, but, even so, it can lead to adsorption and accumulation of metals leached in cells, which can reduce the recovery capacity, and reduce the time in the growth delay phase (first stage) (Minimol et al. 2020).

The average spent bioleaching is the dissolution of metals from solids in the cell-free supernatant that contains extracellular proteins and secondary metabolites (Ilyas et al. 2013; Natarajan and Ting 2013). Thus, in this process, extracellular proteins and metabolites, which tend to be secreted only in the presence of metals, would not be available for any leaching action. However, the inhibitory or toxic effect of metals on microbial growth can be prevented. As for the time required for microbial growth, this may be shorter during the production of the medium, as the latency phase period is shorter and the growth rate in the absence of metal-containing residues is higher (Minimol et al. 2020).

To select which type of bioleaching process will be applied, requirements such as metal–microbe interaction involved, inhibitory nature of the metals, and type of microbes chosen must be considered.

Bioleaching is a low-cost and simple technology that can be applied in the removal of mineral compounds and low-quality ore metals, as well as in the treatment of industrial effluents. The technique is applied in biomineral processing, because the process of bioleaching metals by microbes has the possibility of recovering metals from mineral resources not available by conventional mining. Thus, microorganisms act as biocatalysts in the conversion of metallic compounds to their water-soluble forms in the process of bioleaching (Varjani et al. 2018).

8.3.1.9 Phytoremediation

Phytoremediation (from Greek "phyto," plant and Latin "remedium," cure) refers to the use of plants to contain, reduce, confiscate, accumulate, extract, eradicate, and/or decontaminate organic and inorganic contaminants from soils, sediments, surface, and groundwater (Sharma et al. 2018a, b; Enerijiofi 2021; Mishra 2021; Saxena et al. 2021).

It is one of the methods in situ, ecologically correct, it is a process considered inactive, cheap, with low specialization. The main contaminants that can be removed by phytoremediation are solvents, crude oil, hydrocarbons, pesticides, metals, explosives, and landfill leachate (Wang et al. 2017).

The phytoremediation process can be broadly classified based on fundamental processes, type of contaminant, and applicability (Table 8.1).

Thus, in phytoremediation of organics, the plant metabolism contributes to the reduction of contaminants through the breaking, transformation, volatilization, or stabilization of contaminating compounds in water and soil. In this way, phytodegradation breaks down the organic compounds absorbed by the plant, into simpler molecules and incorporates them into its tissues. Plants contain enzymes like oxygenases, dehalogenases, and reductases that can convert and degrade chlorinated solvents, ammunition residues, and others (Ghosh and Singh 2005; Malode et al. 2013; Mishra 2021; Saxena et al. 2021).

8.3.1.10 Mycoremediation

Due to the ability to produce and secrete extracellular enzymes, such as laccases, polyphenol oxidases, lignin peroxidases, which can break down cellulose and lignin, fungi are used to degrade several environmental recalcitrant pollutants (Singh 2006; Jang et al. 2009). Thus, mycoremediation uses fungal mycelia to carry out the biodegradative process, which converts contaminants in less toxic or non-toxic forms, remediating contaminated soils and groundwater (Gadd 2001; Singh 2006; Anderson and Juday 2016; Ali et al. 2017; Kumar et al. 2018c; Enerijiofi 2021).

Fungi are often more tolerant to pollutants and their hyphae are able to penetrate the soil and reach the pollutant much faster than other microbes (Reddy and Mathew 2002; Harms et al. 2011). They end up being more efficient in bioremediation than other microorganisms. White rot fungus has been used for biotransformation of

Table 8.1 Types of phytoremediation

Technique	Description
Phytodegradation	This technique involves the absorption, storage, and decomposition of contaminants by the activity of proteins and enzymes produced by plant tissues and microorganisms present in the rhizosphere (Enerijiofi 2021; Saxena et al. 2021).
Phytoextraction	In this method, plants accumulate and transport pollutants in aerial part and produce a mass of plants containing pollutants that are later transported for recycling and/or disposal (Mishra 2021; Saxena et al. 2021).
Phytofiltration	It uses the absorption capacity and the large surface area of the entire plant to remove the pollutant, for example, heavy metals from wastewater (Malode et al. 2013). The mechanism is similar to phytoextraction and aquatic plants and terrestrial plants can be used in phytofiltration.
Rhizofiltration	In rhizofiltration there is a mutual relationship between the plant roots and rhizosphere microorganisms creating an environment where pollutants are broken down through metabolic activities and secretion of proteins and enzymes from the plant's root system (Malode et al. 2013; Enerijiofi 2021; Mishra 2021; Saxena et al. 2021).
Rhizodegradation or Phytostimulation	Contaminants are degraded by microbial activity in the rhizosphere. It is a slower process than phytodegradation. In this technique, microorganisms consume and digest organic substances such as fuels and solvents (Malode et al. 2013; Saxena et al. 2021).
Phytostabilization	The pollutant in the soil or water is immobilized by absorption or precipitation by the plant roots, reducing their mobility by forming a stable vegetable mass with the pollutant, thus preventing the entry of the pollutant into the environment (Mishra 2021; Saxena et al. 2021).
Phytotransformation	The technique involves the process by which the plant absorbs and transforms highly toxic organic contaminants, from a contaminated site (soil, sediment, and water body) into less toxic forms (Mishra 2021; Saxena et al. 2021).
Phytovolatilization	The technique uses plants to remove volatile pollutants from the environment. Plants absorb pollutants and transfer them to gaseous substances, which are then released into the atmosphere (Malode et al. 2013). Volatile organic compounds are the best candidates for this process to be treated with the aid of evapotranspiration (Mishra 2021).

pesticides, degradation of petroleum hydrocarbons and lignocellulolytic wastes in the pulp and paper industry. Some common types of macroscopic fungi used in mycoremediation are described in Table 8.2.

Mycoremediation can be effective in breaking down some chlorinated compounds and petroleum hydrocarbons, as well as being, capable of stimulating native microbes and enzymes in situ, and bioaccumulating heavy metals, reducing contamination (CRC 2018). It also employs three types of mechanisms (mycosorption, mycodegradation, and mycoenzymes) for the removal of organic compounds

Table 8.2 Common fungi used in mycoremediation. Source: CRC (2018)

Type of fungi	Target contaminants
Button mushrooms	Cadmium
Elm oyster	Dioxins, wood preservatives
King Stropharia	*E. coli* and other biological contaminants
Pearl oyster	Cadmium, mercury, dioxins, polycyclic aromatic hydrocarbons (PAHs); polychlorinated biphenyls (PCBs)
Phoenix oyster	Cadmium, copper, mercury
Shaggy mane	Arsenic, cadmium, mercury
Shitake	PAHs, PCBs, pentachlorophenol
Turkey tail	Organophosphates, PAHs, mercury

(Noman et al. 2019). Some parameters must be considered when applying the mycoremediation technique, such as soil pH, temperature, and availability (or lack) of oxygen. Mycoremediation is a sustainable, low-cost, low-maintenance technique, presenting fast results (usually completed in a short time (weeks/months)) (CRC 2018).

8.3.2 Ex Situ Bioremediation

Ex situ bioremediation involves the collection/transport (excavation or pumping) of polluting substances from the place of origin to a controlled and designed area, where their decontamination will occur through physical–chemical or biological methods (Varjani et al. 2018). Decontamination involves the treatment of the pollutant after it is transported from the area of occurrence to the treatment area, which facilitates microbial degradation. This removal of the pollutant from the place of origin to the treatment area increases operational costs (Azubuike et al. 2016).

Different techniques can be applied for ex situ bioremediation, such as landfarming, composting, biopiling/biopiles or windrows, bioreactors. These techniques are used efficiently in the treatment of contaminated soil or water (Ghangrekar et al. 2020). Ex situ bioremediation techniques can be easier to control and faster, in addition to the possibility of treating a wide range of contaminants and soil types, in comparison with in situ techniques.

When the treatment of contaminated surface or underground water occurs through the extraction of contaminated water from the place of origin to the place of distant treatment, its remediation is known as the "pumping and treatment system." This system can take years to treat the contaminated area, because the complete discharge of the pollutant requires that a large volume of water be treated continuously over a long period of time (Varjani et al. 2018).

8.3.2.1 Landfarming

Landfarming is a biodegradation technique consisting of excavating and extracting contaminated soil and spreading it over a thin area (the soil layer support fixed above the ground) and periodically plowing the soil to stimulate aerobic biodegradation of pollutants by indigenous microbes (Senel and Hanay 2017; Yadav et al. 2017; Mishra et al. 2020).

Landfarming can be applied only for the treatment of 10–35 cm of soil surface (Kumar et al. 2018a) and excavation of contaminants is not recommended if the polluted soil is less than one meter deep (Kumar and Gunasundari 2018). The application of landfarming is advantageous because it has reduced maintenance and monitoring costs, it is simpler and more economical, however, the main disadvantage of this process is the limitation to treat 10–35 cm of top soil (Williams 2006; Yadav et al. 2017; Mishra et al. 2020).

In the landfarming process, nutrients, microorganisms, and moisture can be added to the soil. By providing moisture to the soil through seasonal spraying of water, it forms a barrier around the contaminated soil, controlling erosion and minimizing the formation of dust when plowing the soil to maintain aeration (Kumar and Gunasundari 2018). Plastic or clay liners can also be used on site, before laying contaminated soil, to prevent the leaching of contaminants into groundwater (Senel and Hanay 2017).

Thus, the treatment of contaminated soil occurs through biodegradation combined with aeration and light photooxidation (Senel and Hanay 2017). This type of treatment is more efficient in areas with hot and humid climate, and in a sunny place. Therefore, during periods with colder temperatures (winter), when snow covers the soil, there is a reduction in the biodegradation process (Mishra et al. 2020).

Although land cultivation is considered an ex situ bioremediation technique, in some cases it applies to in situ bioremediation. This technique can be applied in the treatment of sites contaminated by PCBs, aliphatic hydrocarbons, and polycyclic aromatics (Silva-Castro et al. 2012; Kumar et al. 2018b).

8.3.2.2 Composting

Composting is a technique that involves mixing polluted soils with bulking agents, for example, harmless organic additives (organic manure, wood chips, and agricultural waste such as plant waste) (Kumar et al. 2018b). The additives favor the growth, development, and proliferation of microbial populations (bacteria) capable of degrading the pollutants found in the soil to be treated (Adam et al. 2017). Thus, the pollutants will be degraded and converted into a stabilized final product, which can be applied as a soil conditioner to remedy soil contaminated with organic compounds or be safely discarded in the environment (Cai et al. 2017; Saum et al. 2018; Ren et al. 2018; Kumar et al. 2018b).

In the composting process, parameters like the addition of appropriate microbial consortia and nutrients, cultivation, irrigation, as well as bulks materials in the form of organic residues to improve bioremediation, must be considered (Prakash et al. 2015). This process is conducted outdoors and can be carried out by means of composting in open and static windrow systems. In static windrow systems, the piles are aerated by a forced air system, while in open windrow systems, the compost is loaded in elongated stacks (Cunningham and Philip 2000; Girma 2015).

In order to properly compost soils contaminated with dangerous compounds, for example, petroleum hydrocarbons, the composition needs to occur in a thermophilic phase of 50–65 °C. During the decomposition process of organic materials present in the compost, there is an increase in temperature due to heat generated by microbial activities (Prakash et al. 2015; Ossai et al. 2020). Thus, the aerobic process is viable because the heat produced during the biodegradation reactions with the release of oxidative energy can lead to a substantial increase in temperature (Adam et al. 2017). The efficiency of degradation and the growth of microorganisms must be monitored under the level of humidity, aeration, and temperature.

The composting technique can be used to treat explosives (HMX, TNT, and RDX), hydrocarbons, dangerous chemicals, and others (Kumar et al. 2018b).

8.3.2.3 Biopiles or Windrows

Biopiling or stacking technique is a combination of methods of agriculture (landfarming) and composting, in anaerobic cells designed with an irrigation and nutrient system, vacuum pumps and blowers, leachate collection system for bioremediation of polluting components adsorbed to sediments and soil (Wu and Coulon 2015; Benyahia and Embaby 2016; Kim et al. 2018; Mishra et al. 2020).

The technique involves stacking a contaminated excavated soil, which can be placed in piles (biopiles) or rows (windrows) and subsequent soil correction with nutrients, biostimulation, and forced aeration to increase microbial degradation activities (Azubuike et al. 2016; CRC 2018).

As a hybrid system, biopiling is a favorable system to stimulate microbial growth (anaerobic microorganisms and indigenous aerobes). This system increases aerobic creosote catabolism by inoculating air in piles of contaminated soils (Kumar et al. 2018a) and is suitable for the treatment of large volumes of contaminated sediments and soils in a limited space and in addition to remediating contamination in extreme environments (Whelan et al. 2015).

Biopiles can have heights in the range of three to ten feet (about 1 to 3 m) and can be applied in the treatment of surface contaminants in order to control the physical losses of the contaminants by volatilization and leaching (Azubuike et al. 2016; Kumar et al. 2018a). It can also be used to treat soils contaminated with low molecular weight pollutants and petroleum hydrocarbons (phenols, PAH, sand BTEX). However, biopiling can limit the volatilization of low molecular weight compounds (Dias et al. 2015; Ossai et al. 2020).

Biopiles involve some limitations, when compared to other ex situ bioremediation techniques, as they require much more space and when there is an extreme heating of the air, it leads to excessive drying of the soil to be treated, therefore occurring an interruption of microbial activities (Sanscartier et al. 2009).

8.3.2.4 Bioreactors

A bioreactor involves a controlled container to create a three-phase mixture condition (solid, liquid, and gaseous) to increase the rate of degradation of the water-soluble and soil-bound contaminant. Unrefined materials are transformed into specific non-toxic products due to a series of different biological reactions. Thus, bioreactors are used to degrade contaminants under controlled conditions. They are designed containment systems (reactors or aqueous sludges) that are applied for the treatment of polluted solid materials (sediment, soil, and sludge) and water (Vidali 2001).

Bioreactors are used to treat water or soil contaminated with volatile organic pollutants (toluene, xylene, ethylbenzene, and benzene) (Azubuike et al. 2016). Its use in the biodegradation process is much more efficient, faster, and beneficial than in other techniques, because in these systems, bioremediation occurs in a controlled way. Therefore, parameters such as pH, temperature, amount of nutrients, humidity, bioavailability of pollutants, mass transfer, and others are monitored to improve the biological reactions used in the biodegradation of pollutants, increasing efficiency in the bioremediation process (Mohan et al. 2004; Azubuike et al. 2016; Kumar et al. 2018a).

The main disadvantage of using bioreactors is due to the fact that it spends extra safety measures in transporting the pollutant from the place of origin to the treatment site, as well as treating high volumes of contaminants, which increases the cost of applying the technique (Philp and Atlas 2005; Kumar et al. 2018a). Another disadvantage is that contaminated soil requires pre-treatment, or the contaminant should be removed from the soil through physical extraction or soil washing, before being placed in a bioreactor (EPA 2002; Mishra et al. 2020).

8.4 Importance of Bacteria for Bioremediation

Bioremediation has been considered as the remediation of pulled sites employing microbes, and in this context, bacteria play a fundamental role (Azubuike et al. 2016). As discussed above, all the bioremediation approaches have been classified as in situ or ex situ depending on the polluted material sites. Bioremediation is mainly a prospective method for in situ subsurface remediation with a reduced cost and production of secondary pollution compared with other environmental remediation techniques, these are the motivations for the consideration as eco-friendly and cost-effective approach (Wei et al. 2012; Igiri et al. 2018). Furthermore, two main

strategies have been employed to improve the effectiveness of in situ bioremediation, specifically, bioaugmentation that comports the addition of pre-grown microbial cultures to enhance the degradation of unwanted compounds; whereas biostimulation includes the injection of nutrients and other supplementary components to the native microbial population inducing the propagation at a hastened rate (Tyagi et al. 2011). Thus, for the bioremediation the selection of the appropriate strategy to treat a polluted site has become pivotal, three basic principles have to be considered: the pollutant tractability for biological transformation to less toxic products, the physical accessibility of the contaminant to microorganisms, and also the opportunity for optimization of biological activity (Dua et al. 2002). In this context, the wide metabolic diversity of bacteria is crucial for bioremediation of many different contaminated sites.

8.4.1 Removal of Heavy Metals

Microbial activities strongly influence metal speciation and transport in the environment. In bacteria a number of specific resistance mechanisms, indeed, including active efflux and sequestration or transformation to other chemical species become functional at concentrations above the homeostatic or non-toxic levels (Silver 1998).

For this reason, different organisms exhibit diverse responses to toxic ions, which confer upon them a certain range of metal tolerance. A family of metal-chelating proteins named metallothioneins (MTs) represent a typical mechanism for regulating intracellular metal ions. Bacterial MTs were observed to confer resistance to Zn (II) and Cd (II) and have been considered as the main mechanism of tolerance to metals in the bacterial world (Robinson et al. 2001; Blindauer 2011). These mechanisms of resistance to metal ions are often plasmid-borne, which facilitates dispersion from cell to cell. A common example of heavy metal resistance was observed in *Ralstonia metallidurans* CH34 previously classified as *Alcaligenes eutrophus* CH34 (Goris et al. 2001).

The sulfate-reducing bacteria (SRB) represent another important process involved in the bacterial resistance to heavy metals. They have significant ecological functions in anaerobic conditions for complete mineralization of organic carbon particularly in marine sediments. Furthermore, these bacteria play an important role in bioremediation of contaminated sites (Jørgensen 1982; Ayangbenro et al. 2018). Sulfate can be biologically reduced to hydrogen sulfide by SRB with significant ecological functions, but also play an important role in bioremediation of contaminated sites. SRB can react with the dissolved heavy metal ions and transform them into highly stable metals sulfides, which are usually are more stable than the hydroxides produced by chemical treatment. In addition, metal sulfides can be recycled and reused (Jalali and Baldwin 2000; Kiran et al. 2017; Li et al. 2018).

Finally, another class of bacteria named acidophilic chemolithotrophs shown efficient growth at high metal concentrations, these are iron and sulfur-oxidizing bacteria. The metal resistance of these microbes is an adaptation to very acidic

environments, where metal solubility is high including the thiobacillus group of bacteria. Interestingly, when the growth of *Thiobacillus ferrooxidans* is dependent on Fe (II), this bacterium is highly resistant to Al, Cu, Co, Ni, Mn, and Zn (0.1–0.3 M), although it remains quite sensitive to other metal ions (Hutchins et al. 1986).

Different strategies have been contemplated to improve the bioremediation of heavy metals (Kocur et al. 2016; Liu et al. 2018). In this context, microbes represented an optimal strategy, as they have various mechanisms in response to heavy metals that can be applied for bioremediation of various contaminated sites (Kushwaha et al. 2018; Cornu et al. 2017; Ahemad 2019). In fact, as the role of bacteria and plants in biotransformation of heavy metals into non-toxic forms is well documented, the study of these biological elements at the molecular level permits to detect the mechanism of metal accumulation opening to biotechnological implications for bioremediation of metal-contaminated sites (Dixit et al. 2015). However, for the microbial side the success of the bioremediation action in pulled places depends mainly upon the kind of microorganism and the contaminants involved in the process (Kapahi and Sachdeva 2019). The combination of different biological strategies could lead to an improvement in bioremediation performance. Recently the triple treatment combining bacteria, plants, and invertebrate-like earthworms showed best yields in terms of removal rates and soil health improvements (Urionabarrenetxea et al. 2021).

8.4.2 Biodegradation of Polyaromatic and Halogenated Hydrocarbons

For the persistent organic pollutants, different bioremediation approaches need some improvements due to the long period of biological remediation determined by low degradation rates and the insufficient energy source (Atlas and Hazen 2011). Nowadays petroleum derivatives and organic waste are among the main environmental contaminants. Recently, the bacteria–fungi associations have been improved for the oil bioremediation of soils, making the process more robust against environmental changes that can perturb the bioremediation by bacteria (Quintella et al. 2019). To overcome the process velocity problem on the bioremediation of organic pollutants, the association of plants and bacteria, such as endophytic bacteria and rhizosphere bacteria, has been employed to contribute on the biodegradation of toxic organic compounds in contaminated soil with the potential improving of the bioremoval process (Divya and Deepak Kumar 2011; Gkorezis et al. 2016; Arslan et al. 2017).

Halogenated hydrocarbons are synthesized as industrially valuable materials, or produced as a by-product during chemical reactions, or being released as a result of burning of municipal waste; these are ubiquitous in our environment and several bacteria have been isolated and reported acting on their degradation (Akram et al. 2021). The potential of the nitrification process, where ammonia is oxidized to

nitrate, has been proposed for in situ bioremediation of halogenated compounds. In this process, ammonia-oxidizing bacteria play a key role starting the degradation of many halogenated hydrocarbons (Sayavedra-Soto et al. 2010).

Some microbes have evolved enzymes and metabolic pathways to detoxify and utilize halogenated aromatic compounds as their sole carbon sources. The degradation pathways can be simplified into three stages: an initial debranching process to remove additional moieties attached to the aromatic ring, an intermediate pathway dealing with halide removal and activation of aromatic rings by incorporation of oxygen (in the case of aerobes), and a final stage of ring-cleavage reactions to convert aromatic into aliphatic molecules which can be further converted into common metabolites of central metabolic pathways such as pyruvate, acetyl CoA, succinate, oxaloacetate, and acetaldehyde (Pimviriyakul et al. 2020). Among bacteria, *Pseudomonas* becomes a paradigmatic bacterial genus for the catabolism of aromatic compounds and for the bioremediation of toxic pollutants and the valorization of aromatic compounds present in biowaste (Nogales et al. 2017).

8.4.3 Biodegradation of Petroleum and Diesel

Organic pollutants such as petroleum-derived hydrocarbons are bio-transformed in presence of optimal ecological factors and the necessary nutrients by microbial metabolic activities. Most of the cases the biodegradation takes longer than traditional remediation methods, however the complete degradation is often accomplished. Hydrocarbon biodegradation in soil is determined by a variable number of abiotic and biotic factors, such as the pH, temperature, oxygen availability and nutrient content, and survival of hydrocarbon-degrading bacteria including the bioavailability of pollutants to microbial attack (Koshlaf and Ball 2017). To improve the degradation of oily sludge the combination of bioaugmentation and biostimulation treatments has been carried out with positive results (Varjani et al. 2020). Biodegradation of the petroleum hydrocarbons is a complex process that depends also on the nature and the amount of the hydrocarbons present into the contaminate place (Colwell et al. 1977). Bioremediation techniques based on free cell cultures have been largely used to remove compounds from contaminated areas. However, a recent promising technique to be employed in harsh environments included the immobilization of microbial cell systems, presenting the main advantages of possible reutilization of microorganisms and more tolerance to temperature and pH changes (Partovinia and Rasekh 2018). Besides the important chemical-physical factors affecting diesel biodegradation, the bacterial species have been reported as the major factor that affect the biodegradation performance. Specifically-isolated bacteria seem to be a very promising approach to remediate diesel-contaminated environments (Imron et al. 2020). The screening of bacterial strains isolated from diesel-contaminated soils permitted to discriminate biosurfactant (BS) production and biofilm formation abilities as fundamental characteristics of the selected bacterial strains (Balseiro-Romero et al. 2017). The

bacterial bioremediation technologies as well as the fungi and algae bioremediation strategies may not be enough to remediate hydrocarbon-contaminated sites. In addition, in this case, it was comprehensively illustrated that the integration of remediating techniques can improve the degradation of petroleum hydrocarbons with an improving removal efficiency (Naeem and Qazi 2020).

8.4.4 Biodegradation of Plastics

The countless utilization of plastics is the result of their desirable characteristics, including light weight, durability, corrosion resistance, and low price (Raziyafathima et al. 2016). Consequently, the plastic pollution represents a significant environmental concern as a result of the persistence and potential adverse effects on biota. A number of scientific articles showed how bacteria can work on plastic material degradation, describing how microbial characteristics (e.g., biofilm organization) and environmental factors can affect the plastic biodegradation by bacteria (Yuan et al. 2020). Bacterial groups including Gram-positive and Gram-negative strains have been reported acting on plastic degradation (Raziyafathima et al. 2016). Microbial biodegradation of plastics includes the polymers conversion into monomers, due to bacteria were not able to introduce into the cytoplasmic compartment some polymers, so they must first be depolymerized to smaller monomers through physical and natural processes (Swift 1997). Among the natural processes of depolymerization, microbial enzymes were observed acting on polymers before the monomer formation, absorption into microbial cells and completely biodegraded (Goldberg 1995). Strains of the genus *Bacillus* were detected as one of the main components of the North Pacific Gyre plastic fouling community (Carson et al. 2013). Characterization of the extracellular hydrolase enzymes secreted by *Bacillus* strains included lipase, carboxymethyl cellulase, xylanase, chitinase, and proteases with different levels of activity (Dang et al. 2018). The enzymes that are known to degrade plastic polymers usually belong to the class "hydrolases." Enzymes belonging to this class are involved in a catalytic reaction that causes a breakdown of chemical bonds of its substrate in the presence of water. Cutinase, lipase, and PETase (an esterase) are some of the most common enzymes associated with the degradation of plastics according to recent research developed in this area (Kaushal et al. 2021). All these enzymes act on the plastic polymer in a similar manner, causing hydrolytic cleavage of the long carbon chains and then assimilating these smaller subunits into the microbial cell for further enzymatic degradation and release of metabolic products.

8.5 Potential of Marine Bacteria for Bioremediation

Many of the marine pollutants are the result of direct or secondary human activities. Some of these substances are considered biodegradable, but others unfortunately not (Vikas and Dwarakish 2015). Marine bacteria possess a wide variety of bioremediation potentials, which are beneficial from both environmental and economic point of view (Amidei 1997). Diverse marine bacteria have been reported to have bioremediation potential and they can be utilized in the bioremediation process of heavy metals, hydrocarbons, and many other recalcitrant compounds (Fig. 8.1). The utilization of these microbes permits the in situ bioremediation including the direct use of microorganisms in any adverse conditions and possibly without any genetic manipulation (Dash et al. 2013).

It has been already estimated that in 2025, the oceans will contain more than 25 million metric tons of plastic litter (Jambeck et al. 2015). Plastics are considered the main concern in marine environment representing that more than 60% of all floating debris and the amounts increase each year. Chemical–physical factors start the plastic degradation processes forming polymer fragments susceptible to biodegradation (Gewert et al. 2015). Micro-nano plastics enter into the agro-ecosystem through the wildlife arriving into the human body following the food chain; the processes through which the plastic enter into the human body include ingestion or inhalation, causing various problems as consequence.

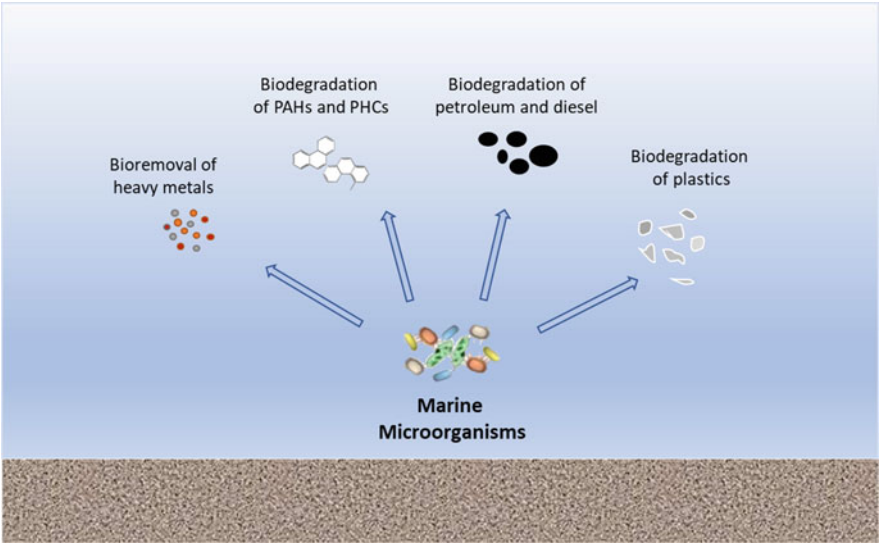

Fig. 8.1 Bioremediation potential of marine microorganisms. Bacteria isolated from different marine environments have been characterized for their ability for bioremoval and bioconversion of heavy metals and biodegradation of polyaromatic hydrocarbons, halogenated hydrocarbons, petroleum and derivatives and plastics

The microbial utilization of the plastic carbon content as energy source with complete degradation/removal of these components has been described as an efficient bioremediation method (Tiwari et al. 2020). Marine bacteria such as *Bacillus cereus, Bacillus sphaericus*, *Vibrio furnissii, and Brevundimonas vesicularis* shown capability to degrade nylon fibers in mineral salt medium at 35 °C and pH of 7.5 under submerged enrichment conditions with the polymer as the sole carbon source (Sudhakar et al. 2007). Efficient degradation of conventional high-density polyethylene (HDPE) can be mostly achieved by plastic-degrading bacterial isolates belonging to the genus *Bacillus* spp. and *Pseudomonas* spp. (Devi et al. 2019). In another research, the marine bacteria *Brevibacillus borstelensis* showed HDPE degrading activity (Mohanrasu et al. 2018). Moreover, other plastic-associated marine bacteria groups and families such as the families *Erythrobacteraceae* and *Rhodobacteraceae* (Alphaproteobacteria), *Flavobacteriaceae* (Bacteroidetes), and the phylum cyanobacteria (such as the *Phormidium* genus) have been frequently observed (Roager and Sonnenschein 2019).

Marine bacteria interaction with eukaryotic microorganisms such as diatoms was reported to be able forming aggregation called marine snow, which bring organic carbon and nutrients to the sea floor (Gärdes et al. 2011). In this context, biofilm formation by marine bacteria could became pivotal for the plastic particle elimination or reduction by the oceanic water column, increasing the speed by which this plastic makes its way to the sea floor. The presence of plastic nanoparticles significantly influenced the formation of biofilms both positively and negatively in a species-specific manner (Okshevsky et al. 2020). Deeper studies on these bacteria together with bacterial engineering approaches may help on this important marine environmental concern. The study on plastic-degrading microorganisms focused also on their searching in deep-sea sediments where temperature decreases below 4 °C (Ravenschlag et al. 1999) and it was possible to isolate microorganisms adapted to cold with unique characteristics (Urbanek et al. 2018). In this interesting environmental context, studies were conducted and detected bacteria with plastic degradation properties, including *Pseudomonas* spp., *Shewanella* sp., *Moritella* sp., *Psychrobacter* sp., *Alcanivorax* sp., *Tenacibaculum* sp. (Sekiguchi et al. 2009, 2011a, b;) *Vibrio* sp., (Raghul et al. 2014), *Clonostachys rosea*, and *Rhodococcus* sp. (Urbanek et al. 2017).

In marine environment oil pollution represents another important concern, it can occur from either catastrophic accidents (shipping disasters or pipeline failures) or natural oil seepages and biota. Various technologies have been developed to contrast these events and one of the reliable and eco-friendly was considered certainly the bioremediation with the objective to minimize the impact on the environment (Catania et al. 2015). When spilled in water, oil cause immediate and long-term repercussion. For this reason, clean-up methods and remediation were contemplated as fundamental to preventing the intoxication and death of a large number of living organisms. In this context, biotechnology applications also play a fundamental role (De Almeida et al. 2016). For petroleum pollution in seawater physicochemical methods alone were earlier defined not enough to contrast the problem (Prince 1997). However, the petroleum hydrocarbons being not soluble in water, it is

difficult to achieve the bioavailability for bioremediation as consequence surfactants are employed to improve the mobility of oil reinforcing the biodegradation process. Thus, an improved hydrocarbon solubility and the emulsification of hydrocarbon–water mixtures permit a better ability of oil-degrading bacteria to use hydrocarbons (Bao et al. 2012). The importance of the microbial biosurfactant production and its use were defined promising for the efficacy, low toxicity, and biodegradability working on the increasing biodegradation and solubilization process of insoluble compounds at extreme environmental conditions including at the common hydrocarbon derivatives, heavy metals, pesticides, and organic/inorganic contaminants (Deepika et al. 2021).

Bacillus genus was characterized as one of the main groups of bacteria able to synthesize biosurfactants as surfactin among the other families of cyclic lipopeptides (Jacques 2011). Moreover, *Bacillus* genus was detected as one of the main studied marine bacteria groups mainly for their easy cultivation in microbiological laboratories (Stincone and Brandelli 2020). Isolation of bacterial strains from seawater contaminated with petroleum derivatives permitted to discover promising biosurfactant-producing isolates belonging to the genus *Bacillus*, indicating their possible use in the bioremediation of marine environments (Durval et al. 2019). Microorganisms producing biosurfactants are efficient accelerators of the hydrocarbon biodegradation process by enhancing their bioavailability and facilitating their degradation by bacteria (Xu et al. 2020).

Another important group of bacteria include those acting on hydrocarbon biodegradation. In this group, a total of 79 genera of marine bacteria were identified and among these the following were the most representative: *Achromobacter, Acinetobacter, Alcaligenes, Actinomycetes, Archrobacter, Bacillus, Cycloclasticus, Coryneforms, Chromobacterium, Flavobacterium, Micrococcus, Microbacterium, Mycobacterium, Nocardia, Pseudomonas, Sarcina, Serratia, Streptomyces, Vibrio, Xanthomonas* (Xue et al. 2015). Features of microorganisms isolated from extreme environments can be employed for enhanced bioremediation of oil hydrocarbons, especially under aerobic conditions in moderate to high salinity conditions. In this condition, strains belonging to various genera have been shown to degrade hydrocarbons but in particular, members of the genera *Halomonas, Alcanivorax*, and *Marinobacter* dominate the literature (Fathepure 2014). The presence of both groups of bacteria, petroleum-degrading bacteria and biosurfactant-producing bacteria, improved the petroleum-degrading rate (Shi et al. 2019). Similarly, polycyclic aromatic hydrocarbons (PAHs) are widespread in marine ecosystems and they are usually resulting from human activities or even natural sources. PAHs adhere to sediments leading to accumulation in coastal and deep sediments and the microbial assemblages play a pivotal role on their degradation (Duran and Cravo-Laureau 2016). The metagenomic analysis of a contaminated coastal sediment allowed to detect three main phyla (Proteobacteria, Firmicutes, and Bacteroidetes) accounting approximately ≥93.0% of the total microbial community (Lee et al. 2018). Bacterial species like *Cycloclasticus spirillensus*, *Lutibacterium anuloederans*, and *Neptunomonas naphthovorans* have also been utilized in enhanced biodegradation of PAHs in marine environment (Hedlund et al. 1999; Chung and King 2001).

Moreover, degradation of PAHs carried out by marine bacteria through mineralization process was reported by *Achromobacter denitrificans, Bacillus cereus, Corynebacterium renale, Cyclotrophicus* sp., *Moraxella* sp., *Mycobacterium* sp., *Burkholderia cepacia, Pseudomonas fluorescens, Pseudomonas paucimobilis, Pseudomonas putida, Brevundimonas vesicularis, Comamonas testosteroni, Rhodococcus* sp., *Streptomyces* sp., and *Vibrio* sp. (Samanta et al. 2002). Recently it was possible to identify *Mycobacterium* and *Sphingomonads* spp. as bioaugmentation and genetic bioaugmentation targets, respectively, due to their positive associations with PAHs and their high abundance and species diversity in contaminated soils (Redfern et al. 2019). Moreover, the marine *Stenotrophomonas acidaminiphila* NCW-702 in biofilm organization was able to degrade PAHs more efficiently as compared to planktonic cells, these findings supporting the efficacy of biofilms over planktonic culture in bioremediation applications (Mangwani et al. 2014).

Industrialization and urbanization activities have increased the flow of toxic metal ions to the marine environments, being harmful when present above critical values. Bacteria from metal polluted habitats are suitable candidates for heavy metal remediation, maintaining the sustainability as they were more capable for quick adjustment to the changing environmental factors such pH, salinity, temperature (Dash et al. 2013; Mohapatra et al. 2017). Bacteria own several mechanisms to act on tolerating and bioremediating high concentration of toxic heavy metals such as: precipitation as phosphates, sulfides, and carbonates through the biomineralization, volatilization via methylation/ethylation/reduction through the biotransformation process, ATP mediated efflux systems, intracellular bioaccumulation, biosorption and sequestration through Extracellular Polymeric Substances (EPS) (Naik et al. 2012).

An important process is represented by the biomineralization that happens continuously on the deep-sea minerals forming polymetallic nodules. In fact, free-living and biofilm-forming bacteria provide the matrix for manganese deposition, and cobalt-rich crusts (Wang and Müller 2009). The marine bacterium *Idiomarina loihiensis* MAH1, growing in environmentally relevant concentrations of uranium, was capable to form uranyl phosphate mineral phases, structurally resembling meta-autunite [$Ca(UO_2)_2(PO_4)_2$ 2–$6H_2O$] precipitated at the bacterial cell surfaces (Morcillo et al. 2014). In a similar way, *Shewanella* sp. strain PV-4, from the microbial mat at a hydrothermal vent of Loihi Seamount in the Pacific Ocean, has been characterized with emphases on metal reduction including Fe (III), Co (III), Cr (VI), Mn (IV), and U (VI) as electron acceptors while using lactate, formate, pyruvate, or hydrogen as electron donor, showing the possibility to be exploited for bioreduction or immobilization of many toxic metals (Roh et al. 2006). More recently, the marine heterotrophic *Pseudoalteromonas* sp. MT33b with strong resistance to Cd, showed as major strategy to eliminate Cd stress the formation of insoluble CdS precipitates and massive biofilm. These characteristics open to possible utilization of bacterium from extreme environments for bioremediation purposes (Ma and Sun 2021).

Bioremediation realized by the marine bacteria *Pseudomonas aeruginosa* CH07 (NRRL B-30604) through the biotransformation process includes enzymatic reduction of toxic mercury (inorganic and organic) to volatile elemental mercury (Hg) in a two-step reaction. The bacterial resistance to Hg has shown activity against other highly toxic heavy metals (De et al. 2006). In this context, most of the Hg-resistant bacteria have shown the *mer* operon mechanisms located either on transposons, plasmids, or bacterial chromosomes (Dash et al. 2014). Bacteria hosting the genes *merT* and *merP*, indeed, encode for two transporters, which are responsible for the transport of Hg (II) into the cytoplasm and the subsequent action of mercuric ion reductase encoded by *merA* reduces Hg (II) to less toxic Hg^0, which diffuses out of the cell through cell membrane (Dash and Das 2012). It was observed that Hg-resistant marine bacteria shown potential for Cd and Pb detoxification, these bacteria were identified as *Alcaligenes faecalis*, *Bacillus pumilus*, *Bacillus* sp., *Pseudomonas aeruginosa*, and *Brevibacterium iodinum*. They were not able to produce any byproducts, and highly efficient even at low metal concentrations (De et al. 2008).

Common inhabitant of the marine environment, *Vibrio harveyi* was reported to possess the potential for bioaccumulation of Cd (Abd-Elnaby et al. 2011), while a consortium of marine bacteria was able to remove Hg by the same mode of action (Von Canstein et al. 2002). More recently, this strategy was detected in culturable marine deep sediment bacteria part of the phyla Firmicutes and Actinobacteria bioaccumulating heavy metals within the cells and/or in EPS (Jroundi et al. 2020). Marine isolates of *Streptomycetes*, phylogenetically inserted into the actinobacteria phylum, show unique growth characteristics including the ability to form spores and mycelia, and relatively rapid colonization of substrates, acting as suitable agents for bioremediation of metals and organic compounds in polluted soil and water (Timková et al. 2018).

Another important process included as bioremediation process of heavy metals carried out by marine bacteria was the absorption, in this case the bacteria together with other marine microbes secrete extracellular polymeric substances (ESP) to facilitate the attachment on surfaces and this led to the formation of structured biofilm communities (Decho and Gutierrez 2017). Metal cations form complexes with EPS resulting in metal immobilization generally promoted by electrostatic interactions between metal and negatively charged components of EPS biopolymers. However, the enzymatic activities in EPS also can assist detoxification of heavy metals (Pal and Paul 2008). The marine environment includes multi-cellular organisms as the sponges able to host huge microbial communities including bacteria, representing an important source of new bacteria with bioremediation capabilities. The sponge-associated Antarctic bacteria *Winogradskyella* sp. strains CAL384 and CAL396, *Colwellia* sp. strain GW185, and *Shewanella* sp. strain CAL606 were studied for their production of EPS with possible application on heavy metals absorption (Caruso et al. 2018a; Giudice et al. 2020). The marine bacteria *Enterobacter cloacae* showed EPS chelating properties with respect to Cd (65%) followed by Cu (20%) and Co (8%) at high heavy metal concentration (Iyer et al. 2005). Interestingly the EPS production by the seawater *Pseudoalteromonas*

sp. MER144 under optimal conditions at different concentrations of Hg and Cd revealed a modulate production of EPS when the heavy metal concentrations increased (Caruso et al. 2018b). Combination of strategies for removal of heavy metals has been shown by marine bacteria as *Rhodobium marinum* and *Rhodobacter sphaeroides* specifically for those involving either bioabsorption or biotransformation (Panwichian et al. 2011). Also, the marine *Bacillus thuringiensis* PW-05 demonstrated its capacity to resist at 50 ppm of Hg as $HgCl_2$ as well as higher concentrations of $CdCl_2$, $ZnSO_4$, $PbNO_3$, and Na_2HAsO_4. Atomic absorption spectroscopy revealed that the isolate can volatilize >90% of inorganic Hg. Moreover, it showed biofilm formation in the presence of 50 ppm $HgCl_2$ and also the possibility to produce EPS under same conditions (Dash et al. 2014).

Another bacterial strategy for an eco-friendly, time saving, inexpensive with easily scaled up for large-scale synthesis to employ in heavy metal bioremediation is the synthesis of metallic nanoparticles either intracellularly or extracellularly (Manivasagan et al. 2016). Marine microorganisms can easily adapt themselves to extreme environmental conditions, it is very essential to explore marine bioresource for the green synthesis of different types of metallic nanoparticles. A strain of *Stenotrophomonas*, isolated from the Mandapam coast, Bay of Bengal in India, was employed for the biosynthesis of Au and Ag nanoparticles by extracellular secretion (Malhotra et al. 2013). In this context, the bacterium *Saccharophagus degradans* was investigated for the synthesis of MnO nanoparticles (Salunke et al. 2015). Microbial extracellular polymeric substances EPS act as capping and stabilizing agents for the biosynthesis of CdS nanoparticles by the marine bacterium *Pseudomonas aeruginosa* JP-11 and its comparison with chemical method were more efficient showing a higher Cd removal efficiency (Raj et al. 2016).

8.6 Engineering Bacteria for Bioremediation

Studies carried out to understand the interactions between xenobiotics and microorganisms and on the fate, survival, and activities of microorganisms in the environment have to intersect with the investigation on biochemical and genetic engineering aspects (Pieper and Reineke 2000). Natural microbes have already shown considerable ability to remove many environmental pollutants with no external intervention. The onset of genetic engineering in the 1980s allowed the possibility of rational design of bacteria to catabolize specific compounds with reduced or no possibilities to be removed (Dvořák et al. 2017). Once identified the genes and their genome location in bacteria that promote bioremediation, the objective becomes modify and incorporate them into a suitable host to be used as a bioremediation agent, in this context at the beginning usually was the *E. coli* (Zylstra et al. 1989). The indispensable incorporation of a gene marker (to discriminate the genetic modification) conferring antibiotic resistance was the simplest way of screening in producing genetically modified bacteria, representing an important concern (Dale et al. 2002). However, during these decades, since the discovery of recombinant DNA

technology many challenges have been encountered in constructing genetically engineered bacteria intended for environmental release and most of them have been resolved.

In general, the literature still counts very few cases where the use of genetically engineered bacteria has been confirmed to be more efficient than natural microorganisms in removal of recalcitrant compounds under natural (in situ) conditions (De Lorenzo 2009). Although the important steps achieved on the field of genetically modified bacteria, deeper studies should be realized to face ethical responsibilities before using such novel strategies for bioremediation (Perpetuo et al. 2011). Among the main concerns necessary to be considered, the ecological impacts of including genetically modified organisms into the environment become fundamental (Rajakaruna and Robinson 2016). Into the field of the engineered bacteria, for example, become necessary the detection and enumeration of these modified microorganisms in complex samples (Widada et al. 2002). Thus, a deeper approach may permit to overcome both environmental concerns and regulatory constraints that have limited the in situ application of genetically engineered microorganisms. Considering that the main objective of this limitation is to avoid the risk of a possible uncontrolled survival/dispersal of engineered microorganisms or recombinant plasmids into the environment, some strategies have been suggested, including the induction of suicide microbes once their mission is completed (Paul et al. 2005; Ezezika and Singer 2010). Furthermore, the integration of microbiological, biological, and ecological acquaintance accompanied by field engineering designs was considered features for effective application of in situ bioremediation of polluted sites by recombinant bacteria (Liu et al. 2019).

Systems biology studies, based on top-down and bottom-up large-scale "omics" tools and mathematical modeling methods, have become pivotal on the engineered bacteria build (Park et al. 2017). The enormous amount of genomics, transcriptomics, proteomics, and metabolomics information, coming from systems biology studies, allow to integrate the data for a more holistic understanding. In this context, novel data mining and analytics approaches, including artificial intelligence can provide breakthroughs where traditional low-throughput experiment-alone methods cannot easily achieve (Helmy et al. 2020). Both integration and mapping of systems biology and metabolic engineering tools and techniques can help to achieve the environmental bioremediation objective, concretely these may permit to pass from a theoretical stage to a practical stage (Dangi et al. 2019).

Various laboratory studies have evaluated engineered bacteria for possible use in bioremediation of contaminated sites. Bacteria genetically modified have been evaluated, for example, to neutralize heavy metals and transform them into less toxic forms (Paliwal et al. 2012; Pratush et al. 2018), to improve the degradation of plastic debris (Kumari and Chaudhary 2020), for petroleum hydrocarbons degradation (Naeem and Qazi 2020), and also acting on the degradation of PAHs (Haritash 2020). Once the metabolic pathways are properly understood, the genetic manipulation could permit the development of both transgenic plants and microbes that may work twice efficiently in direction of the bioremediation of contaminated sites of heavy metal and organic pollutants (Ojuederie and Babalola 2017). Some examples

report that engineered bacteria may be a viable technology for Hg bioremediation from liquid matrices, employing transgenic bacteria expressing metallothionein (*mt-1*) and polyphosphate kinase (*ppk*) genes. It was possible to achieve high Hg resistance and accumulation while reduced Hg volatilization was observed. Specifically, the reduction of the Hg volatility represents an important advantage comparing the reduction at the volatile elementary form (Hg^0) by simple natural bacteria expressing *mer* genes (Ruiz et al. 2011).

Recent advances to face regulatory hurdles and environmental concerns come from engineered bacteria applied to clean up oil spills, showing high efficiency for possible in situ application. The study included the genetic vector detection among environmental bacteria of the contaminated site treated with genetically modified bacteria. A crude oil soil remediation employing *E. coli* transporting three enzymes (almA, xylE, p450cam) resulted in the degradation of 60–99% of target hydrocarbon substrates, from the vector detection it was possible to notice that *E. coli* cells died after five days, while a variety of bacteria received and carried the vector for over 60 days after inoculation. However, it disappeared when the carbon source was removed providing minimal ecosystem disturbance (French et al. 2020).

Some studies have been conducted with bacteria showing the ability to work under ionizing radiation, including marine strains of *Deinococcus* spp., considered for its extremely radio-resistant capacity. This bacterial group has shown DNA repairing proficiency permitting their use in a number of strategies for bioremediation of radioactive waste, collaborating with those capacities such as desiccation, temperature, and metal tolerance (Suresh et al. 2004; Ferreira et al. 1997). For these peculiar characteristics, it has become an organism of choice for bacterial engineering (Brim et al. 2000, 2003). The lyophilized recombinant *Deinococcus radiodurans* genetically modified for PhoN expression showed PhoN activity and uranium precipitation ability as well as other metals like cadmium. PhoN promote the liberation of inorganic phosphate from a suitable substrate molecule like β-glycerophosphate, causing the precipitation of metals as cell-bound metal phosphates and facilitates their easy removal from aqueous solution (Misra et al. 2012).

An important chemical groundwater pollutant is 1,2,3-trichloropropane (TCP) used as degreasing agent with high water solubility that causes large spreading of this recalcitrant component in the environment. Currently, no natural microorganisms are known to be able to mineralize TCP. This necessitates isolation and remediation measures, which may be based on (accelerated) in situ treatment or pump-and-treat methodologies. TCP was chosen as a target for constructing bacteria that use it as a growth substrate since some structurally similar compounds are biodegradable. This was indeed achieved by a combination of protein- and metabolic engineering. In this case, the use of genetically modified bacteria still awaits full-scale application, while successful full-scale application has been achieved for the similar 1,2-dichloroethane by isolating bacteria degrading this compounds and bioremediation activities were carried out with ex situ procedures (Janssen and Stucki 2020).

8.7 Concluding Remarks

Environmental pollution has been widely spread around the world, and the pollution is cumulative, once naturally, it is impossible the auto depuration of all contaminants released in the environment and accumulation in some parts such as in marine water and sediments. Marine pollution is affecting all kinds of systems such as bacterial communities, corals, fish, and other living organisms, even the humans once the ocean has been feeding the human beings since the humanity began. In this scenario, bioremediation became for helping and has efficient strategies for each kind of pollution. Refinery wastes are common contaminants widespread released in the environment and studied in bioremediation. Marine bacteria have been used as efficient biological agents for bioremediation of contaminants as plastics, petroleum, hydrocarbons, and heavy metals. Biodegradation combined with other strategies such as bioaugmentation, biosurfactant production, and biotransformation is very powerful for bioremediation of marine environments with very good clean-up results. Thus, additional studies and applications need to be performed and are necessary to improve bioremediation technologies using marine bacteria.

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Chapter 9
Marine Bacteria for Biofertilizers

Poonam Singh, Kaleemunnisa FNU, and Telma Encarnação

Abstract The fertilizer industry is growing with time and demand. The production of most used nitrogenous fertilizer utilizes methane supplies and sums up to 3% of worldwide greenhouse emissions. Chemical fertilizers have created a large number of environmental dead zones with low oxygens where life forms cannot grow. With a decline in soil quality, the yield of crops go and have gone from minimum to none. Stress caused by abiotic and biotic factors is the constraint that needs to be overcome as it affects the productivity of the crops. Improvement of crop growth under stress depends on unexplored tools of nature. One of such tools is microbe, which are still unexplored and found in enormous quantity. Microbes that produce a variety of metabolites either stimulate the nutrient formation or increase the uptake of nutrients in plants it is symbiotically associated with. The term microbial biofertilizers is associated with the formulations of sufficient densities of strains in active or inactive form of microorganisms, that can be used in rhizospheres for plant growth. The microbes used can be either a single strain or in combination with different specific beneficial effects.

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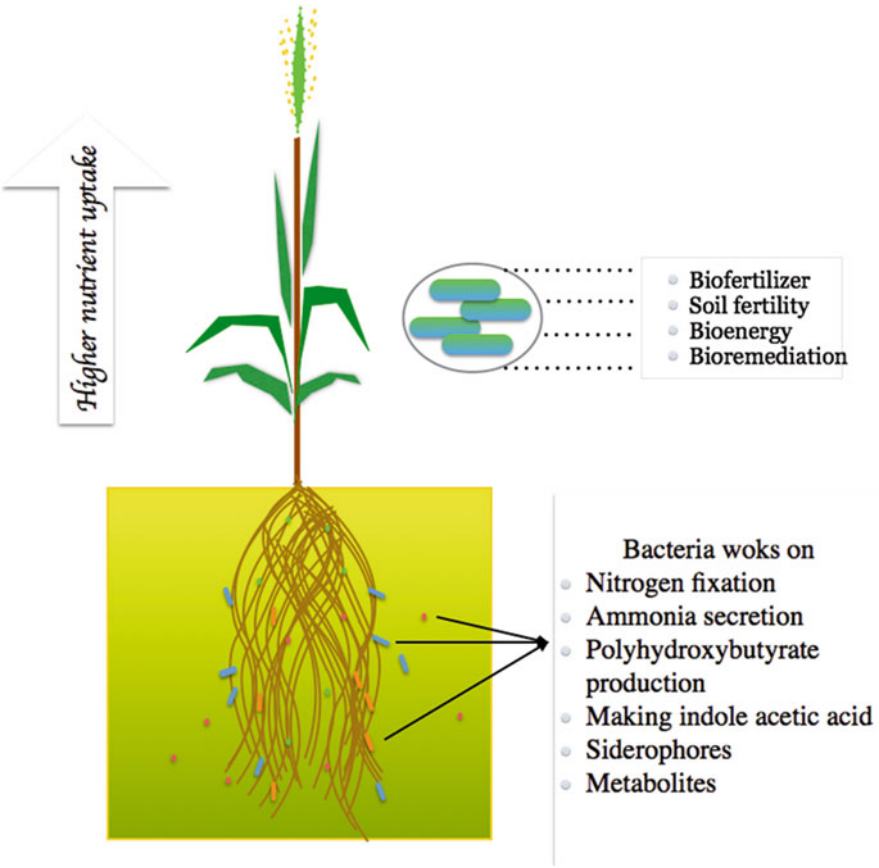


An overview of responses of bacteria as biofertilizer on biochemical properties of the soil

Keywords Biofertilizers · Bacteria · Nutrients uptake · Regulations · Machine learning · Artificial intelligence

9.1 Introduction

Modern agriculture requires expeditious yet responsible measures. The increasing crop productivity for meeting the demand of the growing population has been possible due to synthetic chemical fertilizers and pesticides. The practices followed by farmers have been changing throughout the years with a negligible focus on the problems that are impacting the environment (Mishra et al. 2012; Swapna 2013). This is causing many alarming situations that are going to impact the earth and living

beings not only in the present but also in near future. However, we cannot deny the fact that food security is an important cause and we need to fulfill it. The continuous climate change with increasing temperature and CO_2 levels is a threat to the agriculture (Spiertz 2009).

Living organisms that can deliver nutrients to soil, plant or can help in the germination increasing the biomass of the root is termed as biofertilizer. The use of microbial metabolic ability for degradation/removal of environmental pollutants provides an economic and safe alternative compared to other physicochemical methodologies. Microbes have an ability to alter the microbiota of the soil and living forms around the particular area where they are used as biofertilizer (Hafeez et al. 2006). These microbes might be found in different sources, the most common ones are bacteria, fungi, and cyanobacteria (blue-green algae). These microbes have enzymes that perform the nutrient conversions maintaining the nitrogen and phosphorus balance and hence keeping the soil constitution in equilibrium (Ritika and Utpal 2014).

9.1.1 Marine Organisms as Biofertilizer

The majorities of microalgae are capable to fix the atmospheric nitrogen and are effectively used as biofertilizers. Microalgae or microphytes are microscopic algae invisible to the naked eye. They are phytoplankton typically found in freshwater and marine systems, containing all the essential nutrients needed for plant growth. Symbiotic marine origin form is not only environmentally friendly but also cost-effective (Ravikumar et al. 2005). In general, the molecules produced from marine sources possess no harm to the environment and provide a foundation for organic biomass production. The main factors affecting marine diazotroph distribution in the ocean are oxygen, light, temperature, inorganic N forms, phosphorus (P), Fe, and organic matter (Fu et al. 2014).

9.2 Literature Review

Conversion of molecular nitrogen into ammonia and other nitrogenous compound that can enrich the soil is primarily done by Diazotrophs that include *Rhizobia, Frankia,* and *Azospirillum*. These bacteria provide phytohormones to the host and confer resistance against pathogens. The exchange of signals occurs through diffusion in symbiosis pathway. The soil bacteria are mostly studied in the rhizobia–legume and legume–arbuscular mycorrhiza symbioses (Pankievicz et al. 2021). The evolutionary link has been recognized between these two symbioses.

The ability of rhizobia to fix the nitrogen in the root nodule and maintaining the nutrient uptake during stress conditions, makes it interesting for the crops like soyabean reducing the production cost and reducing adverse environmental impacts.

The impact caused by the microbes can be determined by their ability to promote plant growth in any kind of stress condition. The soil bacteria are known to promote plant growth by providing ammonia, enhancing the uptake of indole-3-acetic acid (IAA), gibberellic acids, cytokinin, plant hormones (Grobelak et al. 2015).

The deep ocean is an unsolved mystery and has organisms that have essential application in different industries and one of them is as natural fertilizers. One of the important evidence to identify nitrogen-fixing bacteria is found in their DNA fingerprints. That contains the gene specifically encoding the protein responsible for nitrogen fixation (Bianchi 2011).

Cyanobacteria is a biological unit to convert solar energy to chemical energy and contributing to the atmospheric oxygen. The capture of photon and by cyanobacteria is a source to generate biofuels and chemicals using sunlight and the greenhouse gas CO_2. The mechanism of how cyanobacteria work as a bio fertilizer in different parts of a plant is shown in Fig. 9.1. *Synechocystis* sp. PCC 6803 is one of the most extensively studied species in research due to its similarity with the plant chloroplasts. The carbon metabolism under variety of conditions makes it suitable for the stress adaption in plants and studying stress using this cyanobacteria species as a model. The genetic modification in *Synechocystis* 6803 has ensured that the species can produce diverse types of chemicals and work as a photosynthetic host (Quiroz-Arita et al. 2019). In absence of nitrogenase it cannot fix N_2 but by producing cyanophycin. Cyanophycin is a polypeptide containing multiple arginine and aspartate residues. However, it is able to store ammonium nitrogen inside of the cell. This generates a possibility to use it as a bio fertilizer by removing the nitrogen from the wastewater. A study done by Krasikov and other researchers found that nitrogen production protein is upregulated, photosynthetic carbon fixation is done in a limited manner, and cells hardly have any enzymatic activity under reduced nitrogen conditions in *Synechocystis* 6803. *Thiobacillus* a widely distributed bacterium

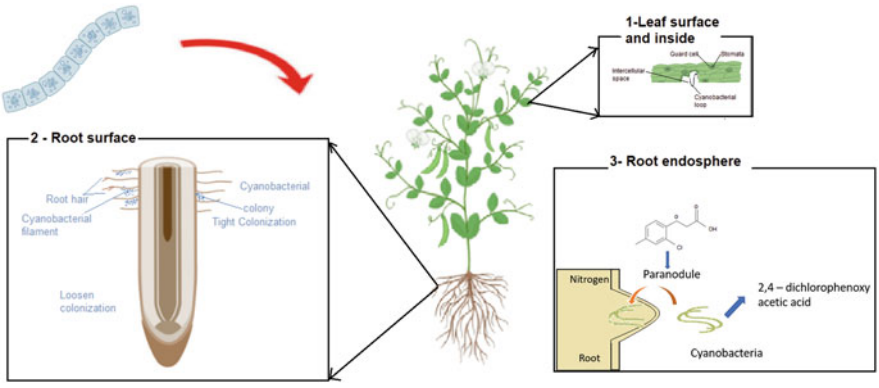


Fig. 9.1 Depiction of working mechanism of cyanobacteria as biofertilizer in the growth of the plant: (1) Colonization of leaf by cyanobacteria. (2) Colonization in the root hair, and root surface. (3) Co-inoculation with 2,4-dichlorophenoxy acetic acid (2,4-D) (synthetic auxin) and Nostoc spp. increases para-nodule formation and nitrogen fixation (Lee and Ryu 2021)

produces nitrous oxide and is found in both soil and water sources. *Thiobacillus* is an autotroph capable of using both sulfur and iron(II) as an electron donor (Hedrich et al. 2011; Krasikov et al. 2012). Canola, rapeseed plant when inoculated with Iron along with *Thiobacillus* sp., along with the application of Zn and Fe micronutrient, showed higher content of zinc and iron in seeds. The yield and oil content in canola was enhanced too (Jashni et al. 2017). Green beans had an increase in their productivity when *Thiobacillus,* sulfur application was used with zinc. The mechanism thought is reduction of the pH soil by providing sulfur, which results in bigger food yield. The maize and wheat production was increased by applying *Thiobacillus thiooxidans* when used with iron and zinc (Davaran et al. 2016; Visser 1997).

Bacillus cereus UW85 and *Pseudomonas protegens* CHA0, the known biocontrol agents, when used together can aim at different pathogenic fungi, producing metabolites against other unwanted growth in plants. The bacteria found on land soil convert N_2 into organic nutrients like ammonium (NH^{4+}) and nitrate (NO^{3-}) that can be utilized by plants. In marine infrastructure, cyanobacteria, blue-green in color are abundantly found, fixing nitrogen. Approximately 90% of natural nitrogen fixation is performed via these organisms also known as diazotrophs. NO_3^- or O_2 and temperature are important to understand the wider latitudinal distribution of different diazotrophs in the ocean tropical surface waters and tropical marine waters (Caulier et al. 2018).

Synechocystis has carboxysome that concentrates inorganic carbon for biomass growth. It is a special organelle which encapsulates Ribulose-1,5-bisphosphate carboxylase (RuBisCO), this enzyme helps with CO_2 fixation. Inorganic polyphosphate can be stored by *Synechocystis* 6803, photosynthetic host for the production of diverse types of chemicals. *phoU* and *sphU* are the phosphate regulator mutant genes that enhance cyanobacterial phosphorus uptake. Mineral fertilizers that contain macronutrients such as nitrogen, phosphorus, and potassium when added increase the crop yield (Tabita 1999).

9.3 Understanding the Mechanisms of Nutrient Uptake

Biofertilizer, Nitragin of *Rhizobium* sp. (Singh and Singh 2019) was used in 1895 by Nobbe and Hiltner. Mycorrhizal fungi inoculants had a positive impact on plant growth promotion (PGP), studied in the 1950s (Koide and Mosse 2004). It has been a great effort to analyze and understand the mechanism of nitrogen fixation via microbes. The difference in location came up as a predominant factor. For example, if the bacteria are growing on coastal or open ocean, deep or shallow, warm or cold water, it makes a variation in rate and order of fixation. N_2 in the rhizosphere and bulk soil can be fixed into atmospheric N_2 in cereal crops such as wheat, maize, rice, and corn by heterotrophic free-living diazotrophic species such as Azotobacter, Azospirillum, and cyanobacteria. In other crops, N availability has been enhanced by free-living diazotrophs (Zeffa et al. 2019).

9.3.1 Nitrogen Fixation by the Bacteria

Only a small number of marine bacteria can convert dissolved N_2 gas into bioavailable ammonia (NH_3) and known as N_2 fixers. This is an intensely energy-requiring process carried out by different kinds of cyanobacteria species. *Trichodesmium, Oscillatoria, Lyngby, Aphanizomenon, Nodularia Richelia, Calothrix Crocosphaera watsonii,* Marine diazotrophs, heterotrophic bacteria, phototrophic bacteria, and *Cyanothece* are the species with nitrogenase complex, which catalyzes N2 fixation. Protein bound by molybdenum and iron constitutes nitrogenase. Oxygen destroys nitrogenase, to cope up with this phenomenon cyanobacteria generate specialized N_2-fixing cells as a protection mechanism. Heterocyst cells are formed that are deficient in oxygen. Time delays are another strategy used against oxidation by proteobacterial diazotrophs and *Trichodesmium* and heterocyst-forming cyanobacteria (Pajares et al. 2019).

9.3.2 Phosphorus: Solubilization and Mineralization by the Bacteria

Phosphorus is one of the main limiting elements and second most important macronutrient for biomass production found in phosphate and sedimentary rock in the marine environment. *Pseudomonas aeruginosa* from marine origin, was reported to the produce pyoverdin type of siderophores, when tested for additional metabolite production, was found to solubilize phosphate. Marine bacteria are capable of solubilizing inorganic phosphorus from insoluble compounds to be assimilated by plants. Marine systems receive phosphorous minerals by continental weathering, however the weathering depends on environmental factors. That includes how old the earth is, erosion conditions, atmospheric composition, phosphorus content in rock, microaggregate fractions from the soil, and biological response. Achromobacter can be found in marine sources. It mineralizes phosphate through production of extracellular enzymes (Kalayu 2019; Rane et al. 2008).

9.3.3 Potassium: Solubilization by the Bacteria

Potassium a vital macronutrient, required in enzyme activation, protein synthesis, and photosynthesis. It is absorbed from the root and helps with the movement of the water, nutrients, and carbohydrates in most agronomic crops. Potassium-solubilizing bacteria minimize health hazards that are resulted by the use of chemical fertilizers. *Serratia marcescens* can exhibit plant growth promoting molecules hence a potential PGPB with diverse activities tested by Sindu et al. The processes like cell osmotic regulation and enzyme activation require potassium, the availability depends on the

quantity in the soil (Janice and Carmen 2007). Potassium is present in the soil in three different forms, in readily unavailable, slowly available, and readily available. The minerals present in soil (feldspar and biotite) have the potassium mostly in readily unavailable form, clay has slowly available, non-exchangeable form to the plants. Water-soluble potassium is readily available and exchangeable present in soil (Teotia et al. 2016).

9.3.4 Oxidation of Sulfur by Bacteria

Sulfur an essential nutrient for the production of proteins, glutathione, chloroplast membrane lipids, coenzymes, and vitamins is mostly found in organic form. The inorganic sulfur that is in the form of sulfate has been depleted recently as a consequence of many environmental factors. Element sulfur based fertilizers are present in the market, but for plants uptake it needs to be oxidized to sulfate. Bacteria are capable of metabolizing sulfur and its compounds (Tang et al. 2009). Hydrogen sulfide (H2S), sulfur, and thiosulfate (S2O32−) are oxidized by bacteria to convert it to sulfate. Thiobacillus, found in marine environment, oxidizes sulfur. The sulfate produced has several advantages when it goes deep into the ground it produces sulfuric acid dissolving metal, concrete, and steel to soluble monomeric and oligomeric compounds. Hence, improving the quality of the soil. Phreagena soyoae is one of the intracellular symbiotic sulfur-oxidizing bacteria (Lin et al. 2018). Purple sulfur bacteria Thiospirillum are strict anaerobes and convert sulfide to sulfur and then sulfate (Pfennig 1975).

9.3.5 Micronutrients Solubilization by Bacteria

Micronutrients are essential for the growth and enzymatic reactions in plants. Processes like photosynthesis, respiration, water oxidation, and oxidative stress protection (Castro et al. 2018) are dependent on the intake of iron, zinc, copper, silicon, cobalt, nickel. If the soil is deficient in micronutrients, it will affect the production of the crop. Silicate classified as a beneficial nutrient by the Association of American Plant Food Control Officials, is found in soil but not readily available. Silicate solubilizing bacteria found in soil, water, marine sediments, and silicate minerals exhibiting their unique property can solubilize the silicate and make it accessible in the form of monosilicic acid for plants (Vasanthi et al. 2018). *Aeromonas, Rhizobium, Enterobacter*, and *Bacillus* are the most studied species.

Iron sequestration is performed by siderophores that possess high affinity and selectivity for Fe(III). They help the plants to survive in iron deficient environment. Siderophores work during many physiological processes such as oxidative phosphorylation and nucleic acid biosynthesis. Insoluble Fe(III) is transported to microbial cells by membrane receptors, after that reduction of Fe (III) to Fe (II) occurs by

redox processes. *Marinobacter* sp. DS40M6 produces the suit of marinobactin siderophores (Martinez et al. 2003). *Synechococcus sp*. and *Vibrio cyclitrophicus* 1F53 have been found to have peptidic hydroxamate siderophore that has an ability to chelate metals via the presence of two oxygen atoms (Chen et al. 2019).

9.4 Regulations

The environmental impact of traditionally used agricultural techniques has moderately led European union to put some regulations on it. The regulatory framework has encouraged better use of microorganisms to improve agricultural practices according to their taxonomic classification and usefulness.

Marine forms maximize the use of the waste and hence play a role in the recycling of the nutrients.

The plethora of marine sources present on this earth remains majorly unexplored giving a subsequent scope for research of the potential application of the extracted components. New advancements have made it possible to investigate the compounds and facilitate the expedition to analyze new components from marine life. Figure 9.2

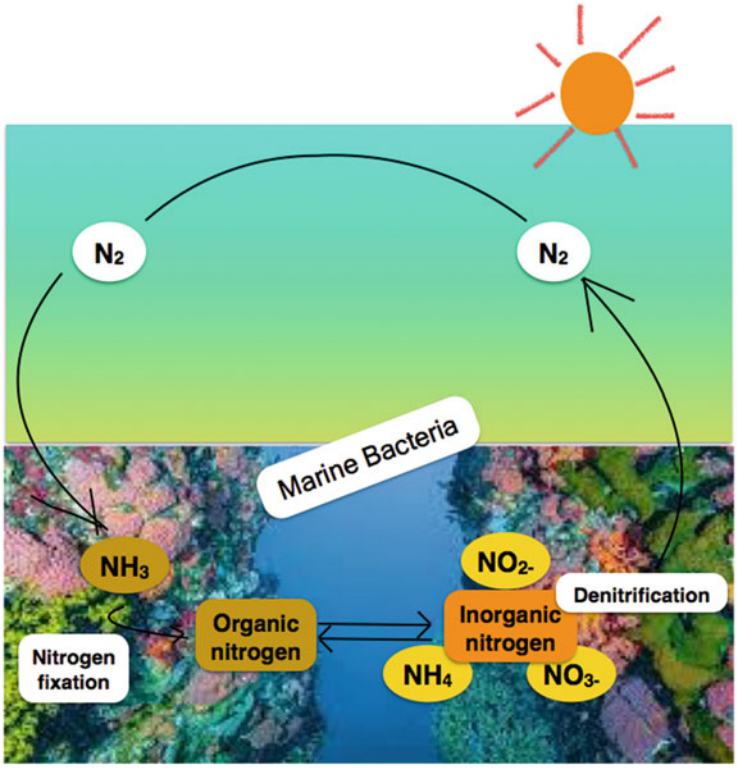


Fig. 9.2 Nitrogen cycle taking place through marine bacteria (White et al. 2020)

is the interpretation of nitrogen cycle in which conversion of nitrogen takes place in different forms via marine bacteria in the ocean and circulating through different ecosystems.

The use of waste from the production of biomass to produce molecules encourages the circular economy. The use of biofertilizers from bacteria should not cause any damage to the environment or human health. To ensure the safety, The European authorities have developed the recent new policy content of the EU Circular Economy Fertilising Products Regulation (EU 2019/1009) on biofertilizer in order to protect humans, plants, animals, and the environment. To trade the fertilizers the products must comply with strict regulations and affix the CE mark. The producers must ensure and demonstrate the labeling requirements. The EU regulation directs the product to be divided into function categories that will determine the specific requirements of quality and safety to be used for intended purposes. There are certain control criteria involved for different categories of the product before making it available to the market. The harmonized quality standards introduce new limit values for contaminants to trade the fertilizers keeping the health attributes into account (Schmidt and Haccius 2020; Saha et al. 2017).

The biofertilizer has to guarantee reduction of soil and environmental risk and provide a high level of soil protection this is regulated with a limit for cadmium that is <1.5 mg/kg. The low cadmium content allows producers to obtain improved level of soil production. The natural source boosts the circular economy and also the use of recycled bio wastes. The use of bacteria as a source of biofertilizers also emphasizes on green innovation reducing the need to use other sources of nutrients for the growth of the plants. The structured framework ensuring the detailed guidelines to use the biofertilizer from bacteria encourages the farmers to use this abundantly available natural source hence avoiding toxic contaminants and elements. The presence of this alternative bacteria based fertilizer in accordance with the market if applied all over the world would remove all the costs in terms of clearance from the national borders. It will persist the homogenous protection of environment and health of the living beings as well (Calvo et al. 2014; Das et al. 2019).

9.5 Limitations

Extensive and long-term application of biofertilizers can cause technological, infrastructural, financial, environmental, human resources constraints. The use of biofertilizers may result in accumulation of salts, nutrients, and heavy metals. This could cause detrimental effects on plant growth. There are very few places where the extraction and purification of the marine bacteria is done; this unawareness can affect the quality of the crop and ultimately impact human health.

Variable concentrations of nutrients in different species and high implementation costs are something to be taken care of otherwise it makes the entire process too expensive. Even after having the desired qualities in a bacterial species it is possible that low transfer of micro- and macronutrients can cause nutritional deficiency. In

comparison to chemical fertilizers large volumes of biofertilizers from bacteria are required for land application, this might be because the process is not yet standardized it is difficult to know exact contents of nutrients (Carvajal-Munoz and Carmona-Garcia 2012).

9.6 Future Outlook

Machine Learning(ML) concept gives computers the ability to think and helps us to solve many problems. ML is a subset of artificial intelligence focused on using algorithms that learn and improve without being explicitly programmed to do so. ML is about learning from existing data to make predictions about the future. It is based on creating models from input datasets for data-driven decision-making. ML uses large datasets to identify (infer) patterns and make decisions (predictions). Automated decision-making is what makes ML so appealing. You can teach a system from a dataset and let the system act by itself to predict future. Currently many researchers' study concluded that there are enormous resources available in the extensive marine environment. These sources play a vital role in the development of renewable sources.

With the data available on the level of nutrients present in the marine bacteria, we can analyze the growth of plant and with the help of Artificial Intelligence algorithms we can predict the growth based on the nutrients content and also classify the nutrients (Larrañaga et al. 2006).

The process of machine learning is as follows (Fig. 9.3):

Machine learning is a method of data analysis, which is a sub section of Artificial Intelligence that automates analytical model building. Using these algorithms that iteratively learn from data, machine learning finds hidden patterns without being explicitly programmed.

These features of ML can be used on the data collected on bacteria from marine source for fertilization to classify the type of species, classification can be done on

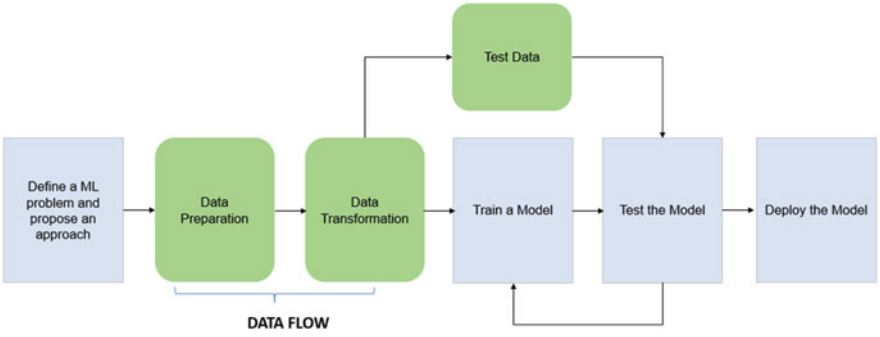


Fig. 9.3 Flowchart of machine learning process

both binary and multivariate, which can help in deciding what kind of crops grows faster with different strain of bacteria. And we can predict the rate of growth at which these fertilizers are helping in achieving cost effectiveness, level of non-toxic achieved and also to check the level of contamination of the ground water. Also, you can check the subspecies of bacteria growing in different weather, temperature, and area (Fig. 9.4).

At the most fundamental level, machine learning is categorized into two main types: supervised learning and unsupervised learning. Supervised learning involves modeling the relationship between measured features of data and some label associated with the data; once this model is determined, it can be used to apply labels to new, unknown data. This is further subdivided into classification tasks and regression tasks: in classification, the labels are discrete categories, while in regression, the labels are continuous quantities. Supervised learning used in applications where historical data predicts likely future events (Goodfellow et al. 2016) (Fig. 9.5).

In supervised learning, an algorithm learns from labeled data. This labeled data is used for training algorithms. The algorithm receives a set of inputs along with outputs. An algorithm learns by comparing its actual output with correct outputs to find the errors. And then it modifies the model accordingly. Once the model is trained, we can test it by new input/data (Fig. 9.6).

There are more than two classes available for classification with the labels for a multi-class classifier. Decision tree is an example of multi-class classification.

Plant growth promoting bacteria (PGPB) contain all the essential nutrients needed for plant growth. The majorities of PGPB are capable to fix the atmospheric nitrogen and are effectively used as biofertilizers. PGPB have a tendency to harbor genetic factor for antibiotic and metal resistance. Through antibiotic resistance property acts as the intrinsic property which attributes to the presence of multidrug efflux pumps, which are involved in performing metabolic processes in bacteria. With the data, available on the level of nutrients present in the PGPB, we can analyze the growth of

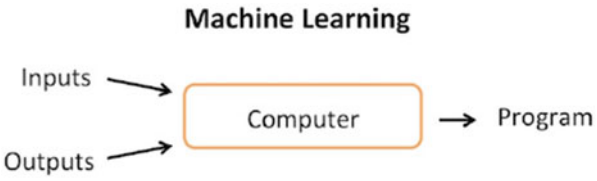


Fig. 9.4 Flowchart explaining the simple working cycle of machine learning

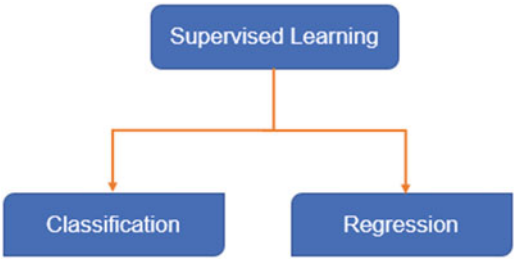


Fig. 9.5 Flowchart of supervised learning

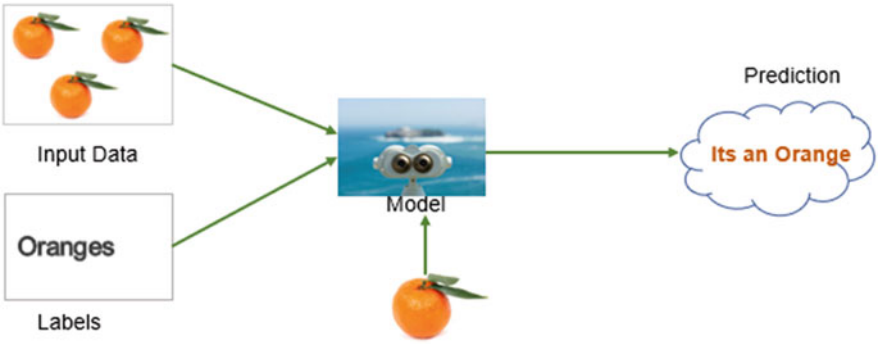


Fig. 9.6 Diagram showing how the data contributes in understanding of a data

Fig. 9.7 Illustration to show how to extract information from Dataset points

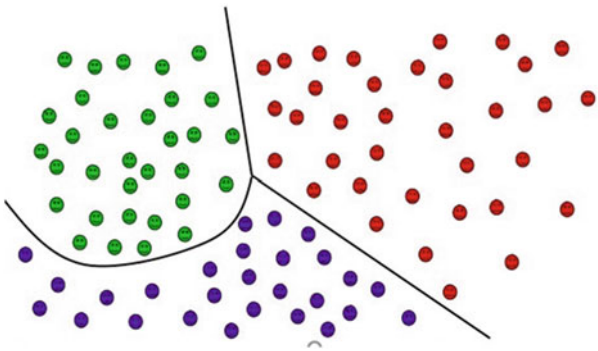
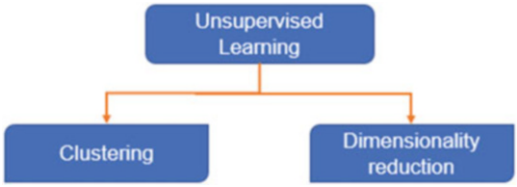


Fig. 9.8 Flowchart of unsupervised learning



plant and with the help of Artificial Intelligence algorithms we can predict the growth based on the nutrients content and classify the nutrients (Fig. 9.7).

Data comes without labels and the task is to find similar data points in the dataset in order to identify any underlying hierarchical structure. Consider a dataset that contains purchase preferences from Amazon users, these users will likely form a cluster that behave in the similar way. They may have similar purchase behavior, they may spend same amount of money, or buy similar type of items. You can think of these different groups as different communities with different preferences. Once these communities are identified you can describe each data point which is each user in terms of the community it belongs to gaining deeper understanding of the data, and also reducing the number of data points that we have to deal with (Larrañaga et al. 2006; Goodfellow et al. 2016) (Fig. 9.8).

9.7 Conclusion

Excessive use of hazardous chemical, pesticides used as fertilizers is posing high risk for imminent agriculture. The alternative to these hazardous chemicals can be biofertilizers, which can provide sustainable agriculture as biofertilizers helps in increasing crop yielding, maintaining, and enduring soil fertility. This can help in meeting the food consumption demand globally. Microbes can help in enhancing the immunity, growth, of crops, by aiding the essential nutrients required in growth of a crops such as nitrogen, phosphorous, potassium, zinc, and silica, to dissolve which are present in the form of insolubilized. The advantage of using biofertilizers is that they are cost-effective, non-toxic, contribute for the reduction of contamination of the ground water, as well as eco-friendly.

Currently, many research studies concluded that there are enormous resources available in the extensive marine environment. These sources play a vital role in the development of renewable sources. Marine scientists aim to evaluate the factor that controls the reservoir of nitrogen in the ocean and its influence on the fertility of the soil. Using a wide range of different techniques causes uncertainty in the estimation of nitrogen fixation rates as they are not comparable among ecosystems.

The global biofertilizer market is set to reach to 3.9 billion in 2025 (Mitter et al. 2021). Even though biofertilizer are considered safe for the environment, they have the drawback of short shelf life and survival of the strains in different environments. In case the necessary requirements are fulfilled during storage and transportation, the entire process is very costly, and mass production is very tricky too. There is no proper guidance for farmers regarding the recommended use.

There is no easy way to deal with the coming challenges but the bio resources that can be exploited with undiscovered marine diversity constituted with culturable microbes might help to overcome them. Especially when Machine Learning can process and analyze the existing and ongoing data making the task organized and simpler.

Not only environmentalists but economists are also concerned with the increase in the cost of crop production due to the application of expensive chemical fertilizers. Availability of nutrients, if maintained and recovered by the ocean-bacteria, can solubilize the minerals in the soil. They can enhance and make the nutrients available for the growth of the plant by N_2-fixing, phosphate-/potassium hence working as biofertilizers.

Biofertilization requires less chemicals in the soil and might be effective in its decontamination of pollutants; therefore, can reduce the environmental impacts associated with agriculture activities.

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Chapter 10
Marine Sponges for Bioremediation Purposes and for Secondary Metabolites Production

Ismail Marzuki and Khairun Nisaa

Abstract Environmental bioremediation is necessary to maintain the balance of the ecosystem to remain friendly and supports the continuity of life in the future. Comprehensive screening of marine sponges, symbiotic bacteria and secondary metabolites produced has been carried out. The activity begins with the identification and characterization of the morphology and histology of sponges. Furthermore, the analysis of phenotype and genotype of symbiotic bacteria continued by exploring the function of several types of bacteria in the biodegradation method of PAHs and bio-adsorption for several kinds of heavy metals. These activities include analyzing secondary metabolite components produced by sponges with specific characteristics and specific behaviour of enzymes in enzymatic reaction mechanisms in several environmental improvement uses. Based on the screening results, it is known that there are 11 types of marine sponges from Kodingareng Keke Island, which are in symbiosis with eight varieties of bacteria from the Bacillus sp., Pseudomonas sp. and Acinetobacter sp. groups. These bacteria can biodegrade PAHs, especially against petroleum sludge, naphthalene and pyrene. Also found 12 types of symbiotic sponge bacteria with the ability to bio-adsorb heavy metals, especially Cr (III), Cr (VI), Mn (II), Mn (VII), Pb, Hg, As, Cu and Ni. Adsorption varies. The interesting part of the results of the bacterial symbiotic test was that three types of symbiotic sponge bacteria were found, which have dual functions as biodegradators of PAHs and bio-adsorbents of heavy metals. The sponges included *Acinetobacter calcoaceticus* strain PHCDB14, *Bacillus pumilus* strain GLB197 and *Pseudomonas stutzeri* strain SLG510A3–8. Therefore, this type of sea sponge is recommended for population propagation through the transplant method.

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10.1 Introduction

Exploration of marine microorganisms to overcome environmental pollution is essential to maintain and maintain the existence of the earth as the best and most comfortable dwelling for every living creature, more specifically in the marine environment, which is an area where various types of ecosystems of life and activities of animals to maintain themselves exist, on earth. It is realized that the actions and dynamics of human life contribute the most in producing various environmental problems today. Environmental pollution in multiple parts of the earth, the largest, is caused by human activities in maintaining their lives, directly and indirectly harming environmental sustainability (Dadrasnia et al. 2020). Human initiatives and efforts to save the environment are not merely desires that are free from interests and without purpose but are indirect efforts to maintain their existence on earth as living beings who want to exist and live for all time (Idah et al. 2018).

Human efforts to save the environment are carried out in various ways. These methods are, for example, by conducting research, experiments and trials both on a micro-scale and on a broad scale, whether carried out in the laboratory or directly carried out in the field. These activities become a necessity of life that humans must carry out because humans are most responsible and have an interest in maintaining the environment to remain a friendly place. Seeing that the living environment of living beings is not simple, comprehensive studies and studies are needed so that the environment remains sustainable throughout time so that the natural balance and dynamics of the ecosystem continue to run naturally (Marzuki et al. 2020a). Actual examples of human activities that contribute to the contaminant component for the environment can be seen in the petroleum mining industry, which produces various types of fossil fuels to meet the needs of life (Lu et al. 2019). There are three main activity stages of petroleum production, namely the stages of production through the processing industry, distribution and use of the product. The processing stage turns out that petroleum exploitation activities always produce by-products in the form of oil sludge or sludge. The distribution stage, which generally uses sea transportation in the form of tankers and distribution pipes, has the potential for incidents of distribution pipe leaks, tanker fires, collisions, sinking ships and other potential incidents that result in oil spills and pollute the environment (Medic et al. 2020). The stage of using this fuel to run production machines, cars, motorcycles and other work equipment that uses fossil fuels produces various types of combustion gases, all of which put a burden on the earth or the potential for environmental pollution (Rua et al. 2018).

Petroleum sludge is composed of two main components, namely hydrocarbon components and heavy metals. Hydrocarbon components contain several sub-components: total solid residue, fixed residue, volatile solid residue and sludge moisture content. These components are suspected of having hazardous and toxic

materials such as polycyclic aromatic hydrocarbons (PAHs), both simple ones such as benzene to heavy ones such as benzo(a)pyrene (Marzuki et al. 2021c). Several types of heavy metals are often found in petroleum sludge, such as lead (Pb), nickel (Ni), copper (Cu), arsenic (As), chromium (Cr), aluminium (Al) and other types of heavy metals. Based on the characteristics of the components of PAHs and heavy metals that make up petroleum sludge, we can state that these components are a severe threat to the air, water and soil environment, so they must be watched out for and prevented from contaminating the environment (Marzuki et al. 2020b; Lajayer et al. 2019). We can find pAHs components in the form of gaseous residues combined with air, liquid, solid and semi-solid. Heavy metal components also include toxic elements to plants, animals and humans (Marzuki et al. 2020c). Generally in the form of particulates that can mix with air, associate with water and soil. Observing the characteristics of PAHs and heavy metals that make up petroleum sludge, We can say that these components are a severe threat to the air, water and soil environment, so they must be watched out for and prevented from contaminating the environment (Yogaswara 2017).

The sea area is a giant container, is the last shelter of the processes that occur in nature. The ocean is also a place for recovery to take home to a balanced state that occurs naturally due to shifts in a balance due to the dynamics of living things that appear on the earth's surface. Pollution that occurs in the atmosphere by heavy metal particulates and other contaminants will eventually end up in the oceans. The same is true for soil contamination by PAHs, heavy metals, microplastics and other components. Pollutants over time, the occurrence of rain that flows following low areas and finally empties into the sea will eventually empty into the ocean (Ziarati et al. 2019a, 2019b; Tereza et al. 2018).

The sea has protected itself to recover the presence of foreign contaminants through natural processes in which dynamics occur, traversed by adaptation between components until the balance is achieved. The problems that exist are contaminants that enter the sea body with the characteristics: (1) large volume, (2) various types, (3) toxic, (4) varying or even non-biodegradable decomposition time. The characteristics of these contaminants are variables that contribute dominantly to the recovery that occurs in the sea. These four variables tend to increase over time so that at a particular time, they pass the tolerance threshold for recovery that happens at sea. This condition causes the capacity and ability, performance and degradability of materials in the sea to decrease. The range of balance that can provide continuity of life is getting wider (Marzuki et al. 2021d).

The effect of hazardous materials in an area creates a chain pressure on various aspects of life, not only on the habitat of a population but also harms environmental productivity in providing support for long-term survival. Of course, this condition cannot be left alone. Herefore, we need solutions and concrete steps to reduce the volume and types of contaminants. In addition, we also need efforts to increase the carrying capacity of the environment for the lives of living things, especially humans, so that they remain in a balanced position between the needs of humans and living things with the availability and production of all conditions that can be provided by the environment (Parhamfar et al. 2020). Environmental management is

determined by technological advances and environmental engineering, either by physical, chemical or biological methods. Aspects of utilizing the wealth of natural materials, both plants and animals, including microorganisms, have a decisive role in environmental management (Orani et al. 2018).

Various types of living things, including plants, animals and microorganisms, can potentially use pollution management and environmental management, which are available and widely distributed both inland areas and in the aquatic environment. Several groups of microorganisms such as fungi, fungi and bacteria are commonly used in environmental improvements to overcome and control the toxic nature of pollutant contaminants. The hydrocarbon, heavy metal and microplastic pollutant components are most often found accumulating in waters, especially the sea. In the marine environment, it is also found that many types of microorganisms have the potential to be used as materials in improving environmental quality (de Kluijver et al. 2021). Bioremediation methods to enhance the quality of the environment contaminated with toxic components using microorganisms have been widely applied. These activities are carried out both on a small scale in the laboratory, as well as on a medium scale and continuously by the company internally, especially in managing the waste produced, so that it meets the requirements for disposal to the accessible environment (Knobloch et al. 2018).

Marine sponge is a marine biota used as a biomonitoring material to determine heavy metal pollution in the marine environment. Determination of the pollution level is carried out by slicing certain body parts of the sponge as a sample. Then preparation is carried out so that the type and content of heavy metals in the sponge can be measured using ICP and or SSA instruments (Akinde and Iwuozor 2012). The measurement data obtained can be used as a basis for determining the level of heavy metal contamination in the waters of the sponge habitat. This example shows that sea sponges are bioindicators of heavy metal pollution. The results showed that several types of sponges could survive in aquatic environments contaminated with heavy metals, presumably due to the ability of these sponges to symbiotic with certain types of bacteria and force them to produce substances that behave enzymes. The substance is then spread on the surface of the sponge body as a mask to protect itself from the stress of heavy metal toxicity (Marzuki et al. 2020b).

Further exploration of sponges, especially their way of life, growth, breeding, self-defence against predators, and their adaptability to the presence of contaminants, is an interesting study to analyze because it holds many secrets of knowledge that need to be learned to be solved. This study also includes a unique feature of sponges: they can associate and symbiotically with several types of microorganisms. In addition, sponges also can nourish the hydrocarbon components. Sponges also play a role in forming parts of metabolically active substances by linking to the ability of environmental bioremediation through biodegradation of hydrocarbon components and bio-adsorption of heavy metals (Schmittmann et al. 2020).

10.2 Potential and Contribution of Marine Sponges in Environmental Bioremediation

Sponges are one of the marine biotas widely used as biomaterials to evaluate and analyze the presence of harmful contaminants in the marine environment. The role of sponges as biomonitoring and bioindicators of the quality of the marine environment is interesting to be studied more systematically. Many aspects need to be analyzed against marine sponges, starting from the way of life, nutrition, growth and symbiotic ability with microorganisms concerning the function of biomonitoring and bioindicators of pollutants and the level of pollution that occurs in the marine environment. A lot of evidence shows that some types of marine sponges can survive and even breed in environments contaminated with toxic wastes, such as hydrocarbon compounds, especially PAHs, heavy metals and microplastics (Marzuki et al. 2021a). The phenomenon of adaptation of sponges to the environment polluted with toxic components gives several assumptions: first, the body of the sponge can carry out the function of detoxifying contaminants; second, sponges can produce mucus substances that are spread on the body surface to protect the penetration of toxic materials; third, marine sponges *Auletta sp., Clathria (Thalysias) reinwardtii*, *Callyspongia aerizusa* are biomonitoring and bioindicators of heavy metal pollution levels (Marzuki et al. 2021b); Fourth, several types of marine sponge symbiotic bacteria of the Bacillus group (*Bacillus licheniformis* strain ATCC 9789, *Bacillus sp*. partial AB353F) and Pseudomonas (*Pseudomonas stutzeri* RCH2, *Pseudomonas stutzeri* strain SLG510A3-8) were able to degrade hydrocarbon components (Marzuki et al. 2020a; Orani et al., 2020a; Orani et al. 2018).

The world's population of sponges is estimated at 15,000 species scattered in seas and lakes, but only 46.67% or ±7000 species have been reported, and only 830 species have been isolated and characterized. Sponges are living organisms with a reasonably old civilization that has existed since ±600 million years ago. Sponges can associate with many different microorganisms, including cyanobacteria, heterotrophic bacteria and unicellular algae (Campana et al. 2021a). Sponges are primitive multicellular animals (metazoans) without natural tissues, unique ways of life, capturing food by filter feeders. Sponges are sponges that reproduce both asexually and sexually. Asexual reproduction occurs by the formation of internal buds or gemmules (Maldonado et al. 2021). Sponges can produce and contain more active compounds than compounds produced by land plants. The facts attached to sponges are an essential and logical argument as multi-functional animals in the role of biota upholding the balance of the ecosystem (Schuster et al. 2018).

Bioindicators of heavy metal pollution in the sea can be determined by analyzing the growth and development of sponges (Siahaya et al. 2013). Several research results show that marine sponges contaminated with several heavy metals do not experience growth and development disorders (Melawaty et al. 2014). These conditions indicate that marine sponges can adapt to the presence of heavy metal contaminants in their habitat. The method of determining heavy metal contaminants in the marine environment is carried out by analyzing the types of heavy metals in the sponge's

body. Meanwhile, the concentration level of heavy metal contamination in the marine environment is carried out using appropriate instruments, for example, analysis using Atomic Absorption Spectrophotometer (SSA). It can also be carried out using Inductively Coupled Plasma, both optical emission type (ICP-OES) and mass spectrometry type (ICP-MS) (Ulli et al. 2016).

Sea sponge, one of the biomaterials for monitoring heavy metal pollution in the marine environment, is an essential contributor to marine natural materials in maintaining the balance and sustainability of the growth and development of ecosystems in the sea. Sponges also act as biodegradators of contaminants containing hydrocarbon components. Sponges also act as biodegradators of contaminants containing hydrocarbon components. Symbiotic bacteria of marine sponges play a role in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) (Marzuki et al. 2021c, d). Pyrene, anthracene, phenanthrene and naphthalene are PAHs that symbiotic sponge bacteria can degrade. Types of PAHs-degrading sponge symbiont bacteria include *Bacillus licheniformis* strain ATCC 9789, a sponge symbiont of *Auletta Sp*; *Acinetobacter calcoaceticus* strain PHCDB14, symbiont *Callyspongia aerizusa*; *Bacillus Sp* isolated from Neopetrosia Sp; *Bacillus pumilus* strain GLB197 isolate sponge *Niphates Sp*; and *Pseudomonas stutzeri* strain SLG510A3-8 isolate the sponge *Hyrtios erectus* (Marzuki et al. 2020b). Bacteria Bacillus sp. AB353f partial symbiosis of Neopetrosia sp. *Bacillus cohnii* strain DSM 6307 symbiont Niphates sp. Several types of sponges suspected to have potential symbiotic bacteria in the biodegradation of PAHs based on the results of identification and morphological characterization include Petrosia (Strongylo Phora) corticata, Clathria (Thalysias) reinwardtii, Callyspongia sp., Coelocarteria singaporensis, Callyspongia (Cladocalina) vaginalis and Callyspongia (Cladocalina) vaginalis. Marzuki et al. 2020a). It was further explained that this type of sponge was thought to have potential symbiotic bacteria through histology of the sponge and analysis of the phenotype and genotype of the symbiotic bacteria. Researchers obtained all the types of sponges mentioned above, about 11 species from three different islands, namely Kodingareng Keke Island, Barrang Caddi Island and Langkawi Island, the administrative area of the Makassar City Government, South Sulawesi Province, Indonesia. These islands are included in the cluster of the Spermonde Archipelago (Marzuki et al. 2021d; Knobloch et al. 2018).

Sponges have an external morphological structure influenced by several general factors, both physical, chemical and biological habitats. Sponges that grow in different habitats have varying growth structures. Types of sponges that live in less stable, shallow, turbid and high waves water environments tend to have creeping growth and are generally shorter. In contrast, the same type of sponges that live in a more stable environment, such as protected growth areas, calm currents and deep waters, tend to grow taller, upright, more symmetrical and have a more extensive body posture (Marzuki et al. 2016; Laport et al. 2009).

The presence of predators, polluting contaminants and competition of sponges with other biota is thought to affect the morphology of sponge growth. The presence of predators such as echinoderms, prosobranchia, opisthobranchia and other types of predators affects the morphology of sponge growth, even forcing sponges to evolve

body structure as a form of adaptation to avoid these predators. Several types of sponges have body structures to drill as a form of disguise and transformation to predatory threats (Yang et al. 2019).

Sponges that live in marine waters that contain coral and are overgrown with algae trigger a competition, where the sponge has a high chance of winning the match if all three are in a relatively deep environment and lack light. Still, the body structure of the growth of such sponges is generally angular (Costa et al. 2020). Sponges that live in marine waters contaminated with hydrocarbons, both aliphatic and aromatic groups, still have a high potential to survive and grow and develop because of their high adaptability to adapt through two mechanisms. First, the nutritional ability of sponges by eating matter. Organic suspended in water flow in body cavities and is sprayed back out. Second, Sponges can have a symbiotic relationship with certain microorganisms, especially bacteria that can selectively and specifically produce enzymes that behave in the form of mucus spread on the sponge body's surface. The function of the mucus is to protect and detoxify the toxic properties of PAHs (Kamaruddin et al. 2021). Sea sponges are also thought to have the ability to survive in areas contaminated with microplastics and produce mucus that can re-glue cracked concrete. Some of the benefits of this marine biota are that sponges deserve the nickname multi-functional biota and have been named green chemistry biota (Obire et al. 2020; Parama Cita et al. 2017).

The presence of predators, polluting contaminants and competition of sponges with other biota is thought to affect the morphology of sponge growth. The presence of predators such as echinoderms, prosobranchia, opisthobranchia and other types of predators affects the morphology of sponge growth, even forcing sponges to evolve body structure as a form of adaptation to avoid these predators. Several types of sponges have body structures to drill as a form of disguise and transformation to predatory threats (Yang et al. 2019).

The internal structure of the marine sponge body at the cellular level is found in several parts such as oscula, surface granules, skeleton and spicules with varying skeletons from each type of sponge. A variety of skeletons are influenced by the growth environment and dynamics of life experienced by the sponge in its growth period. The growth dynamics of sea sponges are influenced by their growth habitat conditions, especially currents, depth, wave height, exposure to sunlight, nutrients, predators and contaminants. These factors also affect the structure and anatomy of cells. The histology of marine sponges based on the catalogue (Krishnamoorthy et al. 1983) is as per Fig. 10.1a–f, for the marine sponge *Niphates sp*., belongs to the family Niphatidae (Duchassaing de Fonbressin and Michelotti 1864).

The interaction mechanism of sponges with bacteria provides a place or sponge host for certain bacteria to carry out cell growth and division activities. Then there is interaction and adaptation to the new environment. If there are interfering components, then the host organism is stimulated to produce active substances or synthesize bioactive compounds as secondary metabolites. The active substance is delivered for self-protection and maintains environmental balance (Campana et al. 2021a, 2021b). The completion and arrangement of substances or chemical components of the active substance are natural. The type of active substance formed is

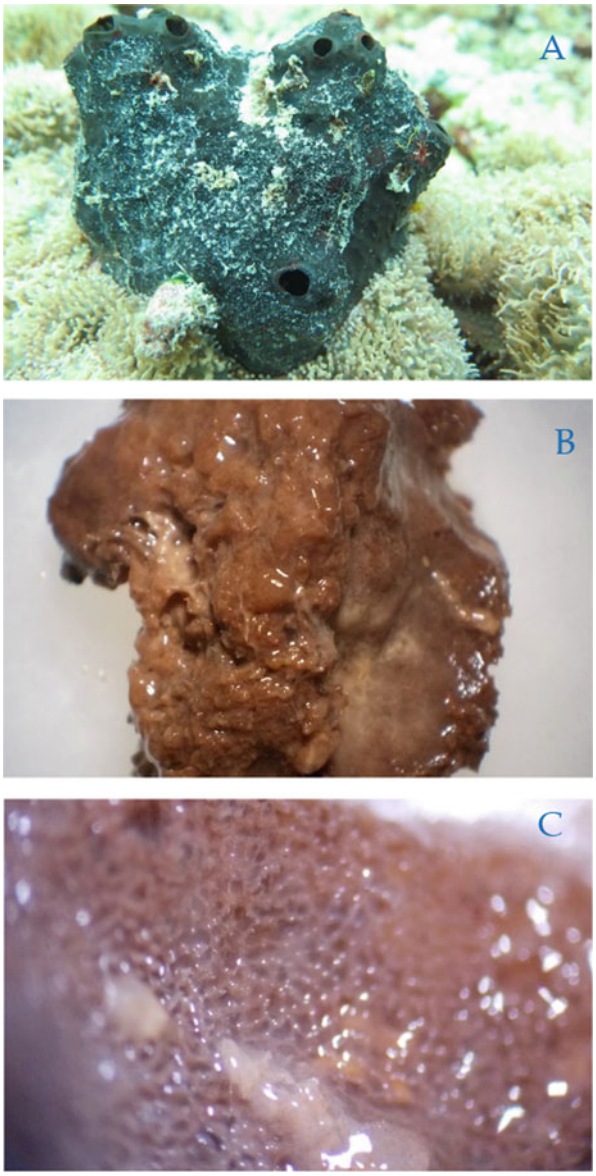

Fig. 10.1 (**a**) Growth from Sligthy globular sponge, with big size oscula. (**b**) Consistency Slippery surface sponge, covered by mudlike slime. Inelastic and brittle body sponge. (**c**) Surface granular sponge surface. (**d**) Skeleton Spicule skeleton with echinating spicule. (**e**) Skeleton tract Paucispicular tract skeleton with high fibre. (**f**) Spicule Small megasclera oxea (magnification 40x)

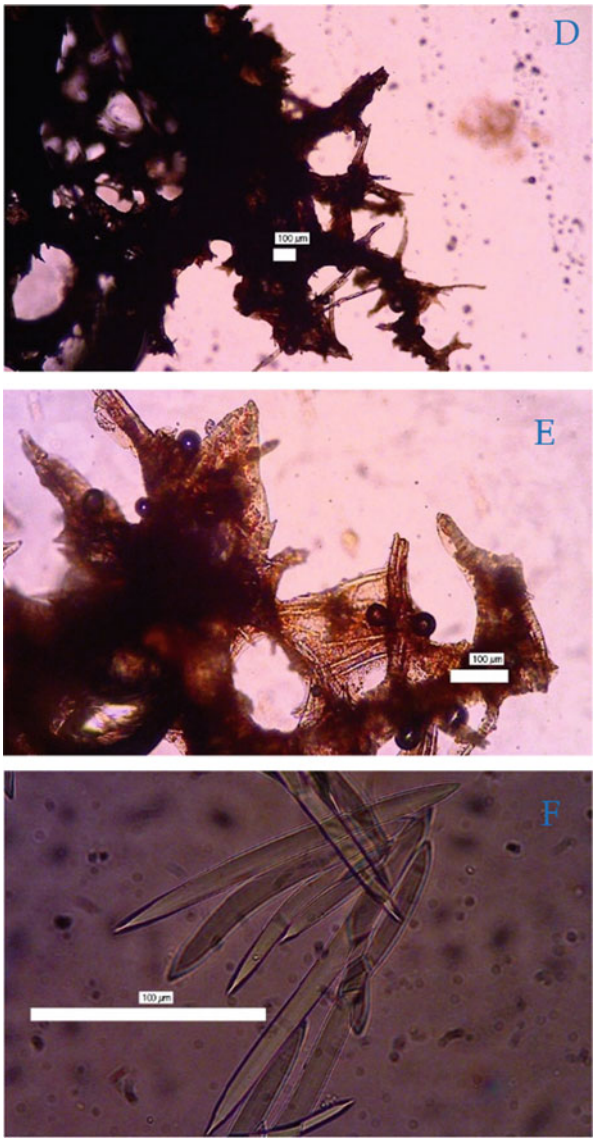

Fig. 10.1 (continued)

influenced by the kind of food and the interfering components in the sponge growth process. Generally, sponge nutrition is rich in new microorganisms with potential pharmacological activity so that sponges can carry out the growth process well, even in extreme environments. On the other hand, microorganisms or bacteria benefit from living on a sponge, i.e. bacteria are protected from the pressure of waves and currents. The interaction between sponges and bacteria occurs as a form of

commensalism symbiosis, in which bioactive compounds are produced (Marzuki et al. 2020a).

Sponge symbiont bacteria have a significant influence on the formation of bioactive substances. This influence can be seen in the role of bacteria as the leading supplier of energy needed by sponges. Screening of symbiotic sponge bacteria was carried out by inoculating bacteria associated with the sponge on NA media overgrown with the test bacteria. This qualitative method can only determine whether the bacteria can inhibit the test bacteria E. coli and S. Aureus without knowing the effectiveness of these bacteria (Gunathilake 2018). It is estimated that 40% of the biomass of some types of sponges is composed of bacterial communities. Cyanobacteria symbiosis can make up one-third of the total mass of living tissue in some types of sponges. The energy needs of some types of sponges are supplied from 48% to 80% of microorganisms (Knobloch et al. 2018).

The bioremediation function of marine sponges on the environment is thought to be played by the active substances in the sponge's body. Sponges produce these chemical components to defend themselves against potential growth disorders. The characters, types and specific properties of these secondary metabolites are formed according to the dynamics of life experienced by sponges during their growth period. These metabolic substances are thought to have several functions and benefits, both in utilization studies in the medical field and environmental bioremediation processes (Obire et al. 2020). Its environmental remediation function is primarily for the application and function of biodegradation of PAHs and heavy metal bio-absorption, including the potential to overcome the presence of microplastics in the environment (Marzuki et al. 2021b). The researcher can achieve knowledge of the role and function of these sponges in the biodegradation of PAHs, bio-adsorption of heavy metals and the absorption potential of microplastic components by conducting a series of studies and analyses. The assessment and analysis activity begins with observing the sponge's growth environment, screening the morphology and histology of potential sponges for environmental remediation functions. The next activity is the isolation of symbiotic bacteria, characterization through phenotypic and genotypic analysis of symbiotic bacteria, to conducting micro-scale tests and field experiments to determine the performance of symbiotic sponge bacteria in the biodegradation of PAHs and bio-adsorption of heavy metals (Marzuki 2020; Rua et al. 2018).

10.3 Search for Potential Sponges for Bioremediation Functions in Polluted Environments

The method of determining potential sponges for the biodegradation function of PAHs was carried out by isolating potential sponge symbiotic microorganisms. The purpose of potential sponges is to select specific types of sponges that meet the following criteria: first, selecting sponges that are suspected of living in areas

contaminated with hydrocarbons; second, the surface of the sponge body is covered by mucus or enzyme behaviour substances or at least dark-coloured sponges; third. The colour of the sponge is generally less bright. The selected sponges were then isolated to obtain bacterial isolates. Isolation of marine sponge symbiotic bacteria using a simple method, as shown in Experiment 10.1.

Experiment 10.1: Isolation of Marine Sponge Symbiotic Bacteria
The selected potential marine sponges were cleaned by spraying the surface using sterile seawater with a ratio of 1 cm2: 5 mL of sterile seawater. The mesohyl sections were ±1× 1 cm in size, crushed and diluted with sterile Phosphate Buffer Saline (PBS) in a ratio of 1:1. Isolate the bacteria on the outer surface using a sterile swab, then wipe in one direction on the outer surface of the sponge. The sterile swab was put into a dilution tube containing sterile PBS and vortexed. The results of the dilution were spread into a petri dish that already contained Seawater Complete (SWC) media, incubated at 26°C for 24–36 h, observed colony growth, bacterial morphology. Selected colonies, separated using a round needle, were purified using the same medium to obtain pure isolates. Purifying bacterial symbionts using the direct plating method was performed by scratching one ose of colonies in a zig-zag direction on a petri dish containing 100% marine-agar media. Incubation temperature 30°C, 1–2 days. Re-scratching on 100% marine agar until a single colony was obtained (Marzuki et al. 2021a).

Marine sponge symbiotic bacteria biodegraded PAHs contaminants by integrating one type (Naphthalene) with a potential bacterial suspension. The complete procedure for biodegradation of marine sponge symbiont bacteria against PAHs is presented according to the method in Experiment 10.2.

Experiment 10.2: Biodegradation of PAHs Using Marine Sponge Symbiotic Bacteria
Determination of potential bacteria is done through preliminary tests. Bacteria that pass the initial test are bacteria that show growth activity on hydrocarbon-contaminated media. Propagation of selected bacterial cells by culture. The bacterial suspension was made and determined the number of cells. Entered 25 mL of bacterial suspension in several degradation reactors (vials), adapted for 1 × 24 h, put 5 mL of contaminant PAHs (naphthalene) with a concentration of 1000 ppm, shaker incubator 200 rpm. Observations and measurements of media degradation parameters were carried out every 2 days for 30 days. Determination of the ability and performance of biodegradation of symbiotic bacteria were analyzed using Gas Chromatography–Mass Spectroscopy (GC–MS) instruments. Biodegradation products were analyzed using Fourier Transform – Infrared Spectroscopy (FT–IR) for serial interaction periods, such as 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 (Lu et al. 2019).

Parameters of PAHs biodegradation by symbiotic sponge bacteria consisted of optical density, pH, temperature, an abundance of gas bubbles and fermentation odour. Optical density was determined using a UV-Vis spectrometer on a mask. For each serial interaction period in multiples of 2 days. Suppose an increase in the optical density (OD) value for the first few days of interaction. In that case, it

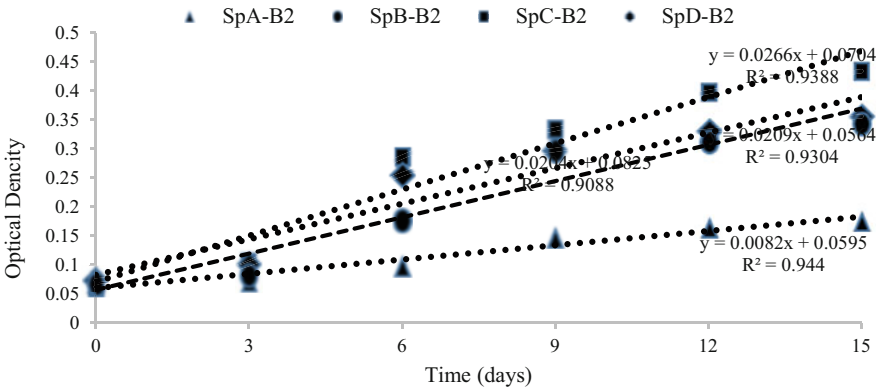

Fig. 10.2 The rate of increase in the value of OD (bacterial cell growth rate) based on the interaction time in days between a sponge symbiotic bacterial suspension and 10,000 ppm naphthalene. Source: Marzuki et al. 2021d)

indicates a bacterial activity in the degradation medium contaminated with PAHs until it enters the saturation period marked by no increase in the OD value in the next few days. The rate of increase in OD value can be identified with the growth rate of bacterial isolates isolated from four different types of marine sponges, namely *Hyrtios erectus* (SpA.B2), *Clathria (Thalysias) reinwardtii*) (SpB.B2), *Niphates sp.* (SpC.B2), and *Callyspongia sp.* (SpD.B2), respectively, interacting with 10,000 ppm naphthalene and 10,000 pyrenes for 15 days (Marzuki et al. 2021d), according to Figs. 10.2 and 10.3.

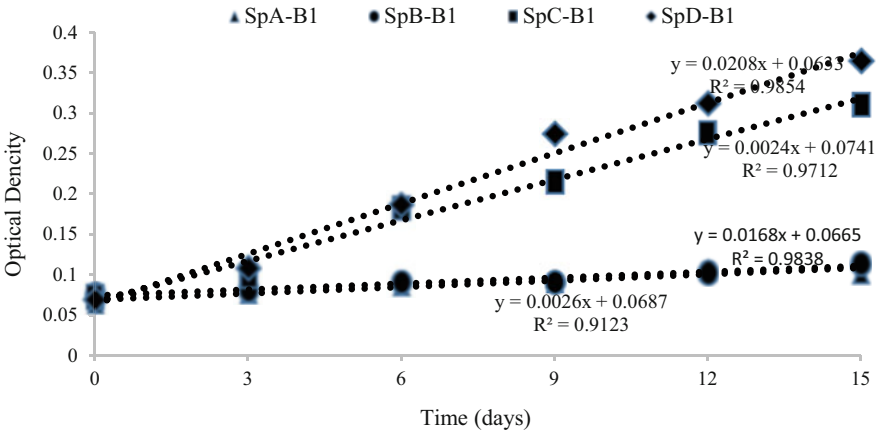

Fig. 10.3 The rate of increase in the value of OD (bacterial cell growth rate) based on the interaction time in days between a sponge symbiotic bacterial suspension and 10,000 ppm pyrene. Source: Marzuki et al. 2021d)

The increase in OD value in the reactor containing PAHs contaminants is directly proportional to interaction time. The rise in OD value indicated an activity of isolates of sponge symbiont bacteria containing PAH. These conditions showed that the bacteria were able to adapt to media containing PAHs contaminants, although the OD values of each isolate were different. The sequence of growth of symbiotic sponge bacterial cells in naphthalene-contaminated media (Fig. 10.2), SpC.B2 SpD. B2 SpB.B2 SpA.B2, while the growth rate of bacterial cells in pyrene-contaminated media (Fig. 10.3) in the order SpD.B2 SpC.B2 SpB.B2 SpA.B2.

Changes in the pH of the degradation medium towards a lower value or tend to be more acidic indicate that there is bacterial biodegradation activity on the hydrocarbon component substrate. The temperature of the degradation media tends to increase generally in the range of ±0.5–2.3 0C after the interaction lasts for several days if there is biodegradation activity in the reactor (Bendouz et al. 2017).

The gas bubbles in the reactor are relatively increased as a sign that there is biodegradation activity. The abundance of gas bubbles formed is directly proportional to the level of biodegradation. Gas bubbles are formed and generally increase after the interaction runs for a few days. The abundance decreases as the biodegradation process in the reactor weaken, indicating a decline in the number of bacterial cells that carry out the degradation process (Marzuki et al. 2020a). Gas bubbles formed as a sign that there are simple organic compounds in the reactor in methane gas, CO_2, NO_2 and other gases resulting from biodegradation or decomposition/ rehabilitation of PAHs components. The smell of fermentation is also part of the biodegradation parameter as an indicator. The degradation that occurs results from the work of bacteria through the mechanism of degradation of enzymatic reactions produced by biodegradative bacteria (Onwosi et al. 2017).

The speciality of bacteria in the PAHs degradation method is the ability of bacteria to destroy the hydrocarbon structure and turn it into an energy source for the activity and survival of bacteria. The biodegradation performance of symbiotic sponge bacteria against PAHs was determined using GC–MS. The data obtained on the chromatogram show the components of the biodegradation product (Marzuki et al. 2020c). The chromatogram data is in the form of component abundance peaks and the potential for new peaks to appear. In general, the abundance of the tested PAHs components as substrates will decrease with the emergence of new peaks. The higher the performance of bacterial biodegradation, the lower the peak height (abundance) of the PAHs test components. The lower the PAHs, the lower the concentration of the degradation components. Figure 10.4 shows an example of a GC–MS chromatogram resulting from the biodegradation of Bacillus SP strain AB353f in symbiosis with the Neopetrosia Sp sponge against naphthalene compounds.

The biodegradation process of PAHs (naphthalene) substrate at 1-day interaction was not seen (Fig. 10.4a). This biodegradation process began to appear after 10 days of interaction between Bacillus sp. strain AB353f and naphthalene (PAHs substrate). An indication between Bacillus sp. strain AB353f and PAHs substrate is indicated by the decreasing peak height of naphthalene in the graph (Fig. 10.4b). Decrease of peak showed that the abundance of the substrate had decreased, meaning that there

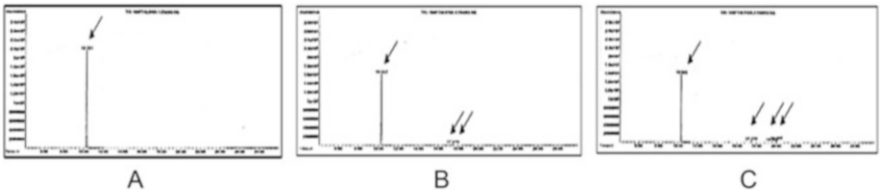

Fig. 10.4 Chromatogram of naphthalene biodegradation of Bacillus SP strain AB353f based on interaction time. (**a**). Interaction time 1 day; (**b**) The interaction time is 10 days, and C. the interaction time is 20 days (Marzuki et al. 2020c)

was a decrease in the concentration of PAHs along with the emergence of two new peaks resulting from biodegradation in the form of simple organic compounds. The new peak increased to three after the interaction period lasted 20 days (Fig. 10.4c), marked by the decreasing peak height of naphthalene which means that the biodegradation process continues. The interaction period in this experiment was up to 30 days. Still, it appears that during the interaction period above 20 days, there was no addition of new peaks, and the peak height of PAHs tended to stagnate. This condition indicates that the biodegradation process is no longer running. It could be because the bacterial cells as biodegradators died due to poisoning by the toxic nature of PAHs or because the bacterial cells could not withstand the increase in the acidic properties of the media in the reactor. The increase in the acidic properties of the reactor media occurred, presumably because components were resulting from the biodegradation of simple organic compounds from the carboxylic acid group (Marzuki et al. 2021c). The results of the GC–MS measurement also provide data on the level of biodegradation. After calculating, the result indicated that the biodegradation rate was in the range of 26% - 46% for using one type of symbiotic sponge bacteria against one type of PAHs (Costa et al. 2020).

Several ways can improve the biodegradation performance of bacteria against PAHs. For example, it increases the number of bacterial cells in the reactor, prolonging the life of bacterial cells by providing nutrients in adding nitrogen, phosphorus and potassium in the degradation reactor. In addition, during the interaction period, oxygen is supplied to prevent an increase in the acidity of the degradation media or the use of several types of bacteria (a consortium of bacteria), which can degrade hydrocarbon components. The use of consortium bacteria to increase the level of biodegradation does not necessarily increase the capacity of these bacteria to degrade, presumably due to competition between bacteria in obtaining energy supplies from the PAH components that have been destroyed. However, the biodegradation of PAHs with consortium bacteria is currently being carried out because of the ease of multiplying bacterial cells and can be carried out very quickly (Bendouz et al. 2017).

Many collections of bacteria that can biodegrade PAHs have been carried out, especially bacteria from the Bacillus and Pseudomonas groups. Both groups of these bacteria can be isolated from several sources, such as soil contaminated with PAHs, seawater contaminated with hydrocarbons, sponges and several other marine biotas.

A collection of PAH-degrading bacteria is known as hydrocarbonoclastic bacteria (Lu et al. 2019).

The mechanism of bacterial biodegradation of hydrocarbon components, both aliphatic and aromatic groups, in principle, is through the oxidation pathway to form alcohol. Alcohol products become aldehydes and finally produce carboxylic acids and a small portion of esters before entering the -oxidation cycle and producing energy for biodegradation activity—next cycle. The biodegradation process also produces by-products in simple organic compounds, such as CO2, methane gas, NO and other gases, depending on the degraded hydrocarbon component. The biodegradation of PAHs is relatively the same as aliphatic components (Medic et al. 2020). The striking difference for the aromatic hydrocarbon component at the stage after the oxidation reaction is that it is preceded by the destruction stage of the aromatic molecular structure so that the biodegradation of PAHs components by suitable specific bacteria takes longer. The resulting degradation rate is generally much smaller than in the degradation of aliphatic hydrocarbon components (Fang et al. 2020). A simple description of the biodegradation mechanism of aliphatic components is presented in Fig. 10.5.

Specific degradation pathways of Bacillus subtilis strain BAB-1684 sponge symbiont Callyspongia sp. against the hydrocarbon components of petroleum sludge waste after 20 days of contact are presented in Fig. 10.6.

Based on the illustration of the biodegradation of hydrocarbon components by the symbiotic marine sponge bacteria (Figs. 10.5 and 10.6), some steps allow the biodegradation process to stop completely. The biodegradation process stopped entirely because the bacterial cells experienced mass death. The biodegradation process can stop completely at the stage of changing the biodegradation products of aldehydes or ketones into carboxylic acids. The formation of carboxylic compounds can cause the interaction medium to become acidic so that bacterial cells have the potential to experience sudden death because they cannot survive at a certain acidity level in the degradation medium (Marzuki et al. 2021a; Yogaswara 2017). This stage is known as the reaction rate stop stage, or the biodegradation process stops completely.

The contribution of marine biota, especially sponges, in maintaining the balance of the marine ecosystem is substantial. The contribution of marine life, especially sponges, in maintaining the balance of the marine ecosystem is enormous. Suppose an in-depth review is carried out on the formation of secondary metabolite

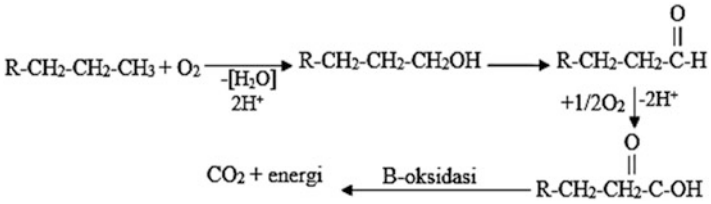

Fig. 10.5 Simple mechanism of biodegradation of aliphatic hydrocarbon components

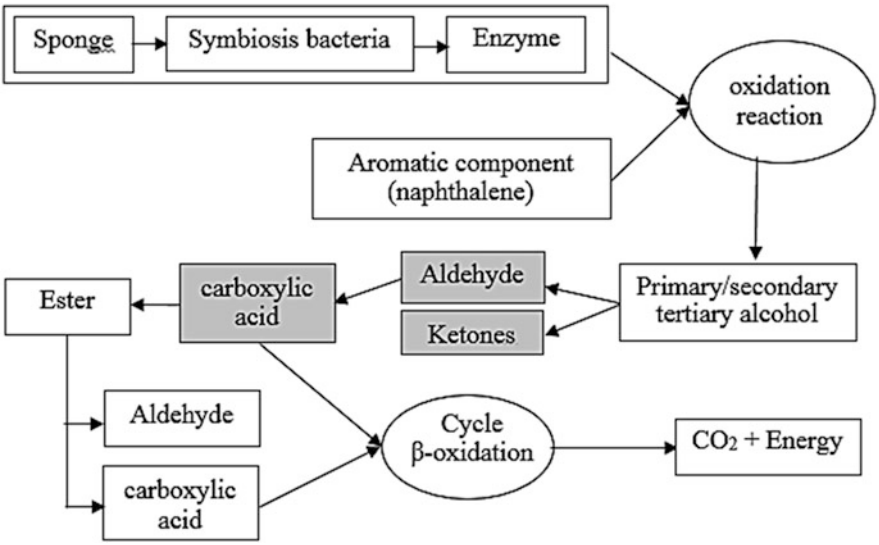

Fig. 10.6 Estimation of a simple path of biodegradation of aliphatic hydrocarbon components of petroleum sludge by the bacterium Bacillus subtilis strain BAB-1684 symbiosis of sponge Callyspongia sp

components of sponges and the effect of these substances on their biodegradability, bio-adsorption and activity of sponge bioactive substances against pathogenic bacteria and fungi. In addition, the active substance of sea sponges is thought to have the potential as primary and secondary raw materials for certain drugs to treat a disease (Shareef et al. 2016). Studies on sponges, symbiotic microorganisms and components of secondary metabolites are interesting to do more comprehensively, including studies related to the competition for the growth of sponges versus other biotas such as corals and algae if all three are in the same area (Schuster et al. 2018).

A simple study of the morphology and histology of marine sponges of the Niphates sp. species obtained from the sea waters around Kodingareng Keke Island has been described in point 10.2 above. Part of the characterization of sponges is to identify and characterize the symbiotic sponge bacteria that are thought to have biodegradation performance against PAHs potentially. Phenotype and genotype characterization of symbiotic sponge bacteria was carried out by taking samples of four isolates of sponge bacteria *Petrosia (Strongylo Phora) corticata* (Sp. 1), Auletta sp. (Sp. 2), *Neopetrosia sp.* (Sp. 3) and *Callyspongia aerizusa* (Sp. 4). (Marzuki et al. 2020a, 2020b, 2020c, Bioflux), are presented in Table 10.1.

The urease test results (Table 10.1) on two isolates (Sp. 1 and 2) showed positive results, meaning that both isolates were able to hydrolyze urea. This ability means that the isolate can produce and possess the enzyme urease. On the other hand, the isolates (Sp. 3 and 4) were negative, meaning that both isolates could not hydrolyze urea or did not have urease enzyme. V-P reagent test isolates (Sp. 1–3) showed a positive reaction, meaning that there were components in the three isolates capable of carrying out the fermentation reaction. In the methyl red test (R-Mr), the four

Table 10.1 Phenotype characterization of sponge symbiont bacteria Callyspongia sp, Biochemical Test method

Biochemical reagents	Media	Sponge bacterial symbiont			
		Sp. 1	Sp. 2	Sp. 3	Sp. 4
Starch hydrolysis	Starch agar	Base	Base	Base	Base
Casein hydrolysis	Milk agar	Acid	Acid	Acid	Acid
Gelatin hydrolysis	Gelatins	–	–	–	–
Nitrate reduction	Nitrate broth	–	–	–	–
Indole	Tryptone broth	–	–	–	–
H_2S	H_2S	–	–	–	–
Reagent methyl red	R-Mr broth	+	+	+	+
Reagent- Voges Proskauer	R-VP broth	+	+	+	–
Citrates	Citrate	+	–	+	–
Urease	Urea broth	+	+	–	–
Glucose	Glucose broth	–	–	–	–
Lactose	Lactose broth	+	+	+	+
Sukrose	Sucrose broth	–	–	–	–
Mannitol	Mannitol broth	–	–	–	–
Catalase	Nutrient agar (NA) slant	+	+	+	–

Note: + (reaction); – (no reaction)
Source: Marzuki et al. (2020a)

isolates showed a positive response, indicating that the isolates could produce acid in glucose fermentation (Marzuki et al. 2015). The catalase test on all isolates resulted in a positive reaction (Sp. 1–3 isolates). The isolates had catalase enzymes that could degrade hydrogen peroxide (H2O2), while the fermentation test used glucose reagent did not show any reaction activity. The lactose test showed that the four isolates gave a positive reaction. The sucrose test resulted in a negative response, meaning that the bacteria that grew in the fermented liquid media were acidic components (Bibi et al. 2016). The results of characterization through gram staining and biochemical tests showed that the two microsymbiont isolates of four different types of marine sponges contained enzymes and could ferment and process carbon from their environment. With the six criteria described above, it is suspected that three isolates (Sp. 1, Sp. 2, Sp. 3) have the potential and ability to degrade, especially to aliphatic hydrocarbon components. Still, it is necessary to test and follow up on analysis further to degrade aromatic hydrocarbons (Khabouchi et al. 2020).

The genotype identification of four isolates from four different marine sponges was carried out to see the pair structure and nitrogen base-pair composition through the Basic Local Alignment Search Tool (BLAST), PCR method. The results of the isolate sequencing were opened through the bioedit programme. The sample bacterial DNA sequences were entered into the BLAST (Basic Local Alignment Search Tool) programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequences were identified with the DNA database. GenBank on the site (Kadhim et al. 2013; Cowden 1976). The results of the alignment of the sample sequences with the GenBank sequences showed a high similarity of the homologous series, which can be seen in the table as presented in Table 10.2.

Table 10.2 BLAST results of Symbiont Sponge bacteria, PCR method

Symbiont code	Sample Sequence	Sequence Gen Bank	Quantity (%)	Difference (%)	Species
Sp. 1	17–972 (955)	608.723–609.690 (967)	944/955 (98.85)	4/955 (0.42)	*Pseudomonas stutzeri* RCH2
Sp. 2	11–985 (974)	524.589–525.563 (974)	956/974 (98.15)	14/974 (0.01)	*Bacillus licheniformis* strain ATCC9789
Sp. 3	15–975 (960)	574.123–575.089 (966)	932/960 (97.49)	16/960 (1.66)	*Bacillus sp.* AB353F.Partial
Sp. 4	21–934 (913)	574.323–575.258 (935)	906/935 (96.90)	12/935 (1.28)	*Acinotobacter calcoaceticus* strain PHKDB14

Source: Marzuki et al. (2020a)

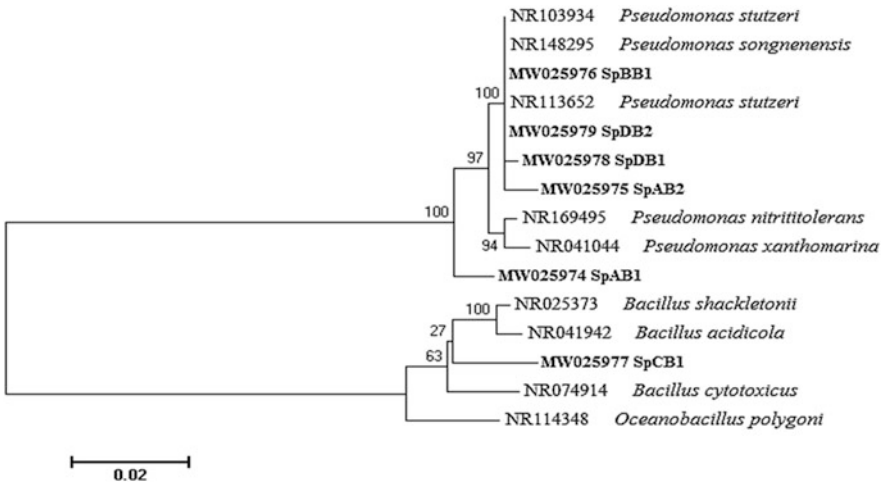

Fig. 10.7 Representative neighbour-joining tree reconstructed with the 16S rRNA sequences of marine sponge symbiont bacteria (Marzuki et al. 2021d)

The results of genetic analysis using PCR found each sponge symbiotic isolate (Table 10.2). There are two types of bacteria with different strains. Each of which is isolates from sponges Auletta sp. (Sp. 2), Neopetrosia sp. (Sp. 3), one group of Pseudomonas, isolates of sponges Auletta sp. (Sp. 2) and one group of Acinetobacter. Which is an isolate from sponge Callyspongia aerizusa (Sp. 4). Based on the results of biochemical tests (Table 10.1) by observing the reaction results of each test medium combined with the results of the identification of the 16S rRNA gene molecule by PCR method (Table 10.2). It is indicated that the four species can carry out chemical reactions to break down hydrocarbon molecules (Maldonado et al. 2021).

Characterization and phylogenetic identification of symbiotic sponge bacteria according to the reconstructed marine sponge symbiotic bacterial phylogenetic tree against 16S rRNA bacteria, three species were well resolved supported by moderate bootstrap values (Fig. 10.7). The first clade, isolates of sponges SpA.B2, SpB.B2, SpD.B2, live in groups with *Pseudomonas stutzeri* and *Pseudomonas songnenensis* with 97–99% homology. The second clade, consisting of SpA. B1/B2, which is positioned as a child clade of the Pseudomonas species. The third clade, SpC isolates. B1 grouped with Bacillus isolates reached 96%.

10.4 Development of the Function of Symbiotic Sponge Bacteria Through Heavy Metal Bio-Adsorption Method

The application of marine sponge symbiotic bacteria in bioremediation of heavy metal-contaminated environments is being developed. Several studies have shown that several types of bacteria can bio-adsorb heavy metals. Heavy metals such as Zn, Pb, and Cd can be absorbed using certain bacteria (Konkolewska et al. 2020); Pb, Ni, Cd and Cr (Alimardan et al. 2016); Cd and Pb (Alaboudi et al. 2018). Even several reports were showing that several types of heavy metals were found in marine sponges, for example, Pb, Cd, Zn, Fe and Cr (Wibowo et al. 2019; Siahaya et al. 2013; Melawaty et al. 2014), where the sponge showed a reasonable growth rate. Pseudomonas bacteria isolated from marine sponges can adsorb heavy metals Cr and Mn (Marzuki 2020).

Almost all types of bacteria that breed in areas exposed to heavy metals can carry out the function of bio-adsorption. The problem that exists for the application of bacteria in the bioremediation of environmental heavy metal contaminants is how to identify and obtain bacteria that are capable and have high performance in the bio-adsorption of heavy metals, including types of heavy metals that can be absorbed. This condition demands an assessment to find out and obtain data on the type of bacteria, the level of absorption and the types of heavy metals that bacteria can adsorb. A data bank on this matter is needed to provide convenience and quick handling of areas exposed to heavy metal, both in the aquatic environment and on land or land, especially ex-mining grounds for land function recovery, for agricultural activities. Alimardan et al. 2016).

Several types of bacteria isolated from sea sponges were stated to be able to absorb several types of heavy metals, including *Bacillus sp.* strain AB353f against heavy metals Cr and Mn (Marzuki et al. 2020b; Marzuki 2020), *Bacillus pumilus* strain GLB197 *and Pseudomonas stutzeri* strain SLG510A3-8 against heavy metals Cr(VI) and Cd(II) (Marzuki 2020). Types of bacterial isolates without known species that are symbiotic sponges have been reported to absorb several types of heavy metals. It is including *Petrosia (Strongylo Phore) Corticata*, *Neopetrosia sp*., *Callyspongia aerizusa*, *Niphates Sp*, *Hyrtios erectus* and *Auletta sp.* to heavy metal Nickel (Ni), Copper (Cu), Lead (Pb), Chromium (Cr) and Mercury (Hg) (Angela and Marzuki, 2021; Wibowo et al. 2019).

The general method of tracing bacteria has the function of bio-adsorption of heavy metals: first, identification of marine sponges that live in waters exposed to heavy metals. Second, sampling several sponges and isolate the associated bacteria. Third, the isolates obtained were tested for their bio-adsorption ability on certain heavy metals by placing some bacterial colonies on media engineered to be contaminated with several types of heavy metals, incubating and observing the growth activity of the isolates. Fourth, potential isolates from possible test results were identified and phenotype and genotype characterization. Fifth, make stock, catalogue

of isolates related to types of heavy metals that can be absorbed (Gebregewergis 2020).

Researchers have carried out trials of stock bacteria that have been characterized against certain metals to see their bio-adsorption ability and performance in an engineered environment contaminated with several heavy metals in known concentrations. During the contact process between bacterial suspensions and heavy metals in an engineering environment, bacterial cell growth was observed by measuring the optical density of the interaction medium based on the contact time in days.

Experiment 10.3: Bio-adsorption of Heavy Metals Using Marine Sponge Symbiotic Bacteria

The Bio-adsorption ability of marine sponge symbiotic bacteria to heavy metal as a sewage contaminant can be determined by several procedures. First, waste is made contaminated with heavy metals, for example, Cr (III), Cr (VI), Mn (II) and Mn (VII), each with a concentration of 250 ppm and a volume of 1000 mL. Second, determine isolates of marine sponge symbiotic bacteria with bio-adsorption potential. For example, *Bacillus licheniformis* strain ATCC 9789 (BS) and *Acinetobacter calcoaceticus* strain PHKD B14 (AC), both types of bacteria was cultured on NA media, then incubated 1 × 24 O'clock. Each culture was suspended in a solution of 1,000 mL of physiological 0.9% NaCl solution. Prepare 10 reactors, each filled with 100 mL of isolate suspension, adapted for 1 × 24 h in a new environment, (3) each reactor is added 10 mL of heavy metal contaminants, such as Cr (III) or Cr (VI). The reactor is in the Shaker incubator at 150 rpm. Observation of bacterial cell growth (optical density) was carried out every 3 days. The Observation used a spectrophotometer and determined the level of bio-adsorption of heavy metals by measuring media absorption using AAS, then plotted in Eqs. (10.1) and (10.2) to determine the capacity and efficiency of bio-adsorption (Marzuki 2020).

$$\mathrm{Q} = \frac{C1 - C2}{C1} x\, V \tag{10.1}$$

$$\%E = \frac{C1 - C2}{C1} \times 100\%; \tag{10.2}$$

Based on Experiment 10.3 that has been carried out, it was obtained an overview of the growth of BC and AC bacterial cells in media contaminated with Cr (III), Cr (VI), Mn (II) and Mn (VII).

The experimental results showed that the growth of BS and AC bacterial cells was more dominant in media exposed to Cr(III) than in media contaminated with Cr (VI) (Fig. 10.8). The growth of BS cells in Cr(III) and Cr(VI) contaminated media was maximum during the first 3 days of contact and relatively low after 6 days of contact onwards (Fig. 10.8a). The growth of AC bacteria cells in media exposed to Cr (III) and Cr (VI) was relatively the same as BS bacteria. Only the first 3 and 6 days of contact in Cr (III) media were much more dominant than in Cr (VI) media which sloped from childhood. First 3 days of contact (Fig. 10.8b). The difference in the growth rate of bacterial cells in these two types of media is influenced by the ability

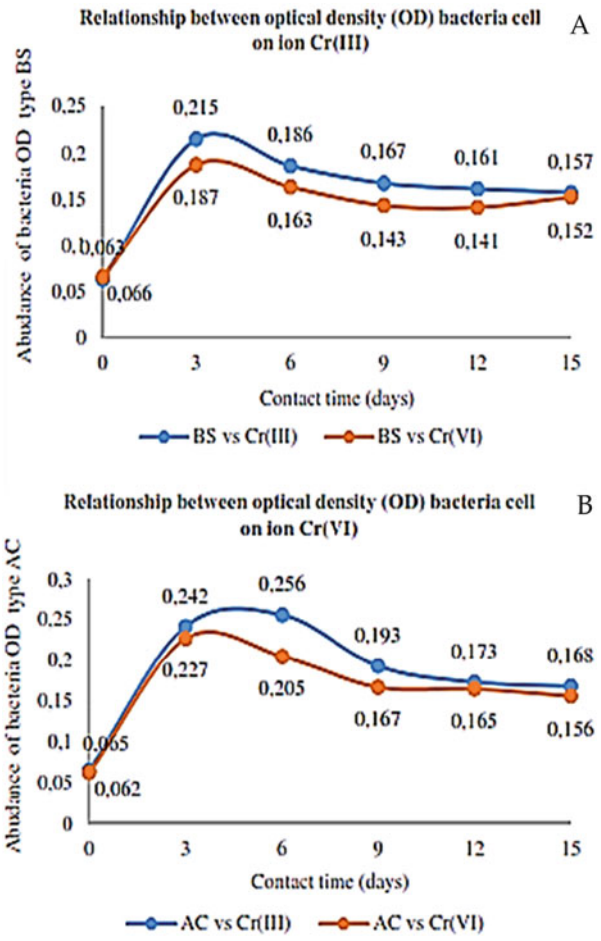

Fig. 10.8 (**a**) Growth of BS bacterial cells in Cr (III) and Cr (VI) contaminated media. (**b**) AC bacterial cell growth in Cr(III) and Cr (VI) contaminated media

of the bacterial cells to adapt to media with different levels of toxicity. It was seen that both types of bacteria BS and AC were relatively underdeveloped in media that had a higher level of toxicity (Marzuki et al. 2021b).

The treatment was the same, and the types of symbiotic bacteria were also the same. Still, Mn (II) and Mn (VII) replaced the types of heavy metal contaminants, showing that the growth of BS and AC bacteria cells did not change significantly. The growth of BS bacteria in Mn (II) media was more dominant in a wider range than in Mn (VII) media. However, the maximum growth of both types of bacteria occurred during the first 3 days of contact (Fig. 10.9a). While the dominant growth of AC bacteria cells in Mn (II) media happened at a more extended contact period, namely on days 3–6 compared to Mn (VII) media and the following contact period, the growth of AC bacterial cells in Mn (II) and Mn (VII) media was relatively the same and slopes (Fig. 10.9b) (Marzuki 2020).

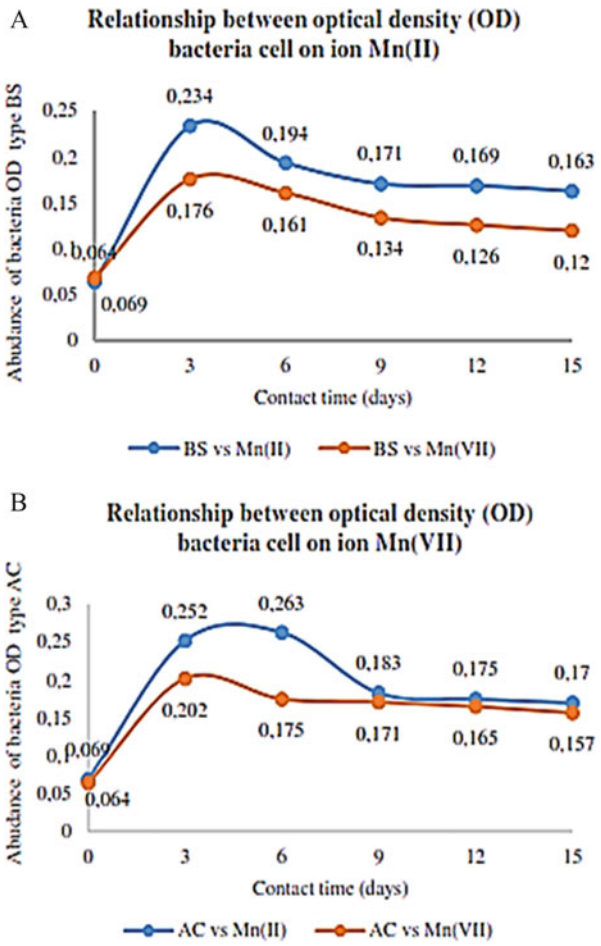

Fig. 10.9 (**a**) Growth of BS bacterial cells in Mn (II) and Mn (VII) contaminated media. (**b**) Growth of AC bacterial cells in Mn (II) and Mn (VII) contaminated media

The bio-adsorption capacity was calculated using Eq. (10.1), while the bio-adsorption efficiency was determined using eq. 2, based on absorption data measured using Atomic Absorption Spectrophotometer (AAS). The bio-adsorption capacity of BS and AC bacteria to heavy metal contaminants Cr (VI) was relatively the same, as was the bio-adsorption efficiency shown by the two types of bacteria (Fig. 10.10a, b). Still, in the same picture, the curved line of bio-adsorption capacity (blue colour) does not coincide with the curve line of bio-adsorption efficiency (orange). These conditions indicate that the bio-adsorption performance of BS and AC bacteria against toxic heavy metals Cr (VI) does not reach 50%; Cr (VI) contaminants were chosen because the toxicity level is higher than Cr (III) (Marzuki 2020).

The bio-adsorption capacity of BS and AC bacteria against heavy metal contaminants Mn (II) was relatively the same, including the bio-adsorption efficiency

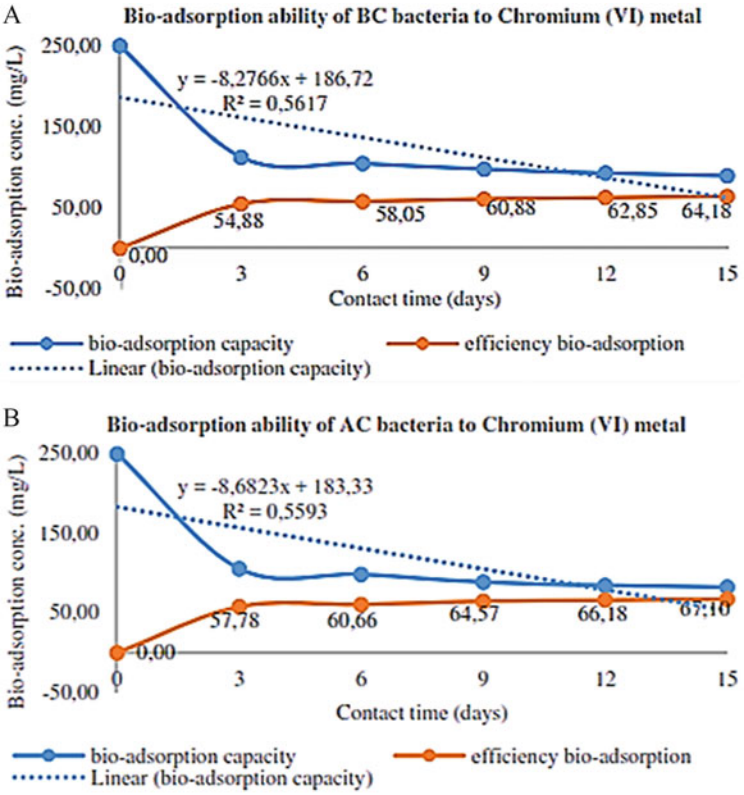

Fig. 10.10 (**a**) Bio-adsorption capacity and efficiency of BS bacteria against Cr(VI) contaminants. (**b**) AC bacteria bio-adsorption capacity and efficiency against Cr(VI) contaminants

performance shown by the two types of bacteria (Fig. 10.11a, b). What is different from the bio-adsorption version of these two types of symbiotic sponge bacteria is the performance of the bio-adsorption capacity shown against heavy metal contaminants Mn (II) exceeds 50%. The two types of bacteria used as bio-adsorbents for heavy metals are Bacteria Bacillus sp. AB353f partial (BS) is an isolate of the sponge *Neopetrosia sp.,* while the bacterium *Acinetobacter calcoaceticus* strain PHCDB14 (AC) is an isolate of the marine sponge *Callyspongia aerizusa* (Marzuki et al. 2021b).

The performance and level of bio-adsorption of heavy metals by symbiotic bacteria of marine sponges have not shown maximum achievement in environmental remediation. So it is necessary to make efforts and developments to obtain bio-adsorption results of heavy metals bio-adsorption capacity reaching 75–90%. The approach that can be taken to improve the bio-adsorption performance of heavy metals is by tracing the types of bacteria that have high bio-adsorption performance against heavy metals. A consortium of bacterial cells that can bio-adsorb heavy

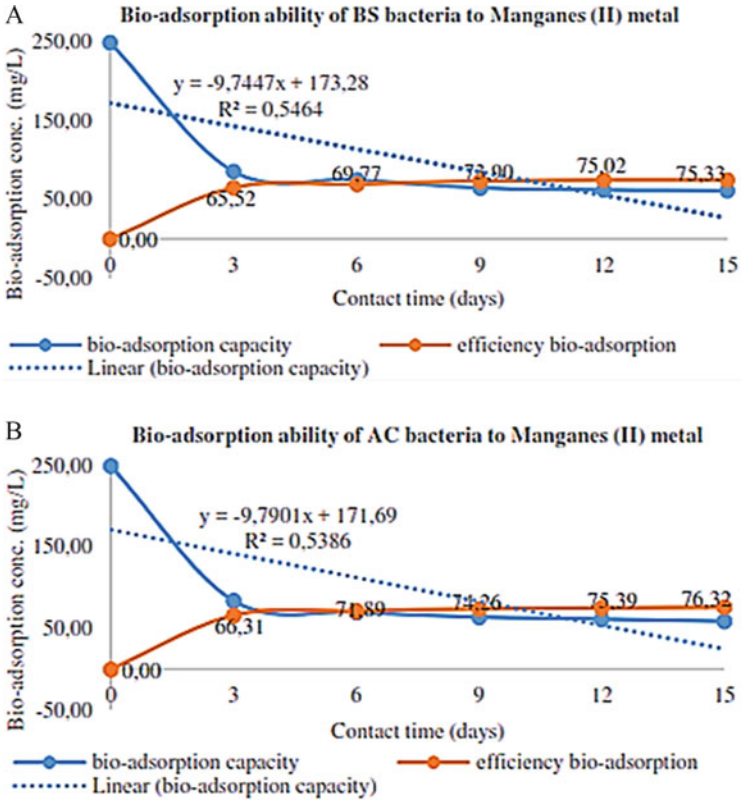

Fig. 10.11 (**a**) Bio-adsorption capacity and efficiency of BS bacteria against Mn(II) contaminant. (**b**) AC bacteria bio-adsorption capacity and efficiency against Mn(II) contaminants

metals; waste engineering and modelling interactions between bacterial suspensions and heavy metal contaminants, interaction time, bacterial nutrient supply; and other modifications aimed at improving the bio-adsorption performance of heavy metals by bacteria (Orani et al. 2018).

Efforts to improve the bio-adsorption performance of sponge symbiotic bacterial cells against heavy metals, especially the use of consortium bacteria, are currently underway by our research team. But, the researcher cannot publish the results and achievements of the bio-adsorption obtained because they are still in the experimental stage and process. Nevertheless, we offer recommendations for the method that we are currently running and the detailed procedure in Experiment 10.4. An overview of the development of heavy metal bio-adsorption methods using several types of heavy metal bio-adsorbent bacteria for utilization in waste exposed to several kinds of heavy metals aims to improve performance maximal bio-adsorption and bioremediation on various types of heavy metal contaminants in parallel (Sobrinho et al. 2020).

Experiment 10.4: Application of Marine Sponge Symbiotic Bacteria Consortium Against Heavy Metal Extreme Waste

The heavy metal bio-adsorption procedure using a consortium of sponge symbiotic bacteria isolates was carried out in several stages. (1) Manufacture of artificial waste contaminated with several types of heavy metals (Pb, Hg, As, Cr and Cu). Each type of heavy metal made a solution of each concentration of 1000 ppm as much as 1000 mL. The heavy metal solution is mixed in a sizeable homogenized portion (extreme waste). (2) Determine five types of potential sponge symbiotic bacterial isolates, the cells of each selected isolate were propagated by culture and incubated for 1×24 h. The cultured isolates were suspended in a 1000 mL solution containing physiological 0.9% NaCl. (3) The five types of mixed isolate suspension in one large container, homogenized (consortium bacteria). (4) Prepare ten reactors, each filled with 500 mL of bacterial suspension of the consortium, adapted for 1 x 24 hours in an incubator. (5) Each reactor is put in 100 mL of extreme waste and a shaker incubator at 200 rpm. (6) Observations and measurements of bacterial cell growth were carried out every 2 days, and measurements of the absorption of each type of heavy metal were to determine the bio-adsorption achievement of the consortium bacteria based on the contact period.

Other developments related to the exploration of the dual function of sponge symbiotic bacterial cells in the biodegradation of PAHs as well as the bio-adsorption function of heavy metals can be carried out by carrying out a similar procedure in Experiment 10.4 above, but with differences in the type of waste and the bacterial consortium used. The engineering waste is made to be super extreme. In addition to containing PAHs contaminants (the type and concentration of PAHs are known), the waste also includes several types of heavy metals (the type and concentration of heavy metals are known). This super extreme waste is homogenized (Bertollino 2019). This extreme super waste modification resembles petroleum waste, which contains several types of heavy metals and containing PAHs. The bacterial consortium was made by having ± six types of symbiotic sponge bacteria, a combination of three types of bacteria that can biodegrade and three types of bacteria with the ability to bio-adsorb heavy metals. The selected sponge symbiotic bacterial cells were multiplied by the culture method, made a suspension, mixed in one container, homogenized, and adapted first. This dual-function bacterial consortium suspension is ready to have interacted with super extreme waste (Wibowo et al. 2019).

The parameters observed for this method include the optical density of bacterial cell growth, changes in pH values, shifts in interaction temperature values, whether there is the formation of gas bubbles in the interaction medium, and whether there is the formation of a fermentation odour. At the same time, the instrumentation recommended for use is GC–MS, FT–IR, and SSA. GC–MS is an instrument in determining the biodegradation process by looking at the abundance of PAHs, the types of simple organic compounds as biodegradation products. FT–IR is an instrument to assess the kind of biodegradation product, whether the alcohol group is an aldehyde, ketode, carboxylic acid and other organic components. In comparison, SSA is an instrument to determine the bio-adsorption capacity that occurs by

determining the uptake of each heavy metal present in the extreme super waste and compared with the initial concentration (Ziarati et al. 2019b).

Bioremediation methods to change and produce friendly environmental quality are the dream of every citizen on this earth. Still, this dream is sometimes difficult to realize because, directly or indirectly, at the same time, we produce by-products, by-products and wastes that are pressing environment so that the environment is massively under continuous pressure and at its natural balance point (Sabdono and Radjasa 2008). Nature provides biomaterials that have a role and function in environmental bioremediation, one of which is sea sponges that can form a symbiosis with various types of bacteria. Symbiotic sponge bacteria have a biodegradation function against PAH components. It includes a heavy metal bio-adsorption function and a strong suspicion that several types of symbiotic sponge bacteria have dual biodegradation and bio-adsorption functions. A collection of several types of symbiotic marine sponge bacteria that have the same role in both the biodegradation of PAHs and the bio-adsorption of heavy metals is called a bacteria consortium (Parhamfar et al. 2020). The term for the type of bacteria with the ability to biodegrade hydrocarbons is known as carbonoclastic bacteria. The type of bacteria that has the capacity for bio-adsorption of heavy metals is metallo clastic bacteria. The term for the symbiotic bacteria of marine sponges can biodegrade hydrocarbons and bio-adsorption of heavy metals called metallo-carbonoclastic bacteria (Marzuki et al. 2021b).

The role and contribution of marine sponges in environmental bioremediation are evident. Including the content of secondary metabolite components possessed by marine sponges have an essential role in biodegradation and bio-adsorption, including the potential utilization of sponge secondary metabolites as biomaterials in overcoming exposure to bioplastics and primary materials in the manufacture of drugs and various other functional potentials. The potentials inherent in sponges, so that marine sponges are recommended for screening to explore potential and develop utilization for the common good of humans and the environment (Saputra et al. 2019). One of the recommendations for marine sponge population is those development efforts to maintain and increase the sponge population in their habitats. Keeping the population of marine sponges can be carried out through the transplant method. Especially the type of marine sponge has been confirmed to have symbiosis against several bacteria in bioremediation and sponges that have been identified as capable of producing chemical components bioactive (Bibi et al. 2016).

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Chapter 11
Genetic Modification: A Gateway to Stimulate the Industrial Production of Biofuels

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Abstract Recent years have seen an explosion in the use of advanced biotechnology techniques in academic and industrial activities to modulate microorganism pathways for the production of fuels or chemicals. Synthetic biology is adopted for biofuel production, and it needs scientific evidence to support the fundamentals and risk assessments. Biofuel is derived from biomass of a plant, animal waste, or microalgae. These materials can be replenished after some time; hence they are a renewable source of energy. Risk assessment is considered significant to maintain and comply with regulatory frameworks existing around the world. The use of scientific tools such as enzymes and microbes itself needs review and approval. Risk profiles are done for the toxicity, infectivity, and strategies. Recent years have seen an exciting increase in developing strategies for the use of advanced biotechnology techniques to enhance the productivity of existing biosynthetic pathways in microbes by cutting off the competing pathways. The biomass is pretreated to speed up the process of obtaining biofuel. The mutant, after genetically modifying the enzymes, produces cellulases and hemicellulases in higher levels. Functional analysis can confirm the changes in several transcription regulatory elements. Generally, successful engineering is demonstrated with an enhanced supply of amino acids.

Keywords Biofuels · Genetic engineering · Lignocellulose · Enzymes · Fungus · Artificial intelligence

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11.1 Introduction

The geographical production of fuel from fossils is a gradual process; an alternative is to obtain the fuel using biomass or living beings, which is termed biofuel. The times have changed, and there is a constantly increasing need for petroleum (Lazarus and Van 2018). The natural sources of the fuel industry are not present in all the countries. Fuel exhaustion has become a significant problem putting the countries unrest and dependent on buying fuel from the enriched countries (Pfaltzgraff and Clark 2014). The readily available biomass that is used directly to convert to biofuel is recovered from wood and grass. Biofuels produce heat energy which generates electricity after being run in a generator. No corner in the world is left to be negatively impacted by the effects of petroleum extraction, refining, transportation, and use. Therefore, biofuels are increasingly getting attention as an alternative source of energy (Nehring 2009; Zou et al. 2016).

11.1.1 Biofuels Basic Definition

Biofuel exists in all forms such as solid, liquid, and gas. Liquid and gaseous forms are easier to transport and deliver. Biofuel term is used for ethanol, biodiesel, green diesel, and biogas (Masjuki et al. 2012). The biofuel production is broadly characterized into first-generation, derived from sugars, starches, and oils, and the crops versus second-generation biofuels extracted from lignocellulosic biomass sources. Recently, the effort has been started to derive biofuels from microalgae and cyanobacteria, termed third-generation production (Rodionova et al. 2017); and fourth-generation biofuels which include the genetic modification of the microorganisms for the enhancement of biohydrogen production processes.

It is no secret that biofuels are preferred and viable substitutes over fossil fuels. However, biofuels such as ethanol create a net energy loss when compared to petrol. If we consider the food-based crops, they must be used for feeding the enormous population than used for fuel. As the demand to produce bio crops increases with the demand for organic consumables, soil erosion, deforestation, fertilizer run-off, and salinity are some of the major issues (Larson and United Nations Conference on Trade and Development 2008; Cheng and Timilsina 2011).

11.1.2 Why the Need for Biofuel?

The need for alternative and efficient methods has made researchers find new ways to produce biofuel. Sugar and sugar-derived ethanol is making a significant contribution to satisfying the need at the moment. Sugar fermentation is used for transportation fuel. Starch-derived ethanol fills the energy supply by mainly using corn

grain production. After the conversion of corn grain into ethanol, its burning causes an emission that impacts greenhouse gas emission but not in a net increase in atmospheric carbon dioxide (Lal 2005). The countries are announcing policies and goals to produce their own biofuel and be less dependent on foreign oil. Britain, the USA, and Canada are encouraging to grow primarily biofuel crops and establish cellulosic ethanol refineries major biofuel centers to produce a million gallons of cellulosic ethanol per year (Sims et al. 2010).

11.1.2.1 Bioethanol

Petroleum demand has put much industrial unease in the countries. Ethanol is readily biodegradable, but its use, just like petroleum, produces air and water-borne pollutants. Feedstock production of the crops to get bioethanol reduces the greenhouse gases like carbon dioxide from the environment, which is being used for photosynthesis (Lima et al. 2012). As the cellulosic matter is present in an abundant amount, it is more fitted than starch and sugar for ethanol because of its limited supply. Food supplies rely on starch-based crops such as sugar cane or corn that need specific climatic conditions to grow; hence, cellulose biomass that is not dependent on weather conditions is convenient to produce bioethanol in most countries (Sarkar et al. 2012). Ethanol produced from cellulose-based material has the potential to replace petroleum (In Marcel 2015).

11.1.2.2 Biodiesel

The second most demanding biofuel is biodiesel, which is obtained from soybean, palm oil, and fat of cooking oil. Algae and cyanobacteria have the potential to account for a large amount of fuel per unit area. Biodiesel is used in combination with petroleum and is widely accepted in European countries.

In this chapter, an oversimplified view of the production of biofuels through various resources, their economic analysis, and possible genetic manipulation of the crops to overcome the existing challenges are presented.

11.2 Biofuel from Different Sources

11.2.1 Biofuels from Lignocellulose Biomass

Lignocellulose biomass is universal and widespread renewable biomaterial on our planet. The bioconversion of lignocellulose comes as a feasible strategy when juxtaposed with the other alternative energies. Three components that are rigidly packed with each other in lignocellulose are cellulose, constituting 30–50% part of it, whereas hemicelluloses 20–40%, and lignin 20–30%. Due to the compact

structure, degradation into fermentable sugars and further conversion into fuels and other value-added materials become difficult (Menon and Rao 2012; Sharma et al. 2017).

Cellulose is linked with β, 1–4 linkage of glucose. Cellulase enzymes can break it down into glucose. Cellulose is not found in pure form in nature but with hemicelluloses and lignin, which act as a physical barrier for cellulase to access cellulose (Volynets and Dahman 2011). Cellulosic ethanol is associated with a high cost of bioreactors; the breakdown of lignin and its removal is required to access cellulose biomass by cellulases to obtain the biofuel from a plant source. The use of xylanases with cellulases is more effective as xylanases hydrolyze hemicelluloses and make cellulose available for biomass degradation. This co-acting releases more fermentable sugars from the biomass (Hu et al. 2011); this method is economically more expensive in comparison to ethanol from corn. Genetic engineering offers a substitution to minimize the cost production of cellulosic ethanol. The first approach can be an integration of cell wall degrading enzymes cellulases and hemicellulases in the crop instead of adding them directly in the bioreactor. Secondly, the amount of lignin can be configured, and the pretreatment process can be avoided. Most importantly, the maximization of polysaccharides can boost cellulosic biofuel production (Hu and Catchmark 2011).

11.2.1.1 The Cellulosic Ethanol Production Process

Lignocellulosic biomass harvested from the feedstock crop is transported to a refinery where it is stored. Either this biomass is treated with extreme heat or with chemicals to remove the lignin by breaking it down into the intermediates. The separation of the solid and liquid components is done by filtration, distillation, evaporation, and chromatography. After the enzymatic hydroxylation using bacteria or fungi, it is ready for the conversion to cellulosic ethanol. After the separation of sugar is done, pure ethanol is obtained (Liu et al. 2019; Zheng et al. 2009).

11.2.1.2 Factors Affecting the Production from Lignocellulose Biomass

Cellulosic ethanol production changes with country, region, agriculture, economy, and politics. These complex factors depend on the type of crop produced, its demand, and the transportation fuel used in that area. The support from the community and government regarding breeding strategy is also paramount for conversion to cellulosic ethanol as it gives a high amount of cellulosic biomass. C4 photosynthetic pathway, perennial growth, water usage efficiency, and segregated underground storage nutrients are the prototypical features of a non-consumable cellulose crop (Huang et al. 2009). These might include silver grass, switchgrass, and woody crops. Other edible crops are rice, corn, and sugarcane. The plant cell wall is the source of lignocellulosic biomass, and it determines the structural configuration of the plant; a representation is shown in Fig. 11.1. The various combinations of glucose sugars are

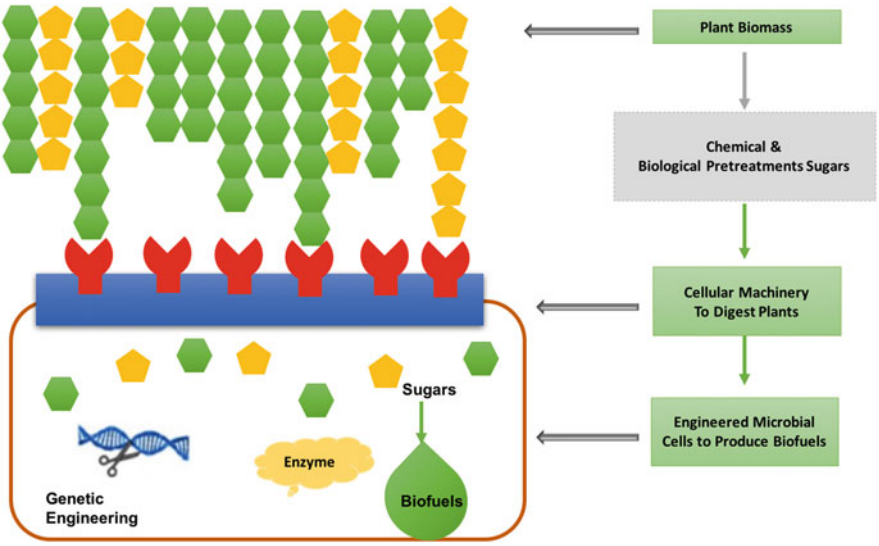

Fig. 11.1 A depiction of the biomass treatment process and genetic engineering for biofuel production

converted to ethanol (Xiros et al. 2013). The cell wall has crosslinked glycan in cellulose, and it is classified in consonance with the type of crosslinks. There are two types: Type I walls have the same amounts of glucan and xyloglucan embedded in a matrix of pectin in dicotyledonous plants; whereas Type II has glucuronoarabinoxylans and lacks pectin and structural proteins in cereals and grasses (Fig. 11.2).

Cellulose, hemicellulose, and pectin are the polysaccharides present in plant primary cell walls; the hydrolysis of their fermentable sugars provides bioethanol production. In trees, the secondary cell wall has three layers, distinguished based on different arrangements of cellulose microfibrils, with only the outermost layer containing the helices. The secondary cell wall of a plant has cellulose, hemicellulose, and lignin, mostly in which cellulose is embedded in lignin in the form of microfibrils (Huang et al. 2009; Sainz 2009; Kenney and Idaho National Laboratory (INL) 2007).

11.2.2 Biofuels from Enzymes

Aerobic and anaerobic microorganisms both produce enzymes cellulose-degrading enzymes. Bacteria and anaerobic fungi produce cellulosomes. It is a complex of cellulolytic enzymes associated with their cell wall. The secretion of cellulases is either free or cell surface-bound (Binod et al. 2019). With the unraveling of new places and areas, cell wall deconstructing enzymes have been and being isolated and

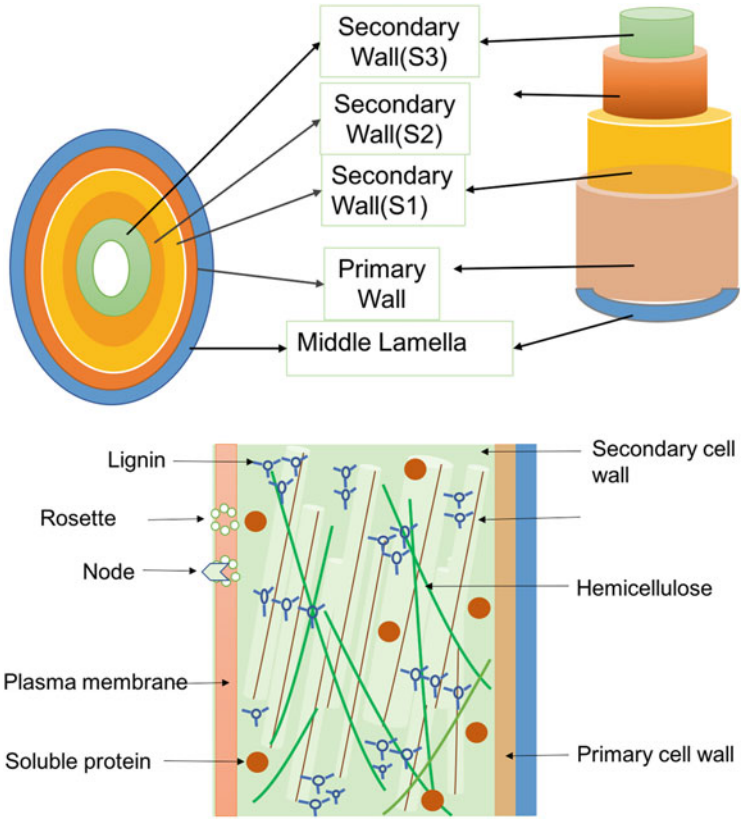

Fig. 11.2 Cell wall primary structure (above) and secondary structure (below) with cellulose microfibrils, hemicellulose, pectin, lignin, and soluble proteins (Sticklen 2008)

characterized from a variety of samples in order to be used in pretreatment investigation. The objective is to achieve higher resistance to the conversion temperatures and a range of pHs. Enzymes cellulases and hemicellulases convert the polysaccharides into fermentable sugars through enzymatic hydrolysis (Himmel et al. 2007).

11.2.2.1 Cellulases

Filamentous fungi produce extracellular cellulases. Due to the ability to produce extracellular cellulose, fungi have been highly researched for cellulase production for a long time. Both fungi and bacteria can degrade biomass; hence the biological method of producing sugars with enzymes is an eco-friendly and accomplishable method (Wilson 2009).

The maximum operating efficiency of the enzymes and their complex composition are always unrepresentative of each other. *Trichoderma viride* and *T. reesei* are amongst the excessively studied microbes (Schuster and Schmoll 2010).

Cellulases are not a single but a group of enzymes composed of endoglucanase and exoglucanases, it also includes cellobiohydrolases and β-glucosidase. Their topologies include β-sheet proteins, β/α-barrels, and α-helical protein. Lignin prevents cell wall hydrolysis by not let enzymes accessing polysaccharides and acting as a barrier. Enzyme production by microbes exposes cellulose to be broken down by cellulases (Schuster and Schmoll 2010). The microbes have the ability to synthesize different functional enzymes effectuating fermentable sugar for biofuels. Cellulases have many applications in industries, and the production of second-generation bioethanol is amongst one of them, it is a low-cost material obtained from lignocellulose. The most abundant renewable biomass; bioethanol production does not create any food insecurity, when lignocellulose is used, hence avoiding any food crops before harvesting. Biological pretreatment concerns with the ligninolytic potential of certain microorganisms that can reduce the recalcitrant nature and cleave it via hydrolytic enzymes. The addition of a molecule with two glucose units into the cultures can increase the cellulase expression. Differential hybridization in many studies showed that gene regulation occurs at transcriptional level (Siqueira et al. 2020; Srivastava et al. 2015).

Abstraction of expression of the glycolytic phosphoglycerate kinase gene, *pgk1,* is the common metabolism observed in cultured media of cellulose. Identification of the functional regions in the promoters of the cellulase genes or the regulatory proteins involved is still a highly debatable topic (Da and Srikrishnan 2012).

11.2.2.2 Factors Affecting the Production from Cellulases

Currently, there are two important factors restricting the production of cellulosic ethanol. The first is the production of strain-specific cellulases, and the other is non-appliance of existing commercial hemicellulases that can increase the output of multiple carbon fermentable sugars. Bioconversion of the complex lignocellulosic material to simple sugars is a complicated process. Genetically modified thermophilic bacteria is the envision for the future as it may lead to enhanced cellulase production through recombinant technology. Isolation of cellulase gene from thermophilic bacteria and its expression in suitable hosts via recombinant technology might enhance cellulase production. It could be done through a classical approach, whole-genome isolation, whole metagenome isolation, or a pre-study with bioinformatics (Verma et al. 2021; Kuhad et al. 2011).

11.2.3 Induction of Cellulase Expression

Plant-based material or cellulose is used in the media to promote high cellulase expression. Expression of cellulose is promoted in cellulose, lactose, and cellobiose that are poor carbon sources rather than in glucose and glycerol (Amore et al. 2013). Cellulase expression is thought to be induced using natural compound sophorose, but the status of cellobiose stays controversial as it needs to be fed in a controlled manner. Cellulase expression might be interfered by the type of nitrogen source that is used. Most of the natural carbon sources used to study cellulase expression offer a competitive growth to other microbes like fungus (Ilmén and Saloheimo 1997).

11.3 Literature Review

11.3.1 Fungus as the Source

When the expression of the cellulases of filamentous fungus Trichoderma reesei QM9414 was studied with genes encoding enzymes cellobiohydrolases and endoglucanases, the steady-state mRNA for cellobiohydrolases was highly expressed. It is also being concluded that cellobiose as a carbon source as an inducer does not show immediate effect and seems to vary depending on the culture conditions, in a study where cellobiose promoted cellulase transcription to a moderate level when compared to that of lactose (Ilmén and Saloheimo 1997). Accumulation of glucose in the culture medium might result in glucose repression of cellulase transcription. The inducing power very much depends on the ratio between carbon source, formation of glucose, and their uptake from the medium. Most of the natural carbon sources used to study cellulase expression offer a competitive growth to other microbes like fungus. Sorbitol and glycerol generally have a neutral effect; it neither promote nor inhibit expression. Glycerol and sorbitol without affecting the fungal growth show a cellulase gene induction in cultures with 1–2 mM sophorose. To understand what is the source of inducing compounds and if they are released from cellulose, the studies have been performed only with glucose and no inducer as a carbon source. If the amount of glucose in a media is subdued, the level of mRNA is found to have a difference in fully induced and repressed states in an actively growing fungus. Without having to add an inducer and still able to cellulase expression after glucose depletion, it can be crucial to keep the fungus alive under starvation conditions (Ilmén and Saloheimo 1997; Margolles-clark et al. 1997).

Aerobic, anaerobic bacteria, and fungi are the models to study cellulolytic enzyme systems. Fungi such as *Trichoderma reese*i, *Penicillium spp*., *Aspergillus niger,* and basidiomycetes secrete extracellular cellulolytic enzymes. Higher fungi also have oxidative systems and can be capable of degrading lignocelluloses through their ligninolytic enzymes.

Fungi are studied for genetic modification reason being their capacity to produce large amounts of extracellular cellulases. Three mainly synergistically acting enzymes in cellulases are cellobiohydrolase/exoglucanase, endo-β-1,4-glucanase, and (c) β-glucosidase. The action of endoglucanase is expedited by lytic polysaccharide monooxygenases. Non-hydrolytic proteins accelerate the action of endoglucanase. Optimization of physical and nutritional parameters can be done by engineering the cellulases, it is also the way to enhance cellulose production. Strain improvement can be approached via random mutagenesis and site-specific mutagenesis in cellulases. One of the most explored fungal strains for commercial cellulase production is *T. reesei* RUT-C30, followed by *Penicillium sp., Aspergillus sp., Myceliophthora,* and *Humicola sp*. (Srivastava et al. 2020).

A study concluded that in a growing fungus, the regulation of cellulase expression depends on glucose repression (Amore et al. 2013). This effect is reversed after the glucose is impoverished and derepression of cellulose occurs with no other inducer present. The use of a mutant can support new ways for biodiesel production; some mutant studies support the theory that separate regulation could exist for different fungal cellulolytic enzymes. This suggests that there is a high possibility that the cellulose enzymes are coordinately expressed. It is always to be mentioned that biologically relevant mRNA levels are not easy to be detected and site-specific proteins might be the ones governing glucose repression or the cellulase expression (Mach and Zeilinger 2003).

11.3.2 Algae as the Source

Photosynthetic algae, both micro and macro, are thought to have the potential to be turned as a possible biofuel resource. Microalgae have the competence to store triacylglycerol and fat that can be turned into biodiesel and ethanol. Due to a high lipid profile, it is believed that crude oil deposits have been created by microalgae over a period of time. Therefore, scientists have a huge interest in understanding and exhilarating the productivity of algae to produce biofuels. What makes microalgae even more interesting is the fact that they are an attractive source of fuel that intake carbon dioxide and keep the environment low on carbon. They grow on marginal land hence are not a competition to terrestrial crops and flourish in waste or saltwater. It means algae does not compete with the resources of food-based crops, which is a problem with lignocellulose-based biofuels. Metabolic pathways of microalgae can significantly be manipulated to produce a greater quantity and a variety of biofuels (Demirbas 2010).

Algae efficiently use photosynthesis to obtain important oils and biomass from carbon dioxide. These oils can be later transformed into feedstocks to produce biofuels, Omega-3 fatty acid oils, and feed for animals feed. Productivity of algae can give an estimate about the approximation of area to fulfill the requirements in a particular country. The protein that comes as a byproduct of fuel production from algae might serve as a very useful food source for protein and other useful products.

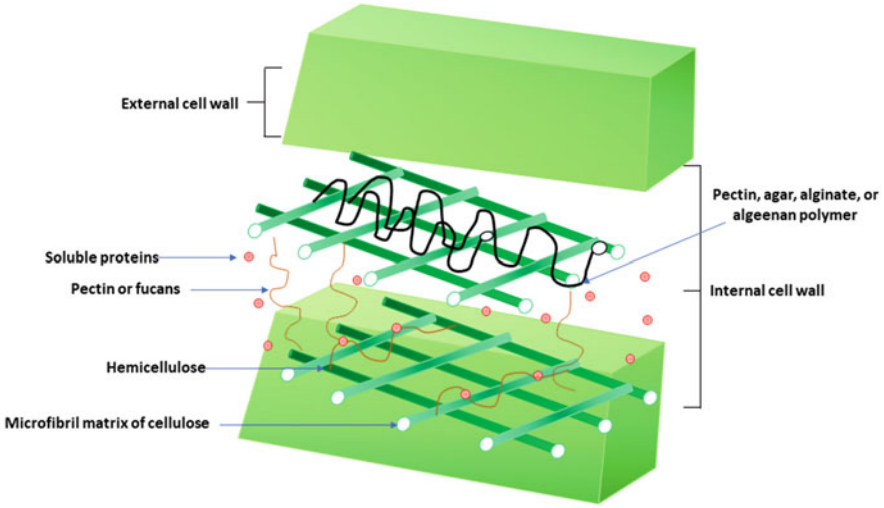

Fig. 11.3 Representation of the structure of microalgae cell wall membrane

The synergistically designed facility takes the edge off liquid fuel hence relaxing the consequences of biofuels production on the land, mitigating global warming, and promoting biofuel production to relieve energy shortage (Demirbas 2011). Microalgae can be aggregated by adjusting the pH; with these adjustment properties of the cell surface, biomass concentration can be modified as per the requirement. After the culture of microalgae, the medium can be recycled, reducing the cost and environmental pollution (Fig. 11.3). The high-pH-induced flocculation method showed an effect on biodiesel production by showing changes in the lipid extraction process and fatty acid profiles of marine microalgae (Castrillo et al. 2013; Liu et al. 2013a).

Nonetheless, and contrary to microalgae, seaweed is a renewable feedstock, and there are some potential concerns and impacts on ecosystems. Polymers in seaweed are mixed sugars, and depolymerization of seaweed polysaccharides is relatively easy, but the conversion of such sugars to biofuels is not an easy task. Excess of seaweed farming can alter natural habitats leading to nutrient depletion and reduction in biodiversity (Kraan 2013).

Economically profitable biofuel production from seaweed is acquired by an efficient conversion of mixed sugars in seaweed hydrolysates. Henceforth, metabolic engineering can be used during the fermentation of sugars. Red marine algae have galactose (up to 23%) as major sugar compound in the hydrolysate Ceylon moss (Wei et al. 2013). Galactose fermentation by engineering *S. cerevisiae* is the eminent way to produce ethanol. The wild-type yeast *S. cerevisiae* is capable of galactose fermentation by controlling the ethanol yield and productivity. However, ethanol production rate and galactose yield are not high when compared to glucose. Also, glucose represses the utilization of galactose by stringent transcriptional repression of GAL genes that are responsible to code enzymes for galactose metabolism.

Hence, excess consumption of glucose and galactose in red seaweed hydrolysates might reduce overall ethanol productivity, which is thought to be improved by metabolic engineering (Johnston et al. 1994).

11.4 Genetic Gateway to Obtain Biofuels

Genetic engineering enables microbe to produce a high number of metabolites. The inherent complexity of the organisms ranges from simple protein structures to folded and globular protein with a variety of medicinal properties. The genetic manipulation requires a preamble of whole-genome sequence to understand and select the desired sites for genetic alterations (Peralta-Yahya et al. 2012). A few examples of such moderation in the gene of the different microbes are mentioned in Table 11.1.

Table 11.1 Improvement of the biofuel production by genetically altering the organisms

Organism	Mutant/gene	Modification	Origin	Reference
T. reesei Qm 6a	RUT-C30	There was an increase by 20 times in cellulase secretion	Fungal	(Peterson and Nevalainen 2012)
Fusarium oxysporum	NTG-19	Cellulolytic activity was 80% more than its parent strain	Fungal	(Kuhad et al. 1994)
Aspergillus nidulans	creAd3	D-glucose metabolism was seen to be improved	Fungal	(Van et al. 1995)
Aspergillus niger DSM 26641	*A. niger* DSM 28712	β-1,4-endoxylanase activity was found to be enhanced by 82%	Fungal	(Ottenheim et al. 2015)
Cellulomonas flavigena	(M4, M9, M11, and M12)	Xylanolytic activities were enhanced	Bacterial	(Mayorga-Reyes and Ponce-Noyola 1998)
Escherichia coli strains	*cydC-D86G, cydC-D86V*	Biofuels and bi-products under ionic liquid stress were higher in the concentration	Bacterial	(Eng et al. 2018)
Chlorella vulgaris	SDEC-3 M	The mutant is supposed to benefit CO_2 biofixation from industrial exhaust gas	Algal	(Qi et al. 2016)
Chlorella minutissima (CM)	CM7	Monounsaturated fatty acids showed an elevation	Algal	(Mehtani et al. 2017)
Saccharomyces cerevisiae	*Saccharomyces cerevisiae* UAF-1	Ethanol production was improved by 12.0% with aeration	Yeast	(Abbas et al. 2017)

11.4.1 Principle

Targeted strain engineering aims at a considerable amount of transformants by homologous integration or deletion of the expression cassette. Nevertheless, introns in the genes and glycosylation convolution cause low-efficiency gene targeting.

Filamentous fungi have the tendency to bring about post-translational modification. The modification includes attachment to a functional group of another molecule by a glycosyl donor and synthesis of organosulfur compounds. They also secrete metabolites that have the ability to expand on a cheaper substrate that makes them suitable for industrial applications. They almost make a good choice to be used as a host for recombinant DNA except for the challenges on non-homologous recombination. By inactivating the double-strand breaks in the DNA pathway this challenge can be resolved (Dellomonaco et al. 2010).

11.4.2 Some Examples from the Previous Studies

Genetic induction or end-product inhibition in microbial cells produces a higher amount of cellulases. Catabolite repression or end-product inhibition in a mutant of *B. pumilus* resulted in a four times higher yield when compared to *Trichoderma reesei* (Kotchoni et al. 2003). RUT-C30 obtained through mutation in *T. reesei* Qm 6a at Rutgers University showed a 20 times increase in cellulase secretion. Most of the available studies state enhancements in cellulase production by mutation without mentioning the changes that might have occurred at genetic level (Peterson and Nevalainen 2012). *Penicillium decumbens* is used in China for the industrial production of lignocellulolytic enzymes. When a comparative genomics analysis by Liu et al. (2013b) was made with the phylogenetically similar species *Penicillium chrysogenum* it was found that the cell wall degradation has advanced with *P. decumbens*. The reason suggested was its strong cellulolytic ability due to more genes involved in cell wall degradation than cellular metabolism, that happens in a medium with cellulose as a carbon source. It has made the lignocellulolytic enzyme system in *P. decumbens* became variegated with hemicellulases and proteins in the cellulose binding site (Liu et al. 2013b). Genetic engineering seems a very efficient method for gene expression by regulating the promoters and can be achieved with minimum changes in the genetic content. Mutagenesis is done to bring the expected changes in the DNA sequences of a gene with specific primers, termed as site directed mutagenesis. The changes are incorporated in a genome by homologous recombination using amino acid sequence primers. However, the sequence in a genome and the site to target are not easy to identify. This process of improving the properties of a protein by alteration in its amino acid sequence increases the secretion of cellulase. Serinine and threonine on the surface of xylanase in *A. niger* BCC14405 were replaced with arginines (Sriprang et al. 2006). The modified enzyme had increased activity than the wild-type strain. As the enzyme activity

also increased half-life of the mutant stability was simultaneously raised. To obtain a hyperthermostability, *Thermotoga maritima* cel5A endoglucanase, when subjected to site directed mutagenesis and CBM engineering, demonstrated 10% higher activity at one site when compared with the native cel5A (Arumugam et al. 2008).

Aspergillus and *T. reesei* are used to express genes from different origins, improving cellulose production. Fungal and bacterial cellulose or carbohydrate binding domains were used from *T. reesei* and *Clostridium stercorarium xylanase* A to be integrated with cel5A. Avicel was used to observe hydrolytic activity in which engineered carbohydrate bonding molecule from both species showed better activity. The activity was linked to binding ability, which was checked via immune gold labeling assay. Mutagenesis of D232A in fungus Macrophomina was used to generate an engineered form for the production of an enzyme with novel substrate requirements. The substrate size of the engineered one was found to be higher than the wild-type 5 b-1,4-endoglucanase but with an equivalent activity on cellohexaose (Druzhinina et al. 2017; Hilden and Johansson 2004). The modified endoglucanase can be used to get complexed carbohydrates by a double decomposition reaction with water present. A study by Liu et al. (2013b) involved *Penicillium oxalicum* mutant JU-A10-T gene with the wild strain 112–2. It has high cellulolytic ability on the processing of decayed organic matter, comparison of whole-genome sequencing, transcriptomes, and secretomes was done. The study revealed that a new lignocellulose-degrading enzyme has emerged (Liu et al. 2013b; Wang and Jones 1997).

11.5 Strategies for Genetic Modification of Microorganisms

Creation, selection, or improvement of strains of desired microorganisms to direct the well-suited output rely on the microbial strains that will be used to catalyze the biosynthesis of the desired compound. Biotechnologies develop befitting strains for fuel by synthetic process.

The imposition of these regulations concerns with the underlying risk and their assessments by the government, taking into consideration proper planning and management. Modification of microorganisms for fuels such as n-butanol, isobutanol, mixtures of alkanes or lipids is evident in number or researches (Keasling et al. 2009; Bhatia et al. 2017).

This is done by overexpression, directed evolution, and codon optimization of key endogenous enzymes to increase the yield of the targeted product (Mythili et al. 2016; Jiménez-Díaz et al. 2017).

The genetic modification deals with the introduction of two or more genes encoding heterologous enzymes to create entirely new biosynthetic pathways or enabling new enzymatic activities of different feedstocks as energy sources. It might also knock out genes encoding enzymes in competing pathways and augment the flow of carbon into a desired pathway. The potential risks are unique to each type of microorganisms, such as in algae; it is possible to have effects on native populations

in order to create or intensify and create a dangerous mutant (Jang et al. 2012; Mary 2011).

To obtain a befitting product, the use of various both prokaryotic and eukaryotic species and strains is common in industrial production. Some of the prevalent and focused microorganisms are yeast fungal and bacterial strains such as *Saccharomyces cerevisiae, Aspergillus* and *Trichoderma, Lactobacillus,* and strains of *Escherichia coli.* The existing strains always have a scope of improvement to have more productivity with genetic engineering for valuable properties (Rubin 2008).

The indication toward the improvisation of carbon fixation enhances pathway in proteins like RuBisCO or alteration in lipid synthesis in algae can help both with the environmental pollution reduction and biodiesel production. To ensure the safety conduct and industrial use of genetically modified organisms, they need to have appropriate risk assessment tools. The methods selected must express, enhance transporter proteins, maximize the carbon flow, and remove toxins and harmful compounds. The change in metabolism should emphasize easier cell lysis and making existing pathways more applicable for commercial purposes. For industrial purposes, fermentation of the microbes must be conducted in a protected environment and must prohibit any exposure or accidental release of the microorganism. Any inherent exposure of the genetically engineered organism that is to be produced commercially. Its exposure into the environment should be assessed for all possible hazards (Jiménez-Díaz et al. 2017; Chen and Dou 2016).

11.6 Regulations for Genetically Modifying Plants and Microbes for Biofuel Production

To fulfill the current demand, the production of renewable fuels or bio-based chemicals is carried out with genetically modified microorganisms. The production includes microalgae, fungus, plants, and cyanobacteria. Researching and finding new biological methods of manufacturing renewable fuels from petrochemical feedstocks foresees a great potential toward more sustainable industrial activities. Without much speculation, genetically modified organisms exhibit certain advantages over other microbiological methods to obtain biofuel. Some of the factors of risk are summarized in Fig. 11.4.

Genetically modified organisms offer remodeled productivity, less operational costs, a variety of feedstocks, and most importantly, substantial carbon footprints. Genetically modified microorganisms (GMM) in most countries require regulatory guidelines before they could enter the market. The government has certain regulations when it comes to genetic engineering of plants and animals. In the late 1980s, risks caused by potential genetically engineered microorganisms to the environment were already getting huge attention. Early scientific reviews laid the foundation for regulatory risk assessments of proposed field tests.

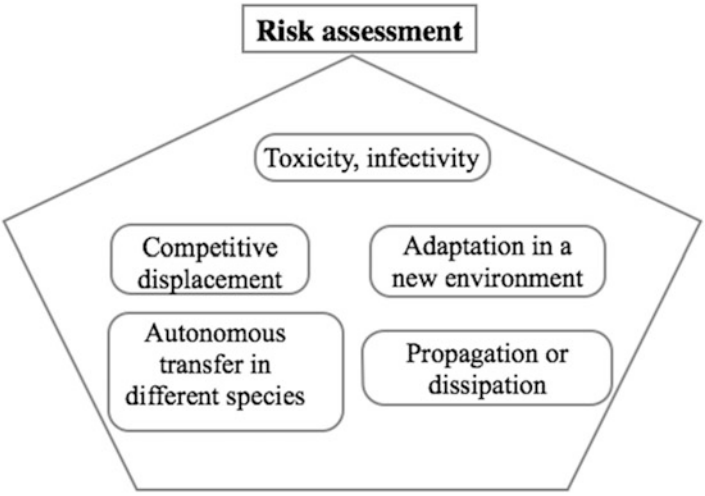

Fig. 11.4 Factors that can influence the risk associated with genetically modifying an organism

EPA and USDA regulations govern the use of modified organisms for the production of fuels or chemicals in the US EPA works under the Toxic Substances Control Act (Glass 2015; Wozniak-Karczewska et al. 2019; Wozniak et al. 2012). The aim of these regulations is to declare it to the agency before merchandising any genetically modified microorganisms.

Ecologists have recommended that engineered strains can perform like non-engineered strains when they are introduced into new environments. This gives a fundamental estimation to predict and monitor the behavior using appropriate risk assessment tools (Viebahn and Chappin 2018). However, we cannot deny the fact that scientific concerns about the potential environmental effects of microorganisms with new traits are reasonable. Tribal Energy Resource Agreements and United States Environmental Protection agency ensure risks are minimized, and it is scalable to establish means of genetic manipulation. The choice of the reactor, its design, and features govern the choice of the starting culture and product.

In the 1990s, there was a standard that was set for the development of biodiesel in order to promote the use of alkyl esters-based biodiesel in pure form or as blends in automotive fuels. The standards vary in the USA and Europe. For the first time in 1999, the American Society for Testing and Materials (ASTM) adopted a provisional specification PS121 for biodiesel. ASTM D6751 was approved in 2002 for middle distillate fuels. ASTM D6751, 2012 onward defines two grades of biodiesel: grade 2-B and grade 1-B. The grades have a strict policy on monoglycerides and cold soak filterability derived from vegetable oils and animal fats. Two automotive standards for biodiesel/diesel fuel are ASTM D975 to allow up to 5% biodiesel to be blended into the fuel, and ASTM D7467, for biodiesel blends from B6 to B20.

In October 2003, the standard for biodiesel EN 14214 was accomplished in Europe for unblended FAME diesel fuel and some biodiesel blends. These standards

set the stepping stone in international standards and became the starting point for biodiesel specifications developed in other countries. Low-level blends are categorized by EN 590. For fatty acid methyl esters fused in diesel engines, EN 14214 makes the regulations.

A category B100 could be used unblended in a diesel engine or blended with diesel fuel to produce a blend conforming EN 590. The changes to cover heating oil applications were inducted by EN14214:2012 to cover blends up to B10. Monoglycerides content was also considered as a separate class. EN 590 covers biodiesel/diesel fuel blends up to B7. The version released in 2004 introduced blending up to 5% of fatty acid methyl ester (FAME) in diesel fuel, which was increased to 7% in 2009. The European biodiesel specification, EN 14214, a European indication that applies only to mono-alkyl esters made with *methanol*, specifies that ester content should be 96.5%, and no additives other than fatty acids can be added. Increased oxidation stability, reducing the sulfated ash limit to 0.005% from 0.02%, and limiting blends to B5 maximum are some of the guidelines for B100 used to make biodiesel/diesel fuel blends used all over the world (ACEA 2009; ASTM 2002; Tasios et al. 2013).

The work carried out in a stepwise and responsible manner can respond to the needs of developing novel sources of energy worldwide. Reducing carbon emissions and avoiding any harmful environmental impacts is the considerable factor that will control, manage, and constrain the genetic engineering of an organism for biofuel production.

Certain uses of GMM could be subjected to FDA regulations because of the production of foods, pharmaceuticals, or other products. However, a standard alternative for companies working with nonpathogenic microorganisms and obtaining ethanol, can be utilization of the excess and waste from the biomass in animal feed.

In the USA, animal feed ingredients are monitored by a non-profit organization that is called Association of American Feed Control Officials. They define if the ingredients are fit for animal feed and pet food.

11.7 Future Outlook

11.7.1 Artificial Intelligence Can Help in Genetic Modification of Biofuels

As we struggle to find out a way to restore diminishing fossil fuel resources, the scientific community is working hard to find alternative sources of fuels; one such form is biofuel, through plantations of certain plants, which takes up to 90 days to grow and be ready processing the biofuel. The goal of the scientific community is to design a plant by a genetic modification that has the ability to produce a large amount of biofuel in less time.

At the molecular level, in a biofuel plant, a gene is responsible for the synthesis of the triglycerides (hydrocarbons). It is necessary to carry out research to identify this gene and isolate it (Rao and Pingali 2008), which might take time for the human eye to get the sequence of the gene. By leveraging Artificial Intelligence, it can be done quicker and with limited sources through AI Machine learning and Deep learning models.

Artificial Intelligence applications in biotechnology include analysis required in modification of genetics, drug screening, predictive modeling. These problems have been solved, which are difficult for humans to solve in a short period of time. AI now exists in every field of study, from recognizing a pattern, forecasting, DNA sequencing of tens of thousands of genes, and plays a crucial role in biotechnology (Klyuchko 2017).

11.7.2 Machine Learning in Action

With the sophisticated machine learning models, we can achieve different clinical trial datasets, enable simulated screening, and analyze vast amounts of data. The basic concept connecting machine learning with artificial intelligence and deep learning is represented in Fig. 11.5. Apart from savings on clinical trial costs, with ML models, we can also gain exclusive insights and feed them back into the process. Machine Learning (ML) concept gives computers the ability to think and helps us to solve many problems. Machine learning is the part of artificial intelligence focused on algorithms which has the ability to learn from experience, and when exposed to new data, its accuracy is measured without explicit programming (Oliveira 2019; Kim et al. 2020).

The ML Algorithms take data as an input, and the output is predicted data or actions. The algorithms improve as they are exposed to more data.

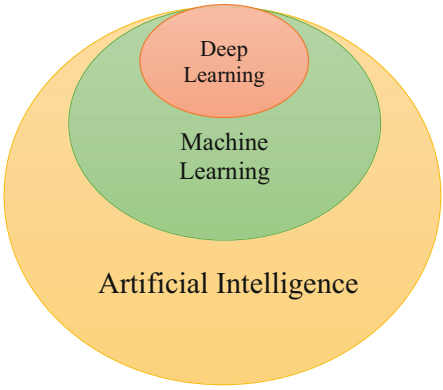

Fig. 11.5 A simple pictorial depiction showing how the artificial intelligence is linked with deep learning and machine learning

As the Dataset grows, the performance of the ML model degrades and may tend to drop its accuracy. Hence, it will be difficult to just work with ML alone. The reasons are

- High dimensions:
  When we have a large number of inputs and outputs, which are nothing but high dimensions, ML is not useful.
- Crucial AI problems:
  Machine learning cannot solve natural language processing, image recognition problems due to their huge data amounts.
- Feature extraction:
  For complex problems, such as object recognition, handwriting recognition, etc., ML will face a big challenge (Jordan and Mitchell 2015).

11.7.3 Deep Learning Methods

Deep learning is an approach of machine learning that has been designed based on the knowledge of the human brain and neurons, a simple depiction in Fig. 11.6. In recent years, deep learning has seen tremendous growth in its popularity and usefulness in the field of biotechnology (Sugomori et al. 2017).

Deep learning is a subset of machine learning methods based on artificial neural networks with multiple layers representation learning in different dimensions, which can be understood with Fig. 11.7.

Machine Learning can be supervised, semi-supervised, or unsupervised. The difference is in supervised learning, we provide labeled data, whereas unsupervised learning machine comes up with its own patterns without labels.

Deep learning algorithms use the concept of multilayer perceptron's. Data is filtered through multiple layers, with each consecutive layer using the output from the previous one to inform its results (Ardabili et al. 2020; Aghbashlo et al. 2021).

Deep learning models will achieve its accuracy level and can provide more data to process. If there is not enough data, then we can apply different deep learning techniques like data augmentation to generate more data for training our model, the more training we provide, algorithm will learn to perform better when unexposed

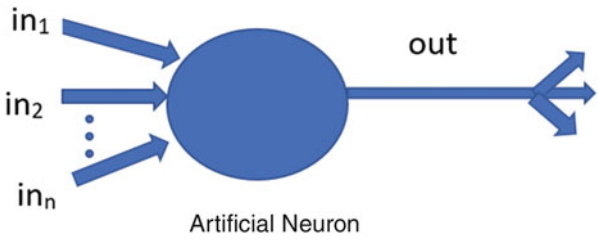

Fig. 11.6 Flow of information in machine learning approach

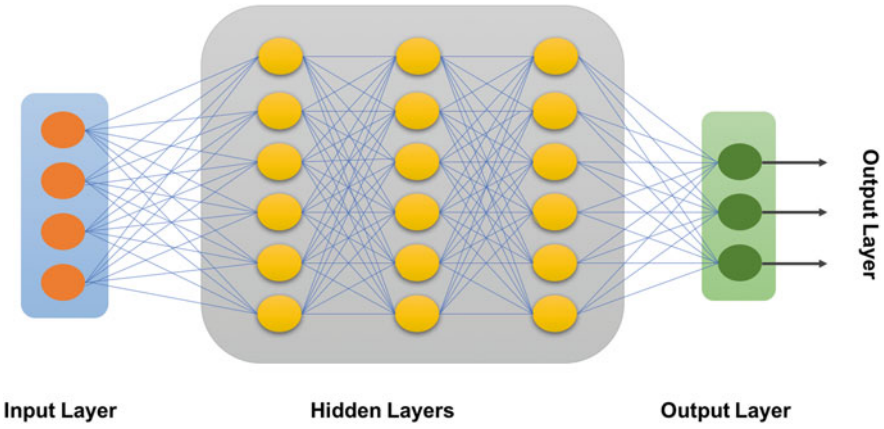

Fig. 11.7 Schematic representation of deep learning methods

data is tested, deep learning models learn from previous results to enhance their capability to make correlations and connections.

- DL algorithms are capable to focus on important features, and this can be achieved through minimal coding.
- DL models are capable to solve high dimensions.
- The main idea behind Deep Learning is to build learning algorithms that mimic biological neurons neural network system.
- Deep Learning is implemented through Neural Network.

Like many other disruptive technologies in Biotechnology, AI is generating much anticipation with Deep Learning's convolutional neural networks, which can work with minimum data as well. Also, a transfer learning model which gained information from a previously trained data can be applied to a new set of data, which can give more accurate results as it has seen certain features of data (Mosavi et al. 2020).

As per American physicist, Kaku and 3M Company (2011), we could be witnessing the next transition from transistors based on silicon to transistors based on atoms. In a decade, Moore's Law will slow down and computer power will level up, and we are going to many such transitions, one of them could be Quantum Computing.

This new field of Quantum Computing is now on the leading edge of computing. World's leading organizations like IBM, Google, etc., are working toward Quantum Supremacy.

As per MIT "One of the goals of quantum computation and quantum information is to develop tools which sharpen our intuition about quantum mechanics and make its predictions more transparent to human minds." The development of Quantum Machine Algorithms has begun for the genetic sample classification.

Unlike classical computing, which is built on either 1 or 0, whereas quantum computing has both the positions at once, that is 1 and 0, and is based on Quantum mechanics (Li et al. 2001; Roe 1998).

There is a future of biotechnology when combined with quantum computing in deriving molecular level properties to modify the genetics of biofuels.

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Chapter 12
"Omics" Technologies in Biodegradation Processes

Sevcan Aydin and Mahmut Çalışkan

Abstract The considerable environmental impact of pollution due to human activities is driving the development of new decontamination and clean-up methods. Hereby, the interactions between the various microbial communities at polluted sites is receiving increased research attention, with the novel omics approaches, opening up new ways to study bioremediation pathways and their mechanisms. This has allowed innovation in the field of new bioremediation as an effective way to combat pollution. In particular, the omics approaches offer great potential to predict the metabolic processes of microbes in environments experiencing pollution. The high-performance analyses provided by these approaches can, amongst others, help track novel organisms for use in bioremediation and offer new, high-quality insights into those molecular pathways that are critical to biodegradation. In short, through multi-omics approaches, the field of bioremediation will benefit from the establishment of new theory-based methodologies to mitigate pollution.

Keywords Bioremediation · Biodegradation · Microorganisms · Metagenomics · Genetic analysis · Horizontal gene transfer

12.1 Introduction

Due to population growth and the accompanying increase in industrial activities, pollution is rising across the world. The growth of industrialization in particular has led to manufacturing processes that produce and incorporate various chemicals for use in high-tech products, including heavy metals, hydrocarbons, and xenobiotics. These substances are not only toxic but also persist in the environment, and thus have a significant adverse effect on ecosystems and organisms. However, there are

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several natural methods that can address these imbalances, with microorganisms representing an effective natural tool with which to remove toxic compounds from contaminated sites. Microbial-mediated biodegradation therefore has considerable potential to naturally restore polluted ecosystems (Shahi et al. 2016a; Aydin et al. 2017; Nwankwo et al. 2021; Sales et al. 2021).

The microbial ecology of an environment, whether pristine or anthropogenically affected, can be assessed using culture-based methods. However, as many of the identified microorganisms of interest to research are not culturable in a laboratory, there remain several challenges in their study. The research trying to culture such microbial communities has so far only been successful with no more than 1% of any given sample of prokaryotes. Moreover, there remains a gap in the knowledge of which factors influence the microbial communities of polluted environments, e.g. their metabolism, growth, or dynamics. Of the abovementioned omics approaches, metagenomics offers a way to conduct genome-level studies of microbial communities, providing substantial insights into the so-called uncultured microbiota (Aydin et al. 2015). The other omics approaches, such as genomics, transcriptomics, proteomics, and metabolomics, have recently also begun to offer an array of advanced techniques designed to treat pollutants in a way that causes no further damage to the environment (Aydin et al. 2022; Malik et al. 2021; Wright et al. 2021).

This chapter sets out to explore to what extent omics techniques can be applied to monitor the processes of biodegradation. In this context, it proposes using a community systems biology approach, integrating the abovementioned multi-omics with bioinformatics as well as simulation tools. The aim hereby is to enhance the predictive ability of models regarding the persistence of toxic chemicals in environmental settings, which should be taken into account and integrated into a tiered assessment strategy regarding the persistence of toxic chemicals. The findings of the research should offer an enhanced understanding of how the composition of the microbial community relates to the catabolic potential and environmental conditions as well as the properties of the chemical under investigation.

12.2 Omics Approaches for Monitoring Biodegradation Processes

Pollution continues to be both a tenacious problem and a significant threat to the health of humans and the environment. While numerous strategies have been applied to address this issue, it is still a challenge, even in terms of simple monitoring, and environmental contamination continues on a global scale. The severity of the issue calls for novel pollution mitigation strategies to ensure environmental and human health, with bioremediation being one of the most promising to emerge in recent years. Bioremediation using microorganisms is receiving increasing research interest, with studies striving to identify ways to effectively restore environments affected

by pollution (Shahi et al. 2016b). Microorganisms present themselves as particularly suitable as they can exist in a variety of environments, even degraded ones, by producing metabolites that have the ability to degrade or even transform contaminants, thereby allowing polluted sites to be naturally restored (Kour et al. 2021). The design of effective strategies hereby depends on modern omics technologies, including metagenomics, proteomics, and transcriptomics, which enable the microbiota's diversity and ecology to be studied to facilitate their implementation in the context of environmental monitoring as well as bioremediation (Aydin 2016).

Strategies based on bioremediation or biotransformation aim to exploit the natural diverse catabolic abilities of microorganisms regarding the degradation, accumulation, or transformation of multitudinous environmentally harmful compounds, such as pharmaceutical substances, heavy metals and polyaromatic hydrocarbons (PAHs). Recent developments in next-generation sequencing (NGS) have enabled key microorganisms to be genomically, metagenomically, and bioinformatically analyzed, allowing hitherto incomprehensible biodegradative pathways to be understood (Shahi et al. 2016c; Tancsics et al. 2021). Similarly, horizontal gene transfer (HGT) is crucial to the processes of microbial biodegradation in that it affects the activities of the microbiota. Specifically, bioremediation relies on functional genes to be transferred between key members of the microbial community. As the availability of macronutrients, among various other abiotic factors, influence HGT in polluted settings, the microbiota's capacity for biodegradation is dissimilar between sites. Hence, a thorough knowledge of which mobile elements and functional genes lead to HGT would further aid the potential for the bioremediation of a polluted area. In addition, as the biodegradation abilities of the microbial community dictate to what extent HGT can occur in a contaminated site, assessing HGT can be employed to monitor bioremediation to evaluate the biodegradation process (Shahi et al. 2017).

The prior research has evidenced a significant correlation between HGT and the existence of functional genes (French et al. 2020). For instance, in their investigation into the horizontal transfer of the alkB, nah, and phnAc genes in the biostimulation of soils contaminated by petroleum, Shahi et al. (2016b) found that the alkB and phnAc genes transferred when nutrients were highly available. They also demonstrated that HGT was positively linked to nutrient content, with an increase in the carbon to nitrogen ratio from 100:5 to 100:15 correlating with increased rates of HGT. Building on this, Shahi et al. (2016a, b, c) showed that the evaluation of the microbial population and the presence of functional genes could be used to assess the efficiency of existing bioremediation efforts. In this study, they were successful in showing that HGT could serve as a suitable monitoring approach to measure the effectiveness of bioremediation for soils that had been contaminated by petroleum. They also found that this method could further be utilized to assess contaminated sites' potential for biodegradation.

12.3 Conclusion

The combined effects of globalization leading to increased industrialization, population growth, and consumption-based lifestyles have caused non-degradable pollutants to persist in the environment, adversely affecting human health. Due to their environmentally and cost-effective characteristics, methods based on bioremediation are opening up new avenues to mitigate these pollutants beyond conventional physicochemical treatments. Almost all polluted environments contain microbial strains that are capable of degrading the contaminants specific to those environments, with the population density increasing with the increasing presence of such contaminants. As the metabolic effectiveness of the microbiota relies on HGT between microorganisms, choosing the right gene for the degradation process and then monitoring how this gene is horizontally transferred comprise a feasible strategy to assess the effectiveness of bioremediation. The bioremediation potential of specific sites can hereby also be assessed. The necessary information on the genes as well as their genomic structure, metabolic and biological pathways, functions, and evolution can be gathered through the omics approaches. Moreover, a functional characterization of the relevant genes, proteins, coding regions, and products of the metabolism should be conducted to quantify the relevant biological processes of the microbiota. Recent developments in the omics fields, especially genomics, proteomics, metabolomics, transcriptomics, and interactomics, thus provide an array of technical techniques and expertise in this respect.

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Chapter 13
Conclusion: Environmental Protection—Our Common Responsibility

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Abstract Environmental pollution is increasing globally and, together with climate change, is a priority on the environmental, political, business, and scientific agendas. Air, land, and water pollution have an impact on all ecosystems and our lives and can jeopardize our future and future generations.

The importance of policies on public awareness and perception is recognized and can have an effective role in the protection of the environment. Policymakers, companies and industries, civil society, scientists, all sectors of society should be involved for the same purpose; coordinated efforts at an international level are needed to tackle all the challenges planet Earth face.

Therefore, it is crucial to stimulate the discourse, narrative, and debate about environmental pollution and degradation and mitigation strategies.

Keywords Environment · Marine pollution · Civil society · Policies · Public awareness · Climate challenge

Approximately 80% of marine pollution originates in dry land. To address this mighty problem, a decisive coordinated policy is needed, and the UN Environment Programme contributes by hosting a specific initiative, the Global Programme of Action for the Protection of the Marine Environment from Land-based Activities (GPA). After the Manila Declaration of 2012, GPA targeted three priority pollution sources: marine litter, nutrient management, and wastewater through global

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voluntary multi-stakeholder partnerships of governments, intergovernmental agencies, academia, the private sector, and civil society.

Various other international organizations of different types have promoted the development of actions and strategies to achieve sustainability and avoid degradation in marine environments. Typically, such strategies rely on information from expert groups about the causes of degradation and the policy options portfolio to tackle them. These strategies rarely consider trustworthy data on public awareness, their concerns, and priorities. Recent results (Gelcich et al. 2014) show that the level of concern regarding marine impacts is closely associated with the level of public acquired information, with pollution and overfishing being two areas prioritized by the public for policy development. Results also suggest that the overall public understand human generated impacts on marine and are highly concerned about ocean pollution, overfishing, and ocean acidification. Promoting further public awareness, concerns, and priorities can enable scientists and funders to understand how the public relates to marine environments and make the much-needed correspondence between policy and public demand (Gelcich et al. 2014).

Naturally, the vastly comprehensive nature of the social involvement necessary to tackle this massive scale problem implies taking into consideration the importance of awareness raising campaigns. It has been shown (Latinopoulos et al. 2018) that, when environmental knowledge is limited, public information campaigns can be effectively used to help individuals make more informed choices. Also, public information campaigns have a positive effect on the willing to pay (WTP) estimates.

A further problem is that situations are heterogenous, in terms of gravity and characteristics. The way they are seen by the public also varies. This was recently assessed (Brouwer et al. 2017) in terms of the social costs of both marine debris washed ashore and litter left behind by beach visitors along different European coasts. The social costs were estimated based on public perception. Previous studies focusing on the valuation of beach recreation did not make a distinction between pollution sources. This latter distinction is considered important in view of the fact that a large share of the litter originates from beach visitors and requires another type of coastal zone policy intervention than diffuse pollution washed ashore. Assessing how responsible beachgoers feel for the presence of beach litter they partly leave behind themselves and to what extent they are willing to pay for the clean-up of this litter compared to litter washed ashore provides important information for priority setting in coastal policy and management.

Naturally, objective means for marine environment monitoring are also required (Xu et al. 2019). During the past two decades, advanced information and communication technologies have been applied to the development of various marine environment monitoring systems. Among others, the Internet of Things (IoT) has been playing an important role in this area. New technologies including advanced Big Data analytics and their applications in this area have also been introduced. These types of data must be associated to relevant metrics (Roberts et al. 2018) so that evolution of contaminated, endangered, or protected sites is recorded. A new set of metrics has been recently suggested focusing on marine biodiversity in protected

sites. This type of approach also permits to compare networks of protected sites, so as to assess the local and overall level of protection.

It cannot, however, be forgotten that the threats posed by different sources of pollutants still persist upon marine environments, and that these pollutants can be detected even in remote polar regions. A further threat arises from climate change causing sea level rise and shifts and changes in flora and fauna of certain sea areas. Thus, policy prompting environmental awareness must be based on a global perspective in this vastly interconnected Earth.

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